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Plasmid biology of natural *Lactococcus lactis* strains and molecular mechanisms of bacteriophage-host interaction

Vincenzo Fallico

PLASMID BIOLOGY OF NATURAL *LACTOCOCCUS LACTIS* STRAINS AND MOLECULAR MECHANISMS OF BACTERIOPHAGE-HOST INTERACTION

A Thesis Presented to the National University of Ireland, Cork for the Degree of Doctor of Philosophy

by

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Dedicated to Mum, Dad, my wife and my lovely daughter

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DECLARATION

I hereby certify that this material, which I submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

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Date: _____

PUBLICATIONS

- **Fallico, V.**, O. McAuliffe, G.F. Fitzgerald, C. Hill and R.P. Ross. (2009). The presence of pMRC01 promotes greater cell permeability and autolysis in lactococcal starter cultures. International Journal of Food Microbiology. 133:217–224.

- **Fallico, V.**, O. McAuliffe, G.F. Fitzgerald, C. Hill and R.P. Ross. (2011). The potential of lacticin 3147, enterocin AS-48, lacticin 481, variacin and sakacin P for food biopreservation. Pages 100-121. In: Lacroix, C., (Ed.), Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation. Woodhead Publishing Limited, Cambridge.

- **Fallico, V.**, R.P. Ross, G.F. Fitzgerald and O. McAuliffe. (2011). Genetic response to bacteriophage infection in *Lactococcus lactis* reveals a four-strand approach involving induction of membrane stress proteins, D-alanylation of the cell wall, maintenance of proton motive force and energy conservation. (Submitted to Journal of Virology).

- **Fallico, V.**, R.P. Ross, G.F. Fitzgerald and O. McAuliffe. (2011). Plasmids of raw milk cheese isolate *L. lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901 suggest a plant-based origin for the strain (Submitted to Applied and Environmental Microbiology).

- **Fallico, V.**, O. McAuliffe, G.F. Fitzgerald and R.P. Ross. (2011). Novel conjugative plasmids from the natural isolate *Lactococcus lactis* subsp. *cremoris* DPC3758: a repository of genes for the potential improvement of dairy starters (Submitted to International Journal of Food Microbiology).

ABSTRACT

In the last few decades, much research has been undertaken to characterize the antimicrobial and preservative qualities of many bacteriocins produced by lactic acid bacteria (LAB). In addition to nisin and pediocin PA-1/AcH, which have gained wide commercial use as natural food biopreservatives, many other bacteriocins offer promising perspectives in terms of preservation and shelf-life extension of food products. Chapter I reviews the studies detailing the characterization and bio-preservative applications of five bacteriocins, namely lacticin 3147, enterocin AS-48, lacticin 481, variacin, and sakacin P, which have proved to be very effective and should find commercial application in food preservation in the near future.

A transcriptomic approach was used to gain insights into the global molecular response of *Lactococcus lactis* IL1403 at the early stages of infection with the lytic phage c2. The phage presence is sensed as a membrane stress in *L. lactis* IL1403, which activated a targeted response probably orchestrated by membrane Phage Shock Protein C-like homologues, the global regulator SpxB and the two-component system CesSR. The bacterium upregulated genes (*ddl* and *dltABCD*) responsible for incorporation of D-alanine esters into LTAs, an event associated with increased resistance to phage attack in gram-positive bacteria. The expression of genes (*yshC, citE, citF*) affecting both PMF components was also regulated, probably to restore the physiological PMF that was disrupted following phage infection. While mobilizing the response to the phage-mediated stress, the bacterium activated an energy-saving programme by repressing growth-related functions and switching to anaerobic fermentation, probably to sustain the PMF and the overall cell response to phage.

An intriguing abundance and diversity of plasmids was found in 17 natural lactococcal isolates from raw milk cheeses, which prompted the sequencing of the 8-plasmid complement of *L. lactis* subsp. *cremoris* DPC3758. The complete sequences of pAF22 (22,388 bp), pAF14 (14,419 bp), pAF12 (12,067 bp), pAF07 (7,435 bp) and pAF04 (3,801 bp) were obtained, whereas gene functions of technological interest were mapped to pAF65 (65 kb) and pAF45 (45 kb) by PCR. These plasmids encode many phenotypes with the potential to improve the technological properties of dairy starters, including three anti-phage restriction/modification (R/M) systems, immunity/resistance to nisin, lacticin 481, cadmium and copper, as well as six conjugative/mobilization functions. By using cadmium selection and conjugative matings, the R/M plasmids were sequentially stacked into a plasmid-free lactococcal

host, thus generating a food-grade derivative with increased protection against 936- and c2-type phages.

The four plasmid-complement of the raw milk cheese isolate *L. lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901 was sequenced and some genetic features functionally analyzed. The complete sequences of pVF18 (18,977 bp), pVF21 (21,739 bp) and pVF22 (22,166 bp) were obtained whereas 49 of the predicted 50 kb of pVF50 were assembled in three contigs. Each plasmid contains genes novel to *Lactococcus* and typical of bacteria associated with plants, in addition to genes associated with plant-derived lactococcal strains. Among the novel phenotypes are activities of plant cell wall modification and a predicted high-affinity regulated system for cobalt uptake, which is functional as demonstrated following plasmid curing. Additional metal transporters could enhance host ability to uptake growth-limiting amounts of biologically essential ions within the soil. In addition, vast and high homology is shared with the plant-derived pGdh442, which includes phenotypes so far exclusive to this plasmid in *Lactococcus*. This wealth of plasmid-encoded plant-associated markers suggests a plant origin for *L. lactis* DPC3901 and provides for the first time the genetic basis to support the concept of the plant-milk transition for *Lactococcus* strains.

The impact of carrying the 60.2-kb plasmid pMRC01, encoding for an abortive infection bacteriophage resistance system and production of the antimicrobial lacticin 3147, on the metabolism and performance of five lactococcal starters was investigated by comparing growth, acidification and viability rates of parental and transconjugant strains. The burden imposed by pMRC01 on host metabolism results in lower specific growth rates and increased cell permeability and autolysis, as revealed by flow cytometry following live/dead™ staining and measurement of released levels of lactate dehydrogenase. The magnitude of these effects appears to be strain dependent but not related to the production of lacticin 3147. Presence of pMRC01 does not significantly affect the acidification capacity of the starters, which implies that plasmid-improved derivatives of starter cultures can be efficiently used in dairy industry. Also, strains prone to early lysis are often utilized as component of dairy starters due to their ability to accelerate cheese ripening and flavour development.

Microarrays were used to analyze changes in pMRC01 gene expression at an early (15 min) and late (30 min) stage of infection with the lytic phage c2 in order to detect the putative activation of the abortive infection (Abi) system of pMRC01. Results suggest that the functioning of the Abi system could be subjected to a complex regulatory control by the bivalent Rgg-like ORF51 and CopG-like ORF58 proteins. These regulators are likely to modulate the activity of the putative Abi effectors, which might be the constitutive proteins ORF50 and ORF49 exhibiting topology and functional similarities to the two-component Rex system that aborts phage λ lytic growth via membrane depolarization. Similarly to RexA, the membrane-anchored ORF50 might use its protein kinase-like activity to cause a conformational change in the putative channel protein RexB-like ORF49, thereby triggering channel opening and consequent membrane depolarization. ORF58 and ORF51 might control, individually or as a network, the activity of ORF50.

Chapter I

LITERATURE REVIEW

The potential of lacticin 3147, enterocin AS-48, lacticin 481, variacin, and sakacin P for food biopreservation

Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation. Woodhead Publishing Limited. (2011). 100-121

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1. ABSTRACT

In the last few decades, much research has been undertaken to characterize the antimicrobial and preservative qualities of many bacteriocins produced by lactic acid bacteria (LAB). To date, only nisin and pediocin PA-1/AcH have gained wide commercial use as natural food biopreservatives. However, many other bacteriocins also offer promising perspectives in terms of preservation and shelf-life extension of food products. Some of them exhibit narrow-spectrum activity and therefore may be used in applications requiring the selective inhibition of certain food pathogens (i.e., *Listeria monocytogenes*) without affecting the natural beneficial microflora. Others with broad-spectrum activity potentially present wider uses. Additionally, when used in combination with selected hurdles (physico-chemical treatments, antimicrobial agents or peptides), these bacteriocins have proved a highly effective form of preservation and should find commercial application as food preservatives in the near future.

2. INTRODUCTION

Many microrganisms, including lactic acid bacteria (LAB), produce the ribosomally-synthesized peptides known as bacteriocins. These peptides are considered to be natural preservatives and their potential application in the food industry has attracted the interest of both researchers and consumers, in search of foods which are minimally processed, naturally preserved and richer in organoleptic and nutritional properties. Among LAB bacteriocins, only nisin and pediocin PA-1/AcH are extensively used commercially and use of their powder preparations for food preservation is now largely established. However, other bacteriocins have recently emerged that also hold great potential for biopreservation and shelf-life extension. Some of them exhibit narrow-spectrum activity and therefore may be used in applications requiring the selective inhibition of certain food pathogens (i.e., *Listeria monocytogenes*) without affecting the natural beneficial microflora. Others with broad-spectrum activity potentially present wider uses (Galvez *et al.*, 2007, Galvez *et al.*, 2008, Ross *et al.*, 1999, Ross *et al.*, 2000). This chapter will review the studies detailing the characterization and biopreservative applications of five of the most promising bacteriocins: lacticin 3147, enterocin AS-48, lacticin 481, variacin, and sakacin P.

3. The potential of lacticin 3147 for food biopreservation

3.1 History, isolation and generally recognized as safe (GRAS) status of the producing strain

Lacticin 3147 is a plasmid-encoded bacteriocin produced by *Lactococcus lactis* subsp. *lactis* DPC3147, a strain isolated from an Irish Kefir grain during a screening of natural sources for food-grade producers of antimicrobial compounds (Rea and Cogan, 1994). Other lacticin 3147 producers have been successively isolated such as the strain *L. lactis* IFPL105 (Martinez-Cuesta *et al.*, 2000). These, and other lactococci, are considered GRAS organisms, since they have been isolated from natural food sources, and, more importantly, because lactococci from dairy products have a long history of safe use and consumption by humans (Casalta and Montel, 2008).

3.2 Characterization, structure and genetics

Lacticin 3147 is a heat-stable proteinaceous compound produced during the exponential phase of bacterial growth (Ryan *et al.*, 1996). FPLC purification of the bacteriocin from the supernatant of *L. lactis* DPC3147 showed that lacticin 3147 is composed of two peptides (LtnA1 and LtnA2) whose synergistic activity is required for full antimicrobial activity (McAuliffe *et al.*, 1998). LtnA1 is a 30-amino acid peptide with a mass of 3,306 Da, whereas LtnA2 is a 29-amino acid peptide with a mass of 2,847 Da. They are encoded as precursor peptides of 59 (LtnA1) and 64 (LtnA2) amino-acids that are subsequently processed to form the biologically active peptides. Maturation of LtnA1 and LtnA2 involves a series of complex post-translational modifications, which includes serine to D-alanine conversion, dehydration of serines and threonines, lanthionine bridge formation, and leader peptide cleavage (Morgan *et al.*, 2005, Ryan *et al.*, 1999). Lacticin 3147 is therefore classified as a member of Class I lantibiotics ("lanthionine-containing antibiotic"), a unique group of small (< 5 kDa) bacteriocins containing the unusual thioether amino-acids lanthionine (Lan) and β-methyllanthionine (MeLan), which form characteristic intramolecular rings, in addition to a number of dehydrated amino-acids (McAuliffe *et al.*, 2001b). Both lacticin 3147 peptides contain Lan residues (Ryan *et al.*, 1999), but a study of their three-dimensional structures by Nuclear Magnetic Resonance (NMR) revealed that the Lan bridging pattern of LtnA1 closely resembles that of the globular type-B lantibiotic mersacidin, whereas LtnA2 has a more elongated structure similar to type-A lantibiotics (Figure 1a) (Martin *et al.*, 2004). Both peptides also contain D-alanine residues that derive from conversion of L-serine in a two-step reaction having dehydroalanine (Dha) as intermediate (Ryan *et al.*, 1999). It has been demonstrated that this post-translational conversion is vital for optimal production and activity of the lantibiotic lacticin 3147 (Cotter *et al.*, 2005). LtnA1 was found to exhibit independent inhibitory activity (MIC_{50} = 200 nM), which was greatly enhanced by the presence of LtnA2, whereas LtnA2 on its own possessed no activity (Morgan *et al.*, 2005).

The genetic determinants for lacticin 3147 production and immunity are encoded on a 60.2 kb conjugative plasmid, pMRC01, the sequence of which has been fully determined (GenBank accession n. AE001272) (Dougherty *et al.*, 1998). Biosynthesis and immunity to lacticin 3147 is encoded by ten genes organized in two divergently

transcribed operons, *ltnRIFE* and *ltnA$_1$A$_2$M$_1$TM$_2$J* (Figure 1b), stretching over 12.6 kb (Dougherty *et al.*, 1998, McAuliffe *et al.*, 2000a). The larger operon, *ltnA$_1$A$_2$M$_1$TM$_2$J*, is responsible for bacteriocin production, modification and export. The *ltnA$_1$* and *ltnA$_2$* genes encode the precursors that will be processed to give rise to the mature LtnA1 and LtnA2 peptides (Ryan *et al.*, 1999). The products of *ltnM$_1$* and *ltnM$_2$* act as modification enzymes by catalysing the dehydration and thioether-forming reactions, which result in Lan bridge formation. Mutagenesis experiments, where the genes were individually inactivated by frameshift mutations, demonstrated that both modification enzymes are necessary for lacticin 3147 activity, with LtnM1 being required to produce mature LtnA1, and LtnM2 required to produce mature LtnA2 (McAuliffe *et al.*, 2000a). *LtnT* encodes a putative ABC-transporter implicated in the secretion of lacticin 3147 (Dougherty *et al.*, 1998); it also contains a proteolytic domain which is probably involved in the cleavage of the leader peptides during export (Ryan *et al.*, 1999). Finally, *ltnJ* encodes a protein sharing significant homology to zinc-containing alcohol dehydrogenases and shown to be responsible for the conversion of Dha to D-alanine (Cotter *et al.*, 2005).

Lacticin 3147 immunity is regulated by the second operon, *ltnRIFE*, divergently located upstream of the biosynthesis operon. *LtnE* and *ltnF* encode proteins with significant sequence homologies to multicomponent ABC transporters involved in immunity to staphylococcal and lactococcal lantibiotics; this supported the initial idea that they might play a similar role in lacticin 3147 too (Dougherty *et al.*, 1998). Surprisingly, deletion analysis excluded their involvement in bacteriocin immunity, and indicated instead the product of *ltnI* as the sole protein responsible for conferring the host with protection to lacticin 3147 (McAuliffe *et al.*, 2000b). Finally, *ltnR* encodes a 79-residue protein sharing homology with a number of transcriptional repressors of the PBSX (Xre) family that are known to auto-regulate their own production. Interesting observations were made on studying the regulation of lacticin 3147 biosynthesis and immunity. While the promoter controlling lacticin 3147 biosynthesis appears to be constitutive, a stem loop structure within the *ltnM1* gene acts as a rho-independent attenuator that controls the level of transcription of downstream genes in order to maintain the correct stoichiometry between the structural peptides and the biosynthetic machinery (McAuliffe *et al.*, 2001a). The authors also demonstrated that LtnR regulates producer immunity to lacticin 3147 by

binding to a region that overlaps the promoter of the *ltnRIFE* operon and thus repressing its own transcription and that of the downstream immunity genes, *ltnIFE.*

3.3 Spectrum of inhibition and mode of action

The inhibitory spectrum of lacticin 3147 was defined using a panel of 54 indicator strains chosen from a number of gram-positive and gram-negative genera. On agar plate assays, a very broad spectrum of inhibition was observed, closely resembling that of nisin. All gram-positive bacteria tested were inhibited, including the pathogens *Listeria*, *Clostridium*, *Staphylococcus* and *Streptococcus* species. Moreover, a number of gram-positive strains which exhibited reduced sensitivity to lacticin 3147 in agar plate assays, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), penicillin-resistant *Pneumococcus* (PRP), *Propionibacterium acne* and *Streptococcus mutans,* were found to be efficiently killed in broth (Galvin *et al.*, 1999). No activity was detected against gram-negative bacteria (Ryan *et al.*, 1996).

The mode of action of lacticin 3147 has been the subject of extensive studies leading to the first detailed analysis of the synergistic activity of a two-peptide bacteriocin at the molecular level (Martin *et al.*, 2004, McAuliffe *et al.*, 1998, Morgan *et al.*, 2005, Wiedemann *et al.*, 2006). In a three-step model proposed by Wiedemann et al. (2006) (Figure 1c), the membrane-bound cell wall precursor, lipid II, is used as a docking molecule for the formation of defined and stable pores. LtnA1 first binds to lipid II and this binding induces a conformational change in LtnA1 which unveils a previously inaccessible binding site for LtnA2. Thus, the LtnA1:lipid II complex is able to recruit LtnA2 . Interaction of LtnA2 with LtnA1:lipid II results in a stable three-component complex with high-affinity for the membrane that allows LtnA2 to insert deeper into the membrane assuming a trans-bi-layer conformation and consequently forming a defined pore. According to these authors, the affinity of LtnA1 for lipid II is low and strongly increases after addition of LtnA2. The synergistic action of LtnA1 and LtnA2 is therefore essential for stabilizing the interaction with the target membrane and to provide lacticin 3147 with dual mode of action: pore formation and inhibition of cell wall biosynthesis by sequestration of the precursor. By using planar bi-layer model membranes supplemented with lipid II, the

same authors observed that lacticin 3147 was able to form defined pores of a diameter of 0.6 nm. McAuliffe et al. (1998) found these small pores to be selective for potassium ions and inorganic phosphate but not for larger compounds such as amino-acids and ATP; the leakage of ions from sensitive cells is accompanied by a change in electrical charge across the membrane that causes immediate and selective dissipation of the membrane potential ($\Delta\Psi$). In a futile attempt to recover these ions by use of ATP-dependent uptake systems, the cells rapidly consume the available internal ATP leading to the eventual collapse of the pH gradient (ΔpH) and, ultimately, to cell death.

3.4 Applications

The ability of lacticin 3147 to inhibit a wide range of food pathogens prompted a series of studies to evaluate its preservative potential in a variety of food systems, either in the form of *ex-situ* produced bacteriocin or of *in-situ* production by bacteriocinogenic strains. Preparation of a bioactive powdered ingredient containing lacticin 3147 was optimised by growing *L. lactis* DPC3147 in 10% reconstituted demineralized whey powder at a constant pH of 6.5 and spray-drying conditions that maintained optimal bacteriocin activity (10,240 AU/ml). Initial results with this powder were promising, as the lacticin 3147-enriched powder was able to reduce numbers of *L. monocytogenes* ScottA and *Staphylococcus aureus* (Morgan *et al.*, 1999). This led to further investigations in more complex food systems. Addition of 10% lacticin 3147 powder to natural yogurt resulted in a 98% reduction of *L. monocytogenes* Scott A (10^4 cfu/ml) within 5 min at 30°C, and no viable cells were detected after 60 min. When the same challenging parameters were tested in cottage cheese, viable cells of *Listeria* were reduced by 40% within 5 min and by 85% within 120 min at 30°C. Even greater efficacy was observed in powdered soup contaminated with *Bacillus cereus* (10^5 cfu/ml); a 5% bacteriocin powder was sufficient to completely eliminate the pathogen within 1 h, whereas a 1% preparation reduced the bacilli population by 80% within 3 h (Morgan *et al.*, 2001). In minced pork-meat, addition of 1.5% powdered lacticin 3147 decreased the population of *Listeria innocua* by 50% (Soriano *et al.*, 2004). Moreover, the efficacy of lacticin 3147 powder can be increased in combination with selected hurdles. A doubling in bacteriocin activity was observed following treatment of lacticin 3147 preparations with high hydrostatic

pressure (HHP) greater than 400 MPa. The combined use of lacticin 3147 concentrates (1-log reduction) and 250 MPa HHP (2.2-logs reduction) demonstrated greater than an additive effect (6 logs reduction) against *S. aureus* ATCC6538 and *L. innocua* DPC1770 in milk and whey (Morgan *et al.*, 2000). Addition of organic acids (sodium citrate or sodium lactate) also enhanced the activity of lacticin 3147 against food-borne pathogens (*Salmonella kentucky* and *Clostridium perfringens*) and spoilage bacteria in fresh pork sausage (Scannell *et al.*, 2000b).

In-situ production by bacteriocinogenic strains represents another means of exploiting the preservative qualities of bacteriocins via incorporation of bacteriocin-producing cultures into foods. This approach harbours several advantages over the addition of concentrated bacteriocin preparations as it lowers the cost of the biopreservation process and, unlike bacteriocin concentrates which may be considered as additives, no regulatory barrier exists since the bacteriocin is not added to the food but produced during its manufacture (Galvez *et al.*, 2007, Ross *et al.*, 1999). Lacticin 3147-producing cultures are very promising for such applications. The presence of the lacticin 3147 genetic machinery on a conjugative plasmid (pMRC01) has allowed the creation of a vast and heterogeneous array of commercial starter cultures with improved technological features, namely bacteriocin production and phage resistance, by using a food-grade approach. These specialised starters retained acidification properties sufficient for use in Cheddar cheese manufacture, can generate significant levels of bacteriocin throughout the cheese ripening process, and have proved to be very effective in increasing the safety of fermented dairy foods (Coakley *et al.*, 1997, Ross *et al.*, 1999, Ryan *et al.*, 1996). As protective cultures on the surface of mould-ripened (Ross *et al.*, 2000) and smear-ripened cheeses (O'Sullivan *et al.*, 2006), lacticin 3147-producing cultures were capable of controlling deliberately inoculated *L. monocytogenes* Scott A. In both cases, spraying the lacticin 3147 producer on the cheese surface resulted in a 1000-fold reduction of the initial pathogen counts (10^4 cfu/ml). In cottage cheese produced with a lacticin 3147-producing transconjugant (*L. lactis* DPC4275), a 3-log reduction in counts of *L. monocytogenes* Scott A (10^4 cfu/g cheese) was observed after 5 days of storage at 4º C (McAuliffe *et al.*, 1999). In this case, the protective effect was most likely to be associated with residual lacticin seeded into the cheese as the starter does not survive the heating step in cottage cheese manufacture. *L. lactis* DPC4275 was also found to

significantly reduce the levels of *L. innocua* and *S. aureus* in beaker sausage(Scannell *et al.*, 2001), in addition to performing satisfactorily as a single starter for manufacture of salami (Coffey *et al.*, 1998) and fermented sausage (Scannell *et al.*, 2001). In a further step to obtain more stable and long-term production of lacticin 3147 in such systems, *L. lactis* DPC3147 was efficiently immobilised on double-layered calcium alginate beads. Results showed that bacteriocin production from the immobilised cells remained constant for 180 h, compared to production by free cells which had declined after 80 h (Scannell *et al.*, 2000a). This technique may improve the protective efficacy of lacticin 3147 on food surfaces as the immobilizing substrates act as reservoirs in which bacteriocin molecules are protected from inactivation by food components (Galvez *et al.*, 2007).

It is interesting to note that lacticin 3147-producing starters have been also shown to contribute to enhanced cheese quality and aroma while protecting its safety. Cheddar cheese manufactured with *L. lactis* DPC4275 was shown to contain 100-fold less adventitious nonstarter lactic acid bacteria than control cheese after 6 months of ripening, while maintaining unaltered physicochemical and organoleptic qualities (Fenelon *et al.*, 1999, Ryan *et al.*, 1996, Ryan *et al.*, 2001). In addition, lacticin 3147-producing starters were found to be more susceptible to permeabilization and autolysis (Fallico *et al.*, 2009) and this has been shown to be associated with improved cheese flavour due to increased proteolysis and enhanced amino-acid transamination and α-keto acids formation (Martinez-Cuesta *et al.*, 2002, Martinez-Cuesta *et al.*, 1998, Martinez-Cuesta *et al.*, 2006).

4. The potential of enterocin AS-48 for food biopreservation

4.1 History, isolation and GRAS status of the producing strain

Enterocin AS-48 (AS-48) is a plasmid-encoded bacteriocin whose activity was first recognized in *Enterococcus faecalis* subsp. *liquefaciens* S-48, a strain isolated during a screening for the production of bacteriocin-like substances by enterococci (Galvez et al., 1986). A PCR-based study showed that the ability to produce peptides identical or similar to AS-48 is very widespread among enterococci (Joosten *et al.*, 1997). Though some of these variants have been designated differently, e.g. AS-48RJ (Abriouel *et al.*, 2005), enterococcin EFS2 (Maisnier-Patin *et al.*, 1996), enterocin 4

(Joosten *et al.*, 1996), and bacteriocin 21 (Tomita *et al.*, 1997), they all share significant homology, if not identity, with AS-48 at the genetic and/or structural level (Maqueda *et al.*, 1998). However, none of the producing strains concerned have been accorded GRAS status, as enterococci are organisms considered at the crossroads of food safety. They have been ascribed beneficial roles such as producing antilisterial bacteriocins, contributing to ripening and flavour development in some artisanal cheeses, and as probiotics to improve the microbial balance of the intestine and for treating gastroenteritis in humans and animals. However, in contrast to this, there is serious concern about the safety of enterococci as a result of their implication in outbreaks of food-borne illness and their recognised role in bacteraemia, endocarditis, and urinary tract infections (Franz *et al.*, 1999).

4.2 Characterization, structure and genetics

AS-48 is a proteinaceous substance that is extremely thermo-stable (Cobos *et al.*, 2001, Cobos *et al.*, 2002). It is active at temperatures of up to 80 ºC or below freezing throughout pH values ranging from 3 to 8 (Maqueda *et al.*, 2004). Optimal production of AS-48 may be obtained by growing the producer strain at 37 ºC in a complex medium broth supplemented with brain-heart infusion, glucose and magnesium sulphate (Galvez *et al.*, 1986). A simple two-step procedure, consisting of cation exchange followed by reversed phase chromatography, guarantees a recovery of up to 9 mg of highly purified bacteriocin AS-48 directly from a pH-controlled 25-liter culture broth (Abriouel *et al.*, 2003, Galvez *et al.*, 1989a). AS-48 is a 70-amino acid peptide with a mass of 7,149.17 Da and represents the very first example of a cyclic bacteriocin to be described in literature, with the cyclic structure originating from a post-translational "tail-to-head" peptide bond formation (Samyn *et al.*, 1994). AS-48 is a strongly basic peptide (pI close to 10.5) and lacks any cysteine or modified residues, such as Lan or MeLan. AS-48 has been therefore included in the class IId of thermostable non-lantibiotics circular bacteriocins (Nes *et al.*, 2002). NMR determination of the 3-D structure of AS-48 in aqueous solution showed a globular arrangement of five α-helices (α1 to α5) enclosing a compact hydrophobic core (Figure 2a). AS-48 may adopt two different dimeric forms in crystals. The molecules in dimeric form I (DF-I) interact through the hydrophobic helices α1 and α2,

suggesting a DF-I for the peptide when in solution. In contrast, dimeric form II (DF-II) involves the interaction of the hydrophilic helices α4 and α5, with the hydrophobic moiety buried within the membrane and the hydrophilic moiety exposed to the solvent. AS-48 may adoptthe DF-II structure when inserts itself into the membrane (Sanchez-Barrena *et al.*, 2003).

The genetic determinants for AS-48 biosynthesis and immunity were first detected on the 68-kb conjugative and pheromone-responsive plasmid pMB2 carried by *E. faecalis* S-48 (Martinez-Bueno *et al.*, 1990). The gene cluster of AS-48 extends over 10.4 kb of sequence and is composed of ten genes, transcribed in the same direction and organized in two operons, *as-48ABCC$_1$DD$_1$* and *as-48EFGH* (Figure 2b). The bacteriocin structural gene, *as-48A*, encodes a precursor peptide of 105 amino-acids (Martinez-Bueno et al., 1994) whose 70 C-terminal residues coincide with the mature AS-48 peptide (Samyn *et al.*, 1994). The gene products of *as-48B*, *as-48C$_1$* and *as-48D* are thought to form a putative multicomponent system able to carry out the simultaneous maturation (involving removal of the leader peptide and head-to-tail cyclization) and secretion of AS-48. *As-48D$_1$* encodes a small cationic hydrophobic peptide and studies of functional analysis demonstrated that it confers some degree of bacteriocin resistance to the producer (Martinez-Bueno *et al.*, 1998). However, appropriate levels of resistance against AS-48 are reached only when the expression of the *as-48D$_1$* immunity gene is combined with that of the *as-48EFGH* operon, which has been proposed to encode a multi-component ABC system: As-48G would be the ATP-binding domain, As-48E and As48-H the trans-membrane subunits, and As-48F an accessory proteins. This second transporter would be mainly responsible for higher levels of producer self-protection against AS-48, whereas the As-48D1 immunity protein would operate as a second resistance mechanism (Diaz *et al.*, 2003).

4.3 Spectrum of inhibition and mode of action

The inhibitory spectrum of AS-48 is remarkably broad, being highly active against most of gram-positive and some gram-negative bacteria tested (Galvez *et al.*, 1989b). A low concentration (3 and 4 μg/ml) of AS-48 exerted a rapid bactericidal activity followed by gradual bacteriolysis against all species of *Bacillus*,

Streptococcus and *Enterococcus* tested, and in most *Corynebacterium* strains. In contrast, 10 μg AS-48 /ml induced no bacteriolytic effect in *Mycobacterium phlei*, *M. smegmatis* and *Nocardia corallina*, although these acid-fast actinomycetes were among the most sensitive gram-positive bacteria, and the same occurred in *Micrococcus* and *Staphylococcus* species. *Clostridium* species were also strongly inhibited by AS-48 (Maqueda *et al.*, 2004), but *L. monocytogenes* emerged as the most sensitive bacterium, with a minimum inhibitory concentration (MIC) of 0.1 μg/ml at 37 ºC (Mendoza *et al.*, 1999). AS-48 also inhibits some gram-negative species, but at much higher concentrations. These include *Myxococcus* strains, *Rhizobium, E. coli, Agrobacterium, Salmonella, Shigella, Pseudomonas* and *Klebsiella*. Finally, no effect of AS-48 was detected against the eukaryotic organisms *Saccharomyces cerevisiae, Naegleria fowleri* and *Acanthamoeba*, even at concentrations as high as 100 μg/ml (Galvez *et al.*, 1989b), nor against HeLa and MCDK cell lines nor erythrocytes (Maqueda *et al.*, 2004).

On the basis of the two dimeric forms found during the crystallographic study (Sanchez-Barrena *et al.*, 2003), a mechanism for the molecular action of AS-48 has been proposed that does not depend upon membrane potential, but implies an effective insertion into the membrane consequent to a structural reorganization of a hydrosoluble dimeric form of AS-48 at the membrane surface (Figure 2c). The strong dipolar moment of DF-I in solution is suggested to drive the approach of AS-48 to the membrane. The low pH provided by the membrane interface would destabilise DF-I due to the protonation of the glutamic side chains. This process would, in turn, allow the interaction between the carboxylate side chains and the phospholipid polar heads, and the stabilisation of the hydrophobic moiety of AS-48 via the interaction with the phospholipid aliphatic chains. The transition from the water soluble DF-I to the membrane bound DF-II would allow molecules of AS-48 to insert itself into the bacterial membrane via an accumulation of positive charges at the membrane surface that would destabilise the membrane potential leading to pore formation and cell leakage (Maqueda *et al.*, 2004).

4.4 Applications

Enterocin AS-48 has significant potential as a biopreservative in a large variety of food systems, although its efficacy is noticeably decreased in the food environment compared with laboratory media. This has been attributed to the interaction of AS-48 molecules with food components which may result in a higher retention, or slower diffusion, or irregular distribution of the bacteriocin molecules in the food (Galvez *et al.*, 2008, Munoz *et al.*, 2007).

Ex-situ application of a semi-purified preparation of AS-48 (40 μg/g) was shown to reduce viable counts of *S. aureus* by 5 logs in a meat sausage model system (Ananou et al., 2005c). In the same food system, a concentration of 225 AU AS-48 /g reduced *L. monocytogenes* counts below the detection level at 3 days of incubation, but did not prevent listeria re-growth after 9 days. Increasing the AS-48 concentration to 450 AU/g resulted in complete kill of listeria over the same incubation time (Ananou *et al.*, 2005b). In yogurt-type soy-based desserts and in gelatin pudding, AS-48 proved more effective against *L. monocytogenes* with bacteriocin concentrations of 87.5 AU/g sufficient to reduce viable counts below detection levels and avoid regrowth of survivors, whereas a two-fold amount of AS-48 (175 AU/g) reduced viable counts of *S. aureus* by only 1.8 log units (Martinez-Viedma *et al.*, 2009). AS-48 also proved effective in decontaminating raw and processed vegetables and avoiding listeria proliferation during storage. Application of immersion treatments (5 min at room temperature) with AS-48 solutions (25 μg/ml) reduced *L. monocytogenes* counts by 2.0 to 2.4 logs in sprouts. During storage of vegetable samples treated with immersion solutions of 12.5 and 25 μg of AS-48/ml, listeria counts were reduced below detection limits in sprouts and green asparagus over 7 days at 15 °C (Molinos et al., 2005). Addition of 30–60 μg/g AS-48 in Russian-type salad significantly reduced *L. monocytogenes* counts during 1-week storage at 10 °C (Molinos *et al.*, 2009a), whereas, in lettuce juice, AS-48 caused strong inhibition of *S. aureus* and complete inactivation of *L. monocytogenes* and *B. cereus* (Grande *et al.*, 2005b). In tomato paste supplemented with 6 μg AS-48/ml and stored at different temperatures, vegetative cells of *Bacillus coagulans* were reduced by 2.4 (4 ºC), 4.3 (22 ºC) and 3.0 (37 ºC) logs within 24 h storage, and no viable cells were detected in any sample after 15-days storage. AS-48 was also very active against the same pathogen in juice from

canned pineapple stored at 22 ºC, and slightly less active in syrup from canned peaches (Lucas *et al.*, 2006). In apple juice and in commercial apple ciders, 3 μg/ml AS-48 completely inhibited rope-forming *B. licheniformis* as well as exopolysaccharide- and acrolein-producing LAB (Grande *et al.*, 2006a, Martinez-Viedma *et al.*, 2008a), whereas vegetative cells of *Alicyclobacillus acidoterrestris* were inactivated by 2.5 μg/ml AS-48 in several types of fruit juices for up to 3 months (Grande *et al.*, 2005a). Interestingly, AS-48 completely eliminated vegetative cells of *B. cereus* in boiled rice, in a rice gruel, and in a rice-based infant formula dissolved in whole milk where it also prevented the pathogen re-growth for at least 15 days at 37 ºC (Grande *et al.*, 2006b). In gelatin and soy pudding, AS-48 (175 AU/g) reduced viable cell counts of *B. cereus* below detection levels after 8 h at 10 ºC or after 48 h at 22 ºC (Martinez-Viedma *et al.*, 2009).

When used in combination with other antimicrobial hurdles, AS-48 showed increased bactericidal activity or the ability to enhance the efficacy of the selected hurdle. In skimmed milk, a moderate heat treatment (65 ºC for 5 min) and 20 μg AS-48/ml eliminated staphylococci after 6 h of incubation (Munoz *et al*., 2007). Sub-lethally heat-injured cells of *E. coli* O157:H7 were inhibited significantly by AS-48 in apple juice, providing a means to lower the intensity of juice processing treatments (Ananou *et al.*, 2005a). AS-48 also significantly increased the heat sensitivity of *B. licheniformis* and *B. coagulans* spores in cider reducing the time for complete inactivation of intact spores (Grande *et al*., 2006a, Lucas *et al*., 2006). In Russian-type salad, the antilisterial activity of AS-48 (30 μg/g) was strongly enhanced by essential oils, and slightly less in combination with bioactive components from essential oils and plant extracts, with other natural or synthetic antimicrobials (Molinos *et al*., 2009a). In the same food matrix, AS-48 (30 μg/g) acted synergistically with lactic acid, PHBME and Nisaplin™ in reducing below the detection limit for 7 days a *Salmonella enterica* cocktail of strains (Molinos *et al.*, 2009b).

Food biopreservation via application of bacteriocinogenic strains producing AS-48 in situ has been tested in dairy and meat systems with satisfactory results. In Taleggio cheese, the activity of AS-48, produced by strain *E. faecium* 7C5 during cheese manufacture, remained stable for at least 40 days (Giraffa *et al.*, 1995). In a non-fat hard cheese, *E. faecalis* A-48-32 strain produced enough AS-48 to inhibit *B.*

cereus and reduce the cell count of bacilli by 5.6 logs after 30 days of ripening (Munoz *et al.*, 2004), while inhibition of *S. aureus* proved less effective (Munoz *et al.*, 2007). Noticeably, growth of starter cultures used in cheese making was not affected by the bacteriocin-producing strain. In Manchego and Hispano cheeses, growth of *L. monocytogenes* was successfully controlled by enterocin 4, a bacteriocin produced by strain *E. faecalis* INIA 4 and analogue to AS-48 (Nunez *et al.*, 1997). In skimmed milk, AS-48 released by *E. faecalis* A-48-32 effectively inhibited *B. cereus* (Munoz *et al.*, 2004) and *S. aureus* (Munoz *et al.*, 2007) provided that a population of at least 10^6 enterococci was used as inoculum. A 10^7 cfu/g inoculum of AS-48-producing strains proved also effective in controlling growth of *L. monocytogenes* (Ananou *et al.*, 2005b) and *S. aureus* (Ananou *et al.*, 2005c) in a meat sausage model system. The antimicrobial efficacy of in situ produced AS-48 is also enhanced by combination with other hurdles. In apple juice, high-intensity pulsed-electric field (HIPEF) treatment was shown to enhance bactericidal effect of enterocin AS-48 against *S. enterica* (Martinez-Viedma *et al.*, 2008b). In raw-milk cheese, the combination of 300 MPa HHP and AS-48-producing adjunct strain was extremely effective in tackling *E. coli* O157:H7, with results varying according to the time of treatment application (Rodriguez *et al.*, 2005).

5. The potential of lacticin 481 for food biopreservation

5.1 History, isolation and GRAS status of the producing strain

Lacticin 481 is a narrow-spectrum lantibiotic bacteriocin produced by strains of *L. lactis* (O'Sullivan *et al.*, 2002a, Piard *et al.*, 1990). Also named lactococcin (Dufour *et al.*, 1991) and lactococcin DR (Rince *et al.*, 1994) in some early reports, the bacteriocin was first isolated from *L. lactis* subsp. *lactis* CNRZ 481 during a screening for bacteriocin producers (Piard *et al.*, 1990). Considering the GRAS status of *L. lactis* strains, lacticin 481 may be regarded as food-grade and therefore the use of ex situ or in situ produced lacticin 481 for food preservation do not pose any legislative issues.

5.2 Characterization, structure and genetics

Optimal purification of lacticin 481 from broth culture was obtained by sequential ammonium sulfate precipitation, gel filtration chromatography, and reversed-phase HPLC leading to a 107,506-fold increase in its specific activity. A preliminary amino-acid analysis of purified lacticin 481 revealed its lantibiotic nature (Piard *et al.*, 1992), while a combination of sequencing of the structural gene, amino-acid analysis, and bidimensional NMR spectroscopy determined the complete amino-acid sequence and the positions of Lan residues (Piard *et al.*, 1993, van den Hooven *et al.*, 1996). Lacticin 481 has a molecular mass of 2,901 Da and is ribosomally synthesized as a prepropeptide containing an N-terminal leader peptide of 24 residues followed by a 27-residue C-terminal propeptide (Figure 3). The mature lacticin 481 contains the unusual amino-acid dehydrobutyrine, two Lan and one MeLan residues. Overlapping thioether bridges in the molecule result in a globular structure at the C-terminal end, whereas the N-terminal part remains linear. As a result of this conformation, lacticin 481 cannot be strictly defined as a Type A (linear peptides) or a Type B (globular peptides) lantibiotic, though the C-terminal bicyclic ring structure makes lacticin 481 more similar to the globular Type B peptides. A lacticin 481 subgroup was therefore proposed which also includes other structurally distinct lantibiotics such as streptococcin A-FF22, salivaricin A and variacin (Dufour *et al.*, 2007, Sahl and Bierbaum, 1998).

The genetic determinants for biosynthesis and immunity to lacticin 481 are encoded on a 70-kb plasmid and are organized as an operon, *lctAMTFEG*, flanked by two insertion sequence elements to form a putative transposon (Dufour *et al.*, 2000, Dufour *et al.*, 1991). The first three genes, *lctA*, *lctM*, and *lctT*, are involved in the bacteriocin biosynthesis and export (Rince *et al.*, 1994). *LctA* is the lacticin 481 structural gene encoding the prepropeptide of 51 amino-acids. The gene product of *LctM* was shown to interact directly with prelacticin 481, thus suggesting an essential role for LctM in Lan residue formation in lacticin 481 (Rince *et al.*, 1997, Uguen *et al.*, 2000). *LctT* encodes an ABC transporter, containing an N-terminal protease domain, that has the dual function of cleaving the leader peptide and exporting the mature bacteriocin (Rince *et al.*, 1994); inactivation of this gene resulted in production of a truncated form of lacticin 481 (Uguen *et al.*, 2005). The proteins encoded by *lctFEG* genes have hydrophobicity profiles and sequence similarities that

strongly suggest their association into an ABC transporter protecting the lacticin 481 producer strain from its own lantibiotic. Co-expression of the three genes into a lacticin 481-sensitive *L. lactis* strain provided the host with immunity to the bacteriocin; deletion of any of the three genes restored strain sensitivity to lacticin 481 (Rince *et al.*, 1997). No regulatory genes have been identified in the lacticin 481 operon suggesting that this lantibiotic may lack a specific regulator (Dufour *et al.*, 2000). The observation that increasing osmolarity stimulates lacticin 481 production suggests that the bacteriocin may be under the influence of global regulation (Uguen *et al.*, 1999).

5.3 Spectrum of inhibition and mode of action

The inhibitory capacity of lacticin 481 was determined on agar plates using selected strains of lactic acid and food spoilage bacteria. Lacticin 481 exerted a bactericidal effect on all species of *Lactococcus*, some lactobacilli and leuconostocs. Of particular interest is the sensitivity of *Clostridium tyrobutyricum* to lacticin 481, as this spoilage organism is responsible for butyric acid formation and late swelling in Emmental-type cheese (Piard *et al.*, 1990). During trials for food preservation, *in-situ* produced lacticin 481 was also found to control growth of *L. monocytogenes* (O'Sullivan *et al.*, 2003b, Rodriguez *et al.*, 2001).

Few studies have addressed the mode of action of bacteriocins of the lacticin 481 group. Recently, Dufour et al. (2007) postulated that these lantibiotics might share a related mode of action due to their close similarity in terms of sequence and structure; their self-protection system is thought to expel bacteriocins from the membrane to the extracellular medium and this supports the idea of a membrane-targeted action. Lacticin 481 was shown to be membrane-active as judged by interaction with artificial lipid monolayers and to have higher affinity for zwitterionic lipids than for anionic lipids (Demel *et al.*, 1996). In another member of the group, nukacin ISK-1, interaction with artificial lipid monolayers and the role of membrane binding in the bacteriocin mechanism of action have been demonstrated. Based on structural similarity, it is conceivable that lacticin 481 acts in a similar manner. However, the N-terminal lysine of lacticin 481 was shown to be not essential for antimicrobial activity (Xie *et al.*, 2004) and, even the absence of the five N-terminal residues

simply reduced but not abolished its biological activity (Uguen *et al.*, 2005). These observations suggest that although lantibiotics of the lacticin 481 group are likely to share a similar mechanism by acting at the membrane level, their modes of interaction with membranes is probably different.

5.4 Applications

A limited number of studies are available in which lacticin 481 has been applied in food systems. In semi-hard raw milk cheese made with the lacticin 481-producing strain *L. lactis* spp. *lactis* TAB 24, inhibition of *L. monocytogenes* growth was observed; the pathogen counts were reduced by 1.6 logs after 8 h from milk contamination (Rodriguez *et al.*, 2001). The efficacy of lacticin 481 antimicrobial activity is enhanced in combination with other hurdle treatments. In raw-milk cheeses artificially inoculated with *E. coli* O157:H7 (10^5 cfu/ml), combining HHP at 500 MPa with the addition of a lacticin 481-producing adjunct strain completely eliminated the pathogen in 60-day-old cheeses. When pressurization was reduced at 300 MPa, results varied according to the time of treatment application; a higher reduction in *E. coli* O157:H7 counts was obtained when the treatment was applied on day 50 (3.8 logs) than if applied on day 2 (1.3 logs) (Rodriguez *et al.*, 2005). The inhibitory effect of lacticin 481 was also shown to work synergistically with that of other bacteriocins. A lactococcal starter derivative producing both lacticins 3147 and 481 reduced the growth rate of *L. monocytogenes* by almost fourfold and showed an increased antilisterial activity compared to the single bacteriocin-producers (O'Sullivan *et al.*, 2003b).

A recent trend is the application of bacteriocins to enhance the release of intracellular enzymes from starter culture cells in order to accelerate ripening and/or improve cheese flavour. Lacticin 481 produced by *L. lactis* subsp. *lactis* DPC5552 was shown to increase lysis and release of proteolytic enzymes by starter *L. lactis* HP without severely compromising its acid-producing capabilities during a cheddar cheese–making trial (O'Sullivan *et al.*, 2002a, O'Sullivan *et al.*, 2003a). Similarly, lacticin 481-producing cultures promoted early lysis of *Lactobacillus helveticus* cells in Hispanico cheese and increased the proteolytic activity (Garde *et al.*, 2006).

6. The potential of variacin for food biopreservation

6.1 History, isolation and GRAS status of the producing strain

Variacin is a lanthionine-containing bacteriocin produced by *Kocuria varians* NCC 1482 (formerly referred to as *Micrococcus varians*). The bacteriocin was identified in two bacterial strains isolated as part of the natural meat flora from laboratory production trials of Italian-type raw salami fermentations. Purification and characterization of the antimicrobial compound produced by both strains revealed the same bacteriocin, though the strains were not identical and had different plasmid profiles. Considering the traditional use of *K. varians* strains as starters in sausage fermentations, these organisms and/or their metabolites may be applied in food preservation without posing any safety or regulatory issues (Pridmore *et al.*, 1996).

6.2 Characterization, structure and genetics

Variacin was purified from cell-free supernatants by hydrophobic interaction followed by fast protein liquid chromatography (FPLC) (Pridmore *et al.*, 1996)**.** The bacteriocin is completely inactivated by proteinases pronase E, proteinase K, and ficin, but retains its activity after incubation with catalase. Variacin is resistant to heat (100 ºC for 15, 30, and 45 min) and pH values from 2 to 10. Total amino-acid composition of variacin was determined by means of peptide sequencing and mass spectrometry analysis of the FPLC active fractions, returning a peptide molecular weight of 2,658.61 Da. Computer analysis revealed a primary structure sharing significant homology with that of lacticin 481, especially at the amino-terminal sequence, and containing the same number of Lan and MeLan residues. At DNA level, variacin and lacticin 481 share 58.7% overall similarity, basically restricted to the 144-bp bacteriocin-encoding segment. In contrast, different degrees of homology exist at the peptide level (Figure 3). The 22-residue leader peptide of variacin is only 60% identical (75% similarity) to that of lacticin 481 (Pridmore *et al.*, 1996) but it contains the "double-glycine" motif that is typically conserved in bacteriocins of the lacticin 481 group (Dufour *et al.*, 2007, Piard *et al.*, 1993). These observations led to postulate that different processing and transport enzymes may have evolved for a similar bacteriocin in different species (*M. varians* and *L. lactis*). In contrast, the 25-residues pro-peptide of variacin is 84% identical (92% similarity) to that of lacticin

481 and shows the conserved residues (three cysteine, two serines and a threonine) potentially involved in the Lan ring formation; mature variacin differs from lacticin 481 for being two amino-acids shorter and having three conservative amino-acid substitutions (Pridmore *et al.*, 1996). No data has been reported to date regarding the immunity genes for variacin; however, the observation that a lacticin 481-producing strain is sensitive to variacin, while natural producers of variacin are both resistant to lacticin 481 suggests that significant differences probably exist between the immunity mechanisms of the two bacteriocins (Pridmore *et al.*, 1996).

6.3 Spectrum of inhibition and mode of action

The inhibitory capacity of variacin was determined by the agar well diffusion method against selected strains of lactic acid and food spoilage bacteria. Variacin exhibited a wide spectrum of activity inhibiting all gram-positive bacteria tested, including pathogenic and spoilage organisms such as listeriae, staphylococci, and the vegetative cells and spores of clostridia and bacilli. Common to most known bacteriocins produced by gram-positives, variacin did not exhibit inhibitory activity against gram-negative bacteria (Pridmore *et al.*, 1996).

No data is available to date regarding the mode of action of variacin. However, considering its sequence and structural similarity with lacticin 481 (Figure 3), the mechanism of action of variacin most likely resembles that of lacticin 481 (described in paragraph 4.3 of this chapter) and related bacteriocins (i.e., nukacin ISK-1) (Dufour *et al.*, 2007).

6.4 Applications

The biopreservative potential of variacin has only been tested in dairy systems so far (O'Mahony *et al.*, 2001). The authors evaluated the feasibility of applying a spray-dried, fermented preparation of variacin to control the growth of a cocktail of three *Bacillus cereus* strains in chilled dairy foods. These authors obtained a powder preparation of variacin by growing *K. varians* in reconstituted skim milk and yeast extract broth at 30 °C for 18 h, and then spray-drying the cell-free fermentate; the amount of active variacin in the fermented milk powder was 3.1 μg/g (20,000

AU/ml). Subsequently, they produced a range of chilled dairy food formulations containing different percentages of fat, carbohydrate and protein to resemble the composition of commercially available products. These dairy models along with two commercial products (chocolate mousse and vanilla dessert), included to validate the experiments, were added with varying amounts of the variacin ingredient and then challenged with the *B. cereus* spore cocktail over a range of abuse temperatures. Addition of 1% variacin inhibited the growth of *B. cereus* at 8 °C in all products and models. At 12 °C, addition of 3% variacin was required to inhibit outgrowth of the spores, whereas a 1% concentration was no longer sufficient to exert long-term inhibition of the pathogen cocktail. When stored at 30 °C, the use of 3% variacin inhibited spoilage in only two of eight chilled food models.

7. The potential of sakacin P for food biopreservation

7.1 History, isolation and GRAS status of the producing strain

Sakacin P is a small, class IIa bacteriocin produced by certain strains of the GRAS organism, *Lactobacillus sakei*. Production of sakacin P was first reported in *Lb. sakei* LTH 673 (Tichaczek *et al.*, 1992) and subsequently in several other producers (Urso *et al.*, 2006), including *Lb. sakei* Lb674 where it was initially named sakacin 674 (Holck *et al.*, 1994). *Lactobacillus* species have a long history of safe use as food adjuncts, and some species are also part of the human gut microflora. Their addition to fermented foods is known to improve the storage stability, flavour, and texture of the products and, following food ingestion, they exert several beneficial "probiotic" effects at the intestinal and immune-system levels. *Lb. sakei* strains are regularly used as starters in salami fermentations and, recently, a patent application proposed their use in pharmaceutical, feed, food, and cosmetic compositions (Park *et al.*, 2009).

7.2 Characterization, structure and genetics

Maximal production of sakacin P may be obtained by growing the producer strain in a completely defined medium at low temperature without pH control (Moretro *et al.*, 2000). Amino-acid composition analysis and protein sequencing of

purified sakacin P revealed a prepeptide of 61 amino-acids containing a typical "double-glycine" leader peptide (Havarstein *et al.*, 1995), which is enzymatically removed during bacteriocin maturation. Mature sakacin P is a 43 amino-acids peptide containing no unusual amino-acids but an N-terminal YGNGVXC consensus motif, typical of class IIa bacteriocins (Eijsink *et al.*, 1998, Tichaczek *et al.*, 1994). The three-dimensional structure of sakacin P has been determined by NMR spectroscopy. The cationic N-terminal region (residues 1-17) has an S-shaped conformation resembling a three-stranded antiparallel β-sheet and contains four positively charged residues pointing in the same direction. The C-terminal tail (residues 34-43) lacks any apparent common secondary structural motif, but it is able to fold back onto a central amphiphilic α-helix (residues 18-33), thereby creating a hairpin-like structure (Uteng *et al.*, 2003).

Biosynthesis and immunity to sakacin P are regulated by seven genes, *sppIP*, *sspK*, *sspR*, *sspA*, *spiA*, *sppT* and *sppE*, with chromosomal (Holck *et al.*, 1994, Tichaczek *et al.*, 1992) or plasmid (Vaughan *et al.*, 2003) location. Uni-directionally transcribed, these genes are functionally organized in three operons, each one preceded by typical inducible promoters, P_{sppIP}, P_{sppA}, and P_{sppT}. The first operon, *sppIPKR*, contains a three-component regulatory system coupling sakacin P production to its cell density. *SppIP* encodes a 19-residue peptide pheromone responsible for inducing bacteriocin production in non-producing strains. *SppK* and *sppR* encode products homologous to proteins of bacterial two-component signal transduction systems of the AgrB/AgrA family: the 448-residues SppK is 59% similar to histidine kinase protein of the plantaricin A operon, whereas the 248-residues SppR shows 65-68% similarity to response regulator proteins of the same operon (Brurberg *et al.*, 1997, Huhne *et al.*, 1996). The three-component regulatory system acts as a quorum-sensing device (Nes and Eijsink, 1999); the increase in extracellular pheromone consequent to low cell density is registered by the histidine kinase, which activates the response regulator that in its turn induces the *spp* promoters. The second operon consists of the sakacin P structural gene (*sppA*) and a cognate immunity gene (*spiA*) encoding a protein of 98 amino-acids. Finally, the genes of the third operon are involved in transport and processing of the bacteriocin and pheromone precursors. *SppT* encodes a 718-residues peptide homologous to ATP-dependent transporters of the HlyB family, which are involved in the signal-sequence independent transport of

proteins across the bacterial membrane. *SppE* encodes a 458 amino-acids peptide that probably acts as accessory protein for the SppT ABC-transporter; SppE is indeed homologous to HlyD-like proteins, which are implicated in the signal-sequence independent export of haemolysin A in *E. coli*. *SppT* and *sppE* are not only essential for sakacin P production but also for immunity; the transcription of the immunity gene *spiA* is indeed regulated by the extracellular pheromone, whose secretion is associated with the activity of SppT and SppE (Brurberg *et al.*, 1997, Huhne *et al.*, 1996). The efficiency of the pheromone-regulated promoter system of sakacin P has been successfully exploited to obtain high-level inducible gene expression in *Lb. sakei* and *Lb. plantarum*. Plasmid vectors or gene expression systems based on sakacin P promoters and regulatory genes have been shown to produce some of the highest levels of regulated gene expression reported so far in lactobacilli (Mathiesen *et al.*, 2004a, Mathiesen *et al.*, 2004b, Sorvig *et al.*, 2003).

7.3 Spectrum of inhibition and mode of action

The antimicrobial activity of sakacin P was found to inhibit the growth of several Gram-positive bacteria, including food pathogen (*L. monocytogenes* and *E. faecalis*) and spoilage (*Carnobacterium*) species (Tichaczek *et al.*, 1992). The mode of action of this and other pediocin-like bacteriocins has not been fully elucidated to date. According to NMR studies, the primary structure of pediocin-like bacteriocins is formed by a hydrophilic, cationic and highly conserved N-terminal region that forms a three-stranded antiparallel β-sheet supported by a conserved disulphide bridge and an amphiphilic α-helix. The less conserved, hydrophobic/amphiphilic C-terminal stretch has instead a rather extended structure that folds back onto the helical part when it inserts into the target-cell membrane (Fimland *et al.*, 1996, Fimland *et al.*, 2000, Nes *et al.*, 2002). The N-terminal region is thought to mediate the initial binding of pediocin-like bacteriocins to target cells via electrostatic interactions. Mutational analysis of charged residues in the N-terminus of sakacin P showed indeed reduced binding to target cells and bacteriocin potency (Fimland *et al.*, 2006, Kazazic *et al.*, 2002). A mutagenesis approach also highlighted the essential role played by tryptophan residues in determining membrane interaction and antimicrobial activity in sakacin P (Fimland *et al.*, 2002). The main role of the C-terminal region

would be to penetrate into the hydrophobic part of the bacterial membrane, thereby mediating membrane leakage (Fimland *et al.*, 1996, Fimland *et al.*, 1998). However, in some pediocin-like bacteriocins (i.e., pediocin PA-1), it also contains a disulfide bridge capable to influence the target-cell specificity. This bridge is missing in sakacin P, but its introduction by site-directed mutagenesis made sakacin P mutants 10 to 20 times more potent than the wild-type towards certain indicator strains (Fimland *et al.*, 2000). Finally, hydrophobic residues located on one side of the C-terminal amphipathic helix were also suggested to be important for receptor recognition and specificity toward particular organisms (Kaur *et al.*, 2004).

7.4 Applications

Because of the combination of high anti-listerial activity and a narrow inhibitory spectrum, sakacin P is one of the most promising bacteriocins for preservation of foods in which contamination with listeria is a problem (Eijsink *et al.*, 1998). Addition of purified sakacin P completely eliminated *L. monocytogenes* in cold-smoked salmon, despite the bacteriocin being subject to proteolytic degradation in salmon tissue (Aasen *et al.*, 2003). Sakacin P alone is ineffective against *E. coli* but it was shown to enhance synergistically the potency of the fish antimicrobial peptide pleurocidin against the pathogen. A combination of 32 μg/ml sakacin P with 2 μg/ml of pleurocidin resulted in complete inhibition of *E. coli* growth after incubation for about 18 h (Luders *et al.*, 2003).

Effective inhibition of bacterial pathogens in food products was also achieved by using sakacin P-producing lactobacilli. A bacteriocinogenic *L. sakei* strain significantly decreased listeria, fecal enterococci and total bacterial counts in sausages, in addition to performing satisfactorily as a starter during meat fermentation (Urso *et al.*, 2006). In raw beef, the sakacin P producer *Lb. curvatus* CWBI-B28 successfully inhibited *L. monocytogenes*. The same strain proved ineffective against the pathogen in raw chicken meat, but it showed a clear anti-listerial effect when inoculated together with a sakacin G-producing strain (Dortu *et al.*, 2008). In cold-smoked salmon, sakacin P released by *L. sakei* L6790 only had a bacteriostatic activity on *L. monocytogenes*; in this case, addition of a sublethal concentration of purified sakacin P in combination with the bacteriocinogenic culture was necessary to

obtain partial inactivation of the pathogen (Katla *et al.*, 2001). Similarly, combined use of a sakacin P preparation and a sakacin P-producing *Lb. sakei* strain resulted in good inhibitory activity of *L. monocytogenes* in vacuum-packaged chicken cuts over a 4-weeks period (Katla *et al.*, 2002).

8. FUTURE PROSPECTS

In response to the growing demand for safer foods free from chemical additives, research into the application of bacteriocins as biopreservatives in food matrices has been underway for more than 20 years. Since then, numerous bacteriocins have been reported to inhibit spoilage and/or pathogen bacteria in several food systems, but only nisin has been licensed as a food preservative (E234) to date. Currently used in almost 50 countries, nisin is also the only bacteriocin to have received the GRAS status for food in the United States. However, several deficiencies of nisin, such as the instability at neutral to alkaline pH and a spectrum of activity restricted to gram-positive bacteria, call for other bacteriocins to be examined (Settanni and Corsetti, 2008). Some of the bacteriocins described in this chapter, particularly lacticin 3147 and enterocin AS-48, possess qualities which make them ideal candidates as alternatives to nisin. Since the effectiveness of bacteriocins in foods is known to decrease in response to a number of food-related factors (pH, temperature, food composition, structure, microbiota) (Schillinger *et al.*, 1996), a single bacteriocin-based technique is unlikely to alleviate the safety issues associated with a large variety of food products. Therefore, the feasibility of these agents as preservatives should be tested on a "product by product" basis using specific bacteriocins for specific tasks. This explains why the use of bacteriocins as part of hurdle technology has received great attention in recent years. Combination of physico-chemical treatments (i.e., heat, HHP, HIPEF), antimicrobial agents (i.e., chelators, essential oils, phenolic compounds) or other bacteriocins with several bacteriocins has been shown to result in additive or synergistic antibacterial effects (Galvez *et al.*, 2007). Ex situ–produced bacteriocins may also be exploited to prepare bioactive food inserts that represent one of the most interesting preservation technologies for foods stored in modified atmosphere packaging (MAP) at refrigeration temperatures. Many of the examples presented in this chapter have shown how the combination of bacteriocins with selected hurdles often results in a more effective form of preservation, providing

an additional barrier even to the more refractive forms like gram-negative bacteria endospores. While the search for new bacteriocins, exhibiting different antimicrobial properties, will certainly continue in the near future, one of the most attractive directions of future research on food protection remains the exploitation of bacteriocinogenic cultures or their pure bacteriocins within a hurdle technology program. In particular, the combination of bacteriocin-activated packaging films and traditional hurdles holds promise for the extension of shelf-life and improvement of microbiological safety of food products.

9. FURTHER INFORMATION

A number of public databases are now available for the detection and structural/functional analysis of bacteriocins during the *de novo* sequencing of bacterial genomes. BAGEL is a web-based software tool which enables the identification of putative bacteriocin gene clusters in novel DNA sequences using a number of ORF prediction tools and knowledge-based bacteriocin and motif databases (de Jong *et al*., 2006). A typical BAGEL search on a genome sequence results in a set of putative bacteriocin gene clusters ranked according to the presence of significant features in the amino-acid sequences and their genomic context. BAGEL is freely accessible at: http://bioinformatics.biol.rug.nl/websoftware/bagel. A complementary tool to BAGEL for bacteriocin characterization is BACTIBASE, a web-based database containing the calculated or predicted physicochemical properties of 123 bacteriocins produced by both Gram-positive and Gram-negative bacteria. The information in this database allows rapid prediction of relationships structure/function and target organisms of these antimicrobial peptides. BACTIBASE is freely accessible at: http://bactibase.pfba-lab-tun.org. Finally, the manually annotated UniProtKB/Swiss-Prot database stores relevant data regarding the majority of sequenced bacteriocins that include mode of action, 3D-structure, post-translational modification of the precursor protein, and interactions with other proteins, other than cross-references to external databases.

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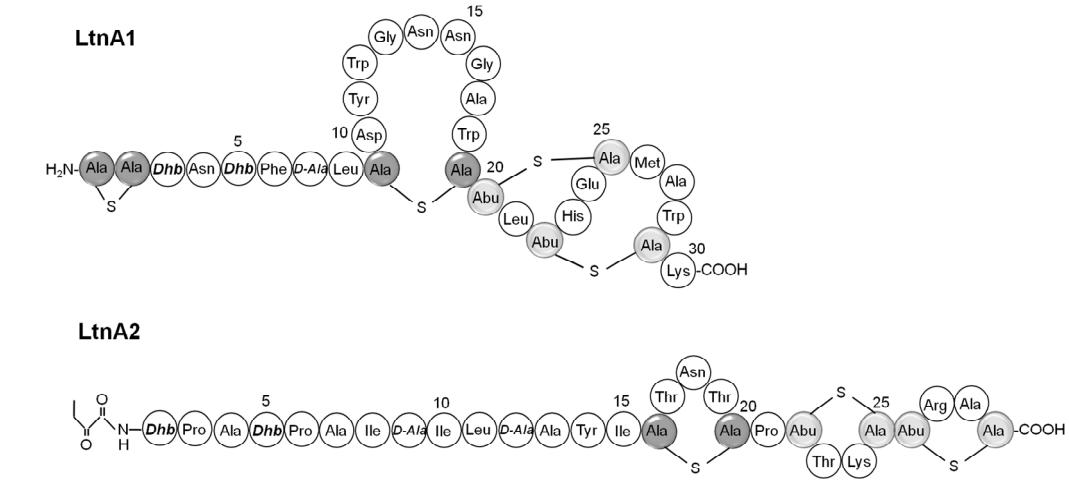
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FIGURES

Figure 1a, b.

(a)



(b)

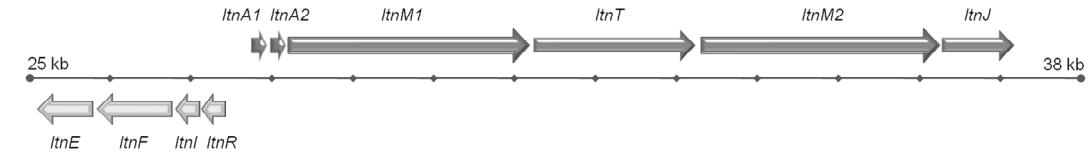


Figure 1c.

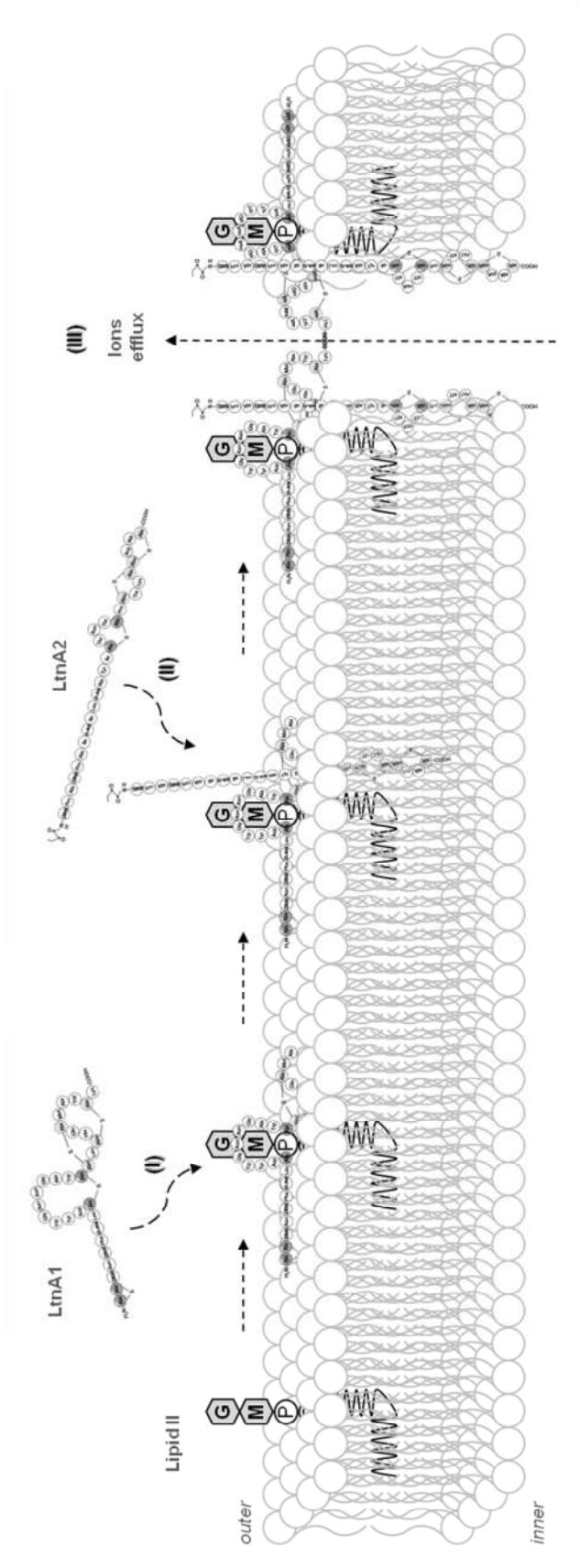


Figure 1. (a) Primary structure of the lacticin 3147 peptides, LtnA1 and LtnA2. Thioether aminoacids are shown in dark (Ala-S-Ala, lanthionine) and light grey (Abu-S-Ala, b-methyllanthionine). Dehydrated amino acids (Dhb, 2,3-didehydrobutyrine) and D-alanine residues are shown in bold and italics (Adapted from Wiedemann et al., 2006). (b) Organization of the gene cluster involved in the production of and immunity to lacticin 3147 (Adapted from McAuliffe et al., 2001a). (c) Three-step model for the action of the two-peptide lantibiotic lacticin 3147 (I) Membrane association of the A1 peptide and binding to lipid II. (II) Binding of the A2 peptide to A1:lipid II and formation of a high-affinity three-component complex. (III) Translocation of the C-terminus of A2 and formation of a defined pore (Adapted from Wiedemann et al., 2006).

Figure 2.

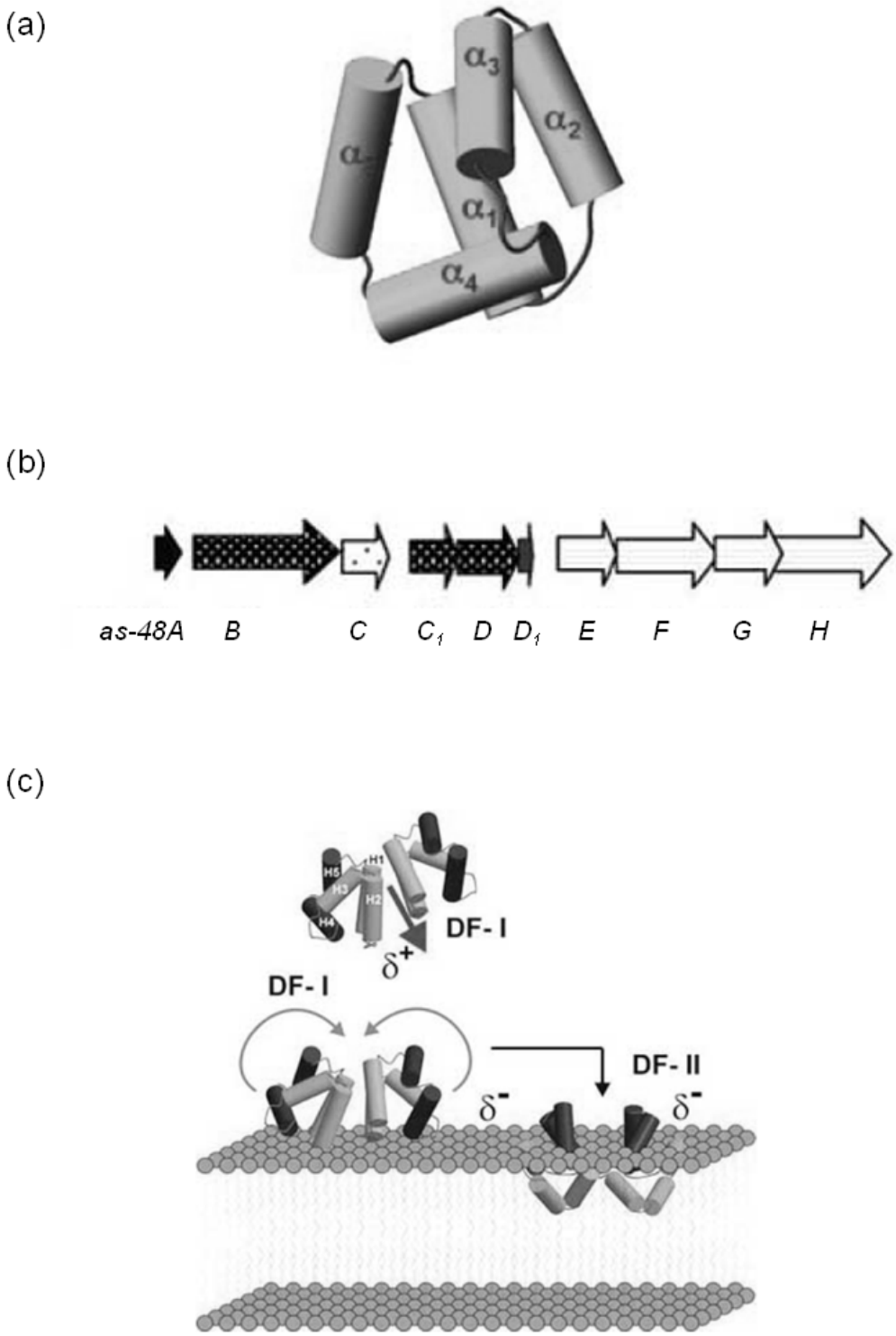

(a)
α3
α2
α1
α4
(b)
as-48A
B
C
C1
D
D1
E
F
G
H
(c)
H5
H1
H3
H2
H4
DF- I
δ+
DF- I
DF- II
δ-
δ-

Figure 2. (a) Three-dimensional structures of enterocin AS-48, with α-helices represented as cylinders. (b) Organization of the gene cluster involved in the production of and immunity to AS-48. (c) Schematic representation of the mechanism for the molecular function of AS-48. The model includes the approach of DF-I to the membrane and the transition from DF-I to DF-II at membrane surface. Hydrophobic and polar helices are depicted in light gray and dark gray respectively. The arrow represents the direction of the intrinsic dipolar moment of DF-I (Adapted from Maqueda et al., 2004; permission to reproduce material granted by Bentham Science Publishers Ltd.).

	Prepeptide sequence						Accession no.	Reference
	Leader peptide			Propeptide				
	-20	-10	-1	+1	+10	+20		
Lacticin 481	MKEQNS**F**NLL**Q**E**VT**ES**ELD** L**IL**G**A**			-KG**GSGVIHTISHEC**N**MNS**W**QFVFTCCS**			AAC72257	Piard *et al.* (1993)
Variacin	MTNA**F**QAL**D**E**VT**DA**ELDAIL**G**G**			- - - **GSGVIPTISHEC**H**MNS**F **QFVFTCCS**			CAA63706	Pridmore *et al.* (1996)

Figure 3. Sequence comparison of precursor peptides (prepeptides) between lacticin 481 and variacin. Identical residues are shown in bold (Adapted from Dufour et al., 2007; permission to reproduce material granted by Wiley-Blackwell).

Chapter II

Genetic response to bacteriophage infection in *Lactococcus lactis* reveals a four-strand approach involving induction of membrane stress proteins, D-alanylation of the cell wall, maintenance of proton motive force and energy conservation

Submitted to Journal of Virology.

ABSTRACT

In this study, we have used whole-genome microarrays to gain insights into the global molecular response of *L. lactis* IL1403 at the early stages of infection with the lytic phage c2. The bacterium differentially regulated the expression of 61 genes belonging to 14 functional categories, including cell envelope (12 genes), regulatory functions (11 genes), and carbohydrate metabolism (7 genes). The nature of these genes suggests a complex response involving four main mechanisms: (i) induction of membrane stress proteins, (ii) D-alanylation of cell wall lipoteichoic acids (LTAs), (iii) maintenance of the proton motive force (PMF), (iv) and energy conservation. The phage presence is sensed as a membrane stress in *L. lactis* IL1403, which activated a cell-wall targeted response probably orchestrated by the concerted action of membrane Phage Shock Protein C-like homologues, the global regulator SpxB and the two-component system CesSR. The bacterium upregulated genes (*ddl* and *dltABCD*) responsible for incorporation of D-alanine esters into LTAs, an event associated with increased resistance to phage attack in gram-positive bacteria. The expression of genes (*yshC, citE, citF*) affecting both PMF components was also regulated to restore the physiological PMF, which was disrupted following phage infection. While mobilizing the response to the phage-mediated stress, the bacterium activated an energy-saving programme by repressing growth-related functions and switching to anaerobic fermentation, probably to sustain the PMF and the overall cell response to phage. To our knowledge, this represents the first detailed description in *L. lactis* of the molecular mechanisms involved in host response to the membrane perturbations mediated by phage infection.

INTRODUCTION

Lactococcus lactis strains are extensively used worldwide for the manufacture of fermented milk products. The efficiency of industrial fermentations, relying on lactococcal starters, is however threatened by their sensitivity to bacteriophage (phage) infection. Lysis of starter bacteria following attack by lytic phages slows down or arrests the fermentation process, and adversely impacts on product quality. Severe phage infection can greatly affect production schedules, as investigations must be carried out to identify the source of the phage and to implement corrective actions, resulting in significant economic losses for the dairy industry (Emond and Moineau, 2007).

Virulent lactococcal phages are ubiquitous within the dairy industry environment and, consequently, a great deal of research has been devoted to improving the phage resistance of *L. lactis* starters over the past decades. Phages derived from failed fermentations have been collected and used in pools to screen for new bacterial strains capable of sustaining such phage challenges. This has led to the selection of highly resilient bacteria and to the isolation and characterization of a myriad of host-encoded natural defence mechanisms. According to the phase of the phage life cycle with which they interfere, these mechanisms have been divided into four groups: adsorption blocking, DNA ejection blocking, restriction/modification (R/M) systems, and abortive infection mechanisms. The majority of these mechanisms are encoded on conjugative or mobilizable plasmids, a feature enabling their transfer to other strains by conjugation. This has allowed the construction of industrial phage-resistant systems where isogenic variants of *L. lactis*, carrying single or multiple natural defence mechanisms, have been incorporated into rotation programs. The combination of culture rotation and anti-phage system pairing has greatly improved the reliability and lifespan of lactococcal starters in the industry (Emond and Moineau, 2007, Garvey *et al.*, 1996). However, the long-term effectiveness of such strategies is always challenged by the evolutionary dynamic of phages that, under protracted selective pressure, are able to evolve and adapt to circumvent the resistance mechanisms. This requires continuous efforts from researchers to provide alternative solutions to the phage threat. New and sophisticated anti-phage approaches may be derived from the wealth of genomic information generated from bacteria and phages with the recent advent of more affordable DNA sequencing

platforms. Used in combination with high-throughput technologies for gene expression profiling such as microarrays, this sequencing data may help to elucidate the molecular mechanisms of the phage-host interactions in gram-positive bacteria by identifying targets for the design of novel anti-phage measures for biotechnological processes.

Lactococcal phages are classified into several groups, but those commonly found in dairy plants belong to three main species, namely 936, P335, and c2 (Jarvis, 1984). The phage infection process requires an initial adsorption to the host cell surface via specific interaction between the phage receptor-binding protein (RBP) and the bacterial receptor, followed by DNA injection. Once inside the cell, the phage DNA takes over the cell metabolic machinery to initiate its own replication followed by packaging of the viral progeny. The early step of the infection process, involving phage adsorption and DNA injection, is therefore crucial to understand the host response to phage interaction at the molecular level. A genome-wide transcriptional profiling of responsive genes may allow for the identification of potential targets (i.e., gene networks, promoters, regulatory elements) for the rational design of new anti-phage strategies for starter cultures.

In this study, we have used whole-genome microarrays to obtain a holistic view of the *L. lactis* subsp. *lactis* IL1403 response at the onset of infection by the lytic phage c2. Our objective was to gain insights into whether any defence strategy is activated by this phage-sensitive strain in response to phage infection. *L. lactis* IL1403 and phage c2 provide an excellent model system for this study as their complete genome sequences are known (Bolotin *et al.*, 2001, Lubbers *et al.*, 1995). Our results suggest that the cell activates a sophisticated response involving induction of membrane stress proteins, D-alanylation of the cell wall, maintenance of the proton motive force (PMF) and energy conservation. Regulation and implications of this response on the chances of cell survival to phage infection are discussed.

MATERIALS AND METHODS

Bacterial strains

L. lactis subsp. *lactis* IL1403 was used for whole genome expression profiling of the host response to phage infection. *L. lactis* subsp. *cremoris* MG1363 served as the sensitive host for propagation of phage c2 at high titers. Lactococcal strains were routinely propagated at 30 ℃ in M17 medium (Oxoid, Hampshire, England) supplemented with 0.5% (wt/vol) glucose (GM17). Solid media contained 1.0% (wt/vol) bacteriological agar (Oxoid, Hampshire, England). Bacterial strains were stocked in M17 containing 40% glycerol at -80 °C. Working cultures were stored at 4 °C and transferred periodically.

Preparation of high-titer stock solutions of bacteriophage c2

Phage c2 was propagated to high titres on the sensitive host *L. lactis* MG1363 as previously described (Jarvis, 1984). Concentration and purification of the phage lysate by polyethylene glycol (PEG) precipitation followed by caesium chloride (CsCl) gradient centrifugation was performed as follows. Briefly, cell debris was removed from the phage lysate by centrifugation at 11,000 x *g* for 30 min at 4 °C. The supernatant was recovered and concentrated by addition of 15% PEG 8000 and 1 M NaCl, followed by slow agitation for 24 h at 4 °C. Phages were then precipitated by centrifugation at 16,700 x *g* for 60 min at 4 °C, and the phage pellet resuspended in 4 ml of TMN buffer (10 mM Tris-HCl, 10 mM $MgSO_4$, 500 mM NaCl) at pH 7.5. Concentrated phages were purified by centrifugation at 400,000 x *g* for 3 h at 4 °C on a discontinuous-step CsCl gradient (1.3, 1.5 and 1.7 g/ml). The high-titer phage preparation was desalted by dialysis against 10 mM Na_3PO_4 buffer (pH 7.0) for 24 h at 4 °C, recovered in 1 ml TMN buffer, and then titrated at 30 °C according to standard procedures (Terzaghi and Sandine, 1975). This protocol consistently yielded phage c2 stock solutions with titers in the range of 10^{13} plaque forming unit (pfu)/ml. Phage stock solutions were stored at 4 °C.

Determination of the Multiplicity of Infection (MOI) value for the phage-host challenge

The phage lytic cycle is estimated to have an average duration of 40 min (Emond and Moineau, 2007, Garvey *et al.*, 1996). To obtain an accurate and homogeneous gene expression profile of the *L. lactis* IL1403 response to phage c2 attack at the chosen time-point (10 min after infection), we determined the MOI value necessary to completely lyse a bacterial population within 40 min. To do this, a number of phage-host challenge tests were performed by infecting a 40-ml culture of *L. lactis* IL1403, growing in mid-log phase (OD_{600} = 0.4), with increasing titers of phage c2. The clearing of the bacterial population was evaluated by measuring the cell density at 600 nm.

Microarray design

The whole genome sequences of *L. lactis* IL1403 (2,310 genes) and phage c2 (39 genes) were used to design 50-mer oligonucleotide probes for each gene which were subsequently spotted onto glass slides in duplicate. Control elements on each array included negative-control probes from unrelated *Arabidopsis thaliana* genes. Oligonucleotide probe design and microarray production were carried out by Ocimum Biosolutions Ltd. (Hyderabad, India).

Total RNA isolation and mRNA enrichment

Total RNA was isolated as follows. An overnight culture of *L. lactis* IL1403 was used to prepare a fresh 0.5% inoculum in 40 ml of GM17 broth, which was propagated at 30 °C to mid-log phase (OD_{600} = 0.4). The IL1403 culture was split in two 20-ml aliquots (experimental and reference), to which were added phage c2 (MOI = 800) (experimental) or TMN buffer (reference), and 10 mM $CaCl_2$ to allow efficient phage adsorption. Following incubation at 30 °C for 10 min to allow phage adsorption on IL1403 cells, samples were immersed in a -80 °C ethanol bath for 5 min to prevent any changes in gene expression profiles. After centrifugation at 11,000 x *g* for 5 min, cell pellets were re-suspended in 200 µl of Tris-EDTA buffer (pH 8.0) containing 6 mg/ml lysozyme and incubated at 37 °C for 10 min. Total RNA was

extracted by using the RiboPure™-Bacteria kit (Ambion, USA) and contaminating DNA was removed with a DNA-*free* kit (Ambion, USA), according to manufacturer's recommendations. Amount and purity of recovered RNA were determined by reading the absorbance at 260 nm for RNA concentration, and the absorbance at 230 and 280 nm for solvent and protein contaminations by means of a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was verified on glyoxal-agarose gels after ethidium bromide staining. Total RNA samples (50 μg) were enriched for mRNA, which provides a superior template for cDNA synthesis, by using the mRNA-ONLY™ Prokaryotic mRNA Isolation kit (Epicentre Biotechnologies, USA). This kit uses a 5'-phosphate-dependent exonuclease enzyme to selectively digest RNA having a 5'-monophosphate.

cDNA preparation and labelling

Reverse transcription of enriched mRNA into single-stranded cDNA was initiated by adding 9 μg of random hexamers (Invitrogen, USA) and 2 μl of a 50X aminoallyl-deoxynucleoside triphosphate stock mixture (25 mM dATP, dGTP, dCTP, 10 mM dTTP, and 15 mM aminoallyl-dUTP). The volume was adjusted to 34 μl with RNase-free water before the mixture was incubated at 70 °C for 10 min followed by 10 min at 25 °C to allow primers annealing. After addition of 12 μl of 5X First Strand buffer (Invitrogen, USA), 3 μl of 0.1 M dithiothreitol (Invitrogen, USA), 3 μl of SUPERase-In (20 U/μl; Ambion, USA), and 6 μl of SuperScript III Reverse Transcriptase (200 U/μl; Invitrogen, USA), the mixture was first incubated at 25 °C for 10 min and then at 42 °C for 24 h. cDNA synthesis was stopped by heating at 95 °C for 10 min and the RNA template was removed by alkaline treatment with 20 μl of 0.5 M NaOH and 10 μl of 0.5 M EDTA at 65°C for 30 min. Ten microliters of 1 M HCl were added to neutralize the cDNA mixture, which was then purified by using the QIAquick PCR purification kit (QIAGEN, Germany) according to manufacturer's instructions, except that the free amines-containing wash and elution buffer were replaced with an ethanol-based phosphate wash buffer (80% ethanol, 5 mM KPO_4, pH 8.5) and a phosphate elution buffer (4 mM KPO_4, pH 8.5), respectively. Purified single-stranded cDNA was dried in a speed vac, resuspended in 60 μl of 0.1 M sodium bicarbonate buffer (pH 9.0), and labelled by incubation with Cy3 (reference)

or Cy5 (experimental) mono N-Hydroxysuccinimide (NHS) ester (GE Healthcare, UK) at room temperature (RT) for 90 min in the dark. Labelling reaction was quenched by addition of 5 µl of 3 M sodium acetate (pH 5.2) followed by incubation at RT for 15 min in the dark. Uncoupled dye was then removed using the QIAquick PCR purification kit (QIAGEN, Germany) and the buffers provided with the kit, according to manufacturer's instructions. The efficiency of labelling was determined by reading the absorbance at 260 nm for cDNA concentration, the absorbance at 550 nm for Cy3 incorporation, and the absorbance at 650 nm for Cy5 incorporation.

Microarray hybridization, scanning, and data analysis.

The two differentially labelled cDNA probes were pooled, dried in a speed vac, and resuspended in 120 µl of a preheated salt-based hybridization buffer (Ocimum Biosolutions, India) at 42 °C. Microarray slides were pre-hybridized in a solution containing 5X saline-sodium citrate (SSC) buffer, 0.1% sodium dodecyl sulphate (SDS), and 1% bovine serum albumin (BSA) at 42 °C for 60 min. After two washes with RT double-distilled water for 10 min, arrays were dried by centrifugation at 1,000 rpm for 5 min. The cDNA probe was denatured by heating at 95 °C for 3 min and cooled on ice for 1 min. After addition of 40 µl of the background blocker KREA*block* (Kreatech Biotechnology, The Netherlands), the hybridization mixture was heated at 42 °C before application to the microarray. The applied probe was kept in place with a coverslip, placed in a metal chamber, and incubated in a water bath at 42 °C for 24 h. Following overnight hybridization, slides were washed under gentle agitation for 1 min in preheated 5X SSC, 0.1% SDS buffer at 42°C, followed by washes at RT for 2 min in 2X SSC, 2 min in 1X SSC, and 1 min in 0.2X SSC. Slides were quickly rinsed in RT double-distilled water and dried in 50-ml conical-bottom tubes by centrifugation at 1,000 rpm for 5 min at RT. Microarrays were scanned with an Affymetrix 428^{TM} Array Scanner (Affymetrix, USA) by using laser lights at wavelengths of 532 nm and 635 nm to excite Cy3 and Cy5 dye, respectively. Images were captured as 16-bit TIFF files by using Affymetrix Array Reader v.1.1 (Affymetrix, USA) and analyzed using ImaGene v.7.5 (BioDiscovery Inc., USA). This software uses a patented automatic segmentation algorithm to differentiate between signal and background values, and a complex tool including seven different criteria for spot fail/pass test to detect and flag low-quality spots. The presence of

additional poor or empty spots not detected automatically was checked manually and flagged spots were excluded from quantification analysis. Background correction was applied globally by using the median of the signal values of all negative-control spots. A locally weighted regression scatter plot smoothing (LOWESS)-based procedure (Yang *et al.*, 2002) was applied globally, using all spots, to normalize background-corrected microarray data. The experiments were biologically replicated twice. Considering that each biological replicate also contained two technical duplicates on the array, the two hybridizations generated eight data points from both channels for each gene. The statistical significance of differential gene expression was calculated with the Significance Analysis of Microarray (SAM) software (Tusher *et al.*, 2001) by using a minimal two-fold change in expression ratio and maximal false discovery rate (FDR) of 1%. The microarray data have been submitted to the Gene Expression Omnibus (GEO) at the website http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE26042.

Quantitative Real-Time PCR (qRT-PCR).

Enriched mRNA (1 μg) was reverse transcribed using 6 ng of random hexamers (Invitrogen, USA), 5 mM dNTP mix, 1X First Strand buffer (Invitrogen, USA), 5 mM dithiothreitol (Invitrogen, USA), 40 U of SUPERase-In (Ambion, USA), and 400 U of SuperScript III Reverse Transcriptase at 55 °C for 1 h. Primers for the qRT-PCR (Table 1) were designed by using PrimerSelect v.8.0.2 from the Lasergene suite (DNASTAR Inc., USA). The qRT-PCR was set up by using SYBR Green I Master mix (Roche, USA), 200 nM of each forward and reverse primer, and 10 ng of cDNA template. The housekeeping *alaS* gene was used as internal control and for normalization of results. The reactions were performed in triplicate by using an ABI PRISM 7000 detection system (Applied Biosystems). Cycling conditions for all amplifications were one cycle of 95 °C for 10 min and 45 cycles of 95 °C for 15 sec, 55 °C for 5 sec, and 72 °C for 15 sec. From the qRT-PCR data, an average cycle threshold (Ct) value was calculated from the triplicate reactions. A mathematical model based on PCR efficiency and crossing point deviation (Pfaffl, 2001) was used to determine the relative amount of target and reference gene transcripts and,

consequently, the average relative expression ratio between phage-treated and untreated samples.

Computational prediction of protein subcellular localization and function.

Predictions of subcellular localization (SCL) of *L. lactis* IL1403 proteins were retrieved from the PSORTdb v.2.0 database. This database includes information from a manually curated dataset of ~2,000 proteins of experimentally verified localization as well as SCL computational predictions of 2,165 proteins, from 140 completely sequenced microbial genomes (96 Gram-negative and 44 Gram-positive organisms), generated by using the PSORTb program. This predictive tool uses the dataset of proteins of known SCL to train multiple SCL predictors and relies on a series of analytical modules to identify typical sequence features known to correlate with specific localizations. In the case of Gram-positive bacteria, four single (cytoplasm; cytoplasmic membrane; cell wall; extracellular) and two multiple (cytoplasm/cytoplasmic membrane; cytoplasmic membrane/cell wall) localization sites are being predicted by PSORTb and included in PSORTdb (Rey *et al.*, 2005). Prediction of topology in putative membrane proteins, including a specification of the membrane spanning segments and the in/out orientation relative to the membrane, was obtained by using the servers TMHMM v2.0 (www.cbs.dtu.dk/services/TMHMM) and TOPCONS (http://topcons.cbr.su.se/index.php). This latter tool uses the prediction from five different topology prediction algorithms (SCAMPI-seq, single sequence mode; SCAMPI-msa, multiple sequence mode; PRODIV-TMHMM; PRO-TMHMM; and OCTOPUS) as input to the TOPCONS Hidden Markov Model (HMM), which gives a consensus prediction for the protein, together with a reliability score based on the agreement of the included methods across the sequence (Bernsel *et al.*, 2009).

Prediction of function in proteins annotated as hypothetical in the genome of *L. lactis* IL1403 was achieved by using the PFAM database (http://pfam.sanger.ac.uk) (Finn *et al.*, 2010), the Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2009), and the InterProScan service (www.ebi.ac.uk/Tools/InterProScan). This latter tool predicts the occurrence of functional domains and motifs/signatures in a protein

by combining 12 different databases and their relative protein signature recognition methods (Zdobnov and Apweiler, 2001).

Fluorometric determination of membrane potential changes in *L. lactis* IL1403 following phage adsorption.

Changes in the membrane potential of *L. lactis IL1403* cells following phage adsorption were estimated by using the lipophilic cationic dye rhodamine 123 (Sigma, St. Louis, USA) and flow cytometry. Rhodamine 123 was prepared as a stock solution of 1 mg/ml in methanol. The uncoupler CCCP (carbonyl cyanide *m*-chlorophenol hydrazone) was used as positive control for lack of activity of the electron transport system. CCCP is a lipophilic weak acid that readily passes through microbial membranes, binds protons on the acidic side of the membrane, diffuses through, and releases them inside the cell. As a consequence, the formation of a proton gradient is blocked, the membrane potential ($\Delta\Psi$) is reduced to zero and ATP is not synthesized by the electron transport oxidative phosphorylation (Novo *et al.*, 1999). Cells were grown at 30 °C to mid-log phase (OD_{600} = 0.4), added of 2 μg/ml rhodamine 123, and then re-incubated for additional 15 min to allow cell uptake of the dye. At this time-point, a 1-ml aliquot was withdrawn for flow cytometric analyses, and the remaining sample was infected with phage c2 (MOI = 800) in the presence of $CaCl_2$ (10 mM) and further incubated at 30 °C to allow phage adsorption on IL1403 cells. One-ml aliquots were then withdrawn after 10, 15 and 25 min, and processed for flow cytometric analyses. Aliquots were centrifuged at 20,800 x *g* for 2 min to remove the growth media. Cell pellets were washed twice in 0.22 μm-filtered 50 mM sodium phosphate buffer (pH 7), containing 0.01% Tween-20, and finally re-suspended in the same buffer to an approximate concentration of 5 x 10^6-10^7 cells per ml. Fluorescence measurements were made with a FACSCanto II flow cytometer (BD Biosciences, San Jose, USA) equipped with two air-cooled lasers, a 20-mW solid state (emission, 488 nm) and a 17-mW HeNe (emission, 633 nm), and five sensors for the detection of forward (FSC) and sideward (SSC) light scatter, green (FL1, 525 nm), yellow (FL2, 575 nm), and far red (FL3, 695 nm) fluorescence. Cell samples were delivered at the low flow rate, corresponding to 300 to 500 cells per sec, until a total number of 10,000 events were acquired by using the FACSDiva

software v.5.0.2 (BD Biosciences, San Jose, USA). Fluorescence signals were recorded by using the following detector settings: FSC, 300; SSC, 300; FL1, 400 with logarithmic amplifications. A threshold was set at FSC and SSC signals of 200 to reduce background noise deriving from cellular debris.

RESULTS AND DISCUSSION

Transcriptional profiling of the *L. lactis* IL1403 response to phage c2 infection.

The life cycle of lytic phages involves different steps that may be organized into four main phases: (i) infection, (ii) early development, (iii) late development, (iv) and lysis. The infection stage comprises the steps of phage adsorption to host surface receptors and injection of the viral DNA, and its duration is phage-dependent (Emond and Moineau, 2007). In lactococcal phages, appearance of viral DNA into the host has been detected between 5 and 15 min after adsorption (Garvey *et al.*, 1995, Garvey *et al.*, 1996, O'Driscoll *et al.*, 2006). Consequently, 10 min after phage addition was chosen as a representative time-point of an early stage of infection to determine the response of *L. lactis* IL1403 to attack by phage c2. In order to obtain an accurate and homogeneous gene expression profile of the host response, we deemed it necessary to use infection conditions ensuring complete lysis of the cell population within 40 min, which is the estimated time for phage c2 to complete its lytic life (Garvey *et al.*, 1995, Garvey *et al.*, 1996, O'Driscoll *et al.*, 2006). A number of phage-host challenge tests were performed, and the MOI required to achieve complete lysis within this time frame was determined to be 800 (Figure 1).

Analysis of the microarray data (Figures 2 and 3) of two independent biological replicates revealed that *L. lactis* IL1403 differentially regulated the expression of 61 genes in response to phage c2 challenge (Table 2), with an even distribution between up- and down-regulated genes. No differential expression of phage c2-related genes was observed, which is indicative that the chosen sampling time reflects a stage prior to phage DNA replication. Almost one third of the regulated genes (19 out of 61) and most of the positively induced genes (17 out of 32) encoded products annotated as hypothetical proteins. The 61 regulated genes belonged to 14 functional categories, mostly representing components of the cell envelope (12 genes), regulatory functions (11 genes), and carbohydrate metabolism (7 genes), which accounted for half of the regulated genes.

L. lactis IL1403 increases expression of genes involved in D-alanylation of cell wall (lipo-)teichoic acids in response to phage infection

Half of the genes regulated by *L. lactis* IL1403 in response to phage attack encode proteins whose putative functions are involved to different extents in biogenesis, modification and regulatory processes of the cell envelope. This finding suggests that the phage presence is mainly sensed as an extra-cytoplasmic stress by the cell. Among these, *L. lactis* IL1403 upregulated three genes (*ddl*, *dltA* and *dltD*) involved in the peptidoglycan biosynthesis pathway. *Ddl* encodes the enzyme D-alanine-D-alanine ligase, which converts D-alanine to its dimer in a reversible reaction involved in both D-alanine metabolism and cell wall biosynthesis. *DltA* and *dltD* belong to the four-gene operon *dltABCD* where they overlap with *dltB* and *dltC* genes, respectively. This is indicative of transcriptional coupling and, indeed, RT-PCR confirmed that *dltB* and *dltC* were up-regulated at levels similar to *dltA* and *dltD* during phage attack (Table 3). Induction of the *dlt* operon leads to increased incorporation of D-alanine esters (D-alanylation) into cell wall teichoic acids (WTAs) and lipoteichoic acids (LTAs) in gram-positive bacteria (Koprivnjak *et al.*, 2006, Neuhaus and Baddiley, 2003). Universally present in these organisms, these phosphate-rich glycopolymers play crucial roles in protecting, connecting and controlling the major envelope constituents, and in mediating host-cell adhesion, inflammation and immune response activation (Weidenmaier and Peschel, 2008). WTAs and LTAs contain D-alanine esters as their main backbone substitutions and the extent of this modification is known to exert profound modulatory effects on cell physiology and host-mediated responses, including a protective function against UV and acid stress (Boyd *et al.*, 2000, Duwat *et al.*, 1997), autolysins (Steen *et al.*, 2005), cationic antimicrobials (Kristian *et al.*, 2005), and phage attack (Raisanen *et al.*, 2007).

As regards this latter function, LTAs have been suggested to serve as phage receptors in *L. lactis* (Spinelli *et al.*, 2006, Tremblay *et al.*, 2006) and in many other organisms including *Listeria, Bacillus subtilis, Staphylococcus aureus*, and *Lactobacillus* (Chatterjee, 1969, Givan *et al.*, 1982, Raisanen *et al.*, 2004, Wendlinger *et al.*, 1996). There is increasing evidence to suggest that the degree of LTA substitutions deeply affects phage binding to bacterial cells, either when the receptor is LTA or a different molecule. Phage-resistant strains of *Lb. delbrueckii* subsp. *lactis*

were found to contain higher levels of D-alanine esters in their LTAs compared to phage-sensitive ones (Raisanen *et al.*, 2007), whereas galactosyl-modified LTAs were suggested to prevent phage adsorption to a *L. lactis* subsp. *cremoris* strain by steric shielding of the actual receptor site (Sijtsma *et al.*, 1990). Lactococcal bacteriophages are known to use cell wall carbohydrates containing rhamnose, glucose or galactose moieties as primary receptors for adsorption (Valyasevi *et al.*, 1991, 1994), and for some, including phage c2, a requirement of a second irreversible binding step to certain membrane proteins has been demonstrated (Monteville *et al.*, 1994, Valyasevi *et al.*, 1991). However, the nature of the primary receptor used by phage c2 remains still unclear. This phage was found to adsorb to rhamnose moieties on the cell wall of *L. lactis* subsp. *lactis* C2 (Monteville *et al.*, 1994), and a similar receptor may be used to infect other related strains such as *L. lactis* IL1403. Rhamnose is also a component of LTAs that have been recently proposed as receptor candidates for other lactococcal phages of the 936 species (Spinelli *et al.*, 2006, Tremblay *et al.*, 2006). Based on the above observations, the up-regulation of the *dlt* operon in *L. lactis* IL1403 in response to phage c2 infection adds more weight to the suggestion of a role for LTAs as receptors for this phage. Indeed, it is tempting to speculate that increased D-alanylation of LTAs may represent the host attempt to evade phage infection. This could be achieved via two different mechanisms: D-alanine esters may sterically impede the already adsorbed phage to access and irreversibly bind the membrane protein, or they may affect the initial (and reversible) adsorption of other phages to the cell surface by modifying the primary receptor. This strategy would resemble an adsorption blocking mechanism and could be very effective at controlling phage infection by rendering the cell impervious to the phage DNA and leaving the cell and its biochemical activities unaltered (Emond and Moineau, 2007). While proving ineffective for most cells of a sensitive host since phage infection would proceed regardless, such mechanism could allow the survival of a small percent of the cell population when facing *in vivo* low titer-phage attacks.

L. lactis IL1403 also regulated the expression of other genes indirectly related to TAs. Repression of the gene *malQ* encoding for the enzyme amylomaltase would switch maltose utilization towards production of D-glucose-1-phosphate, which is the substrate for the activity of the β-phosphoglucomutase (β-Pgm) encoded by the up-regulated gene *pgmB*. β-Pgm catalyzes the reversible transformation of D-glucose-6-

phosphate to D-glucose-1-phosphate, which is required for the synthesis of uridine diphosphate-glucose, a precursor of TAs (Weidenmaier and Peschel, 2008) (Levander *et al.*, 2001). In tune with the up-regulation of the *ddl* and *dlt* genes, this would point to a bacterial activity directed towards strengthening and modifying these surface glycopolymers. Additionally, the strongest up-regulated gene (*yndF*) in *L. lactis* IL1403 encodes a protein that is likely to interact or to be regulated by the *dlt* genes. YndF is a large protein (653 aa) containing the highly conserved LPXTGE motif (PF00746), common to many Gram-positive surface proteins (Cox *et al.*, 2009). This cell-wall anchor domain is shared by Internalins of *Listeria monocytogenes* and M proteins of *Streptococcus pyogenes*, which play a key role in host adhesion and subsequent colonization by these bacteria (Courtney *et al.*, 2002, Sabet *et al.*, 2005). Interestingly, the expression of the M protein was recently observed to be modulated by the D-alanylation pathway in a defective *dltA* mutant of *S. pyogenes* exhibiting reduced virulence (Cox *et al.*, 2009).

L. lactis IL1403 also induced at high levels a number of genes encoding hypothetical proteins with predicted localization on the cytoplasmic membrane. For some gene products, the presence of conserved domains has allowed for speculation regarding their putative function. These included the YeeG and YiaI proteins that are homologous to the LytR family (PF03816) of membrane-bound transcriptional attenuators involved in peptidoglycan catabolism. In *Staphylococcus aureus*, *lytR* modulates a dicistronic operon that is thought to control peptidoglycan hydrolase activity (Brunskill and Bayles, 1996), and was also found to be part of the cell wall stress stimulon induced by a number of antibiotics (Utaida *et al.*, 2003). In contrast, FloL is a large protein (503 aa) that is predicted to be located in the cytoplasmic membrane and to contain a flotillin-like domain (cd03399). A member of flotillins, HlfC (High frequency of lysogenization C) from *Escherichia coli*, is known to play a role in the decision between lysogenic and lytic cycle growth during lambda phage infection (Babuke and Tikkanen, 2007). If FloL plays a similar role in lactococcal phage infection, its repression by *L. lactis* IL1403 might be explained by the cell attempting to deprive phage c2 of a putative host determinant of lysis.

L. lactis IL1403 restores the physiological PMF altered by phage infection

In any bacterial cell, the electrochemical gradient of protons across the cytoplasmic membrane, also termed PMF, is a major store of free energy. A membrane potential ($\Delta\Psi$) and a pH gradient (ΔpH) are the two main components of the PMF, which are usually generated by the activity of primary ion pumps (e.g., F_0F_1-ATPase) and of secondary transporters (e.g., the citrate/lactate exchanger) (Lolkema *et al.*, 1995). In response to the phage stimuli, *L. lactis* IL1403 was found to repress the expression of *yshC* and *citEF* genes, which are actively involved in the primary and secondary mechanisms of PMF generation, respectively. The *yshC* gene encodes a manganese-dependent inorganic pyrophosphatase that plays a key role in the oxidative phosphorylation pathway and has been shown to be essential to *E. coli* growth (Chen *et al.*, 1990). Its activity removes excess pyrophosphate, released in the cell by numerous metabolic pathways, thus providing the phosphate necessary to the F_0F_1-ATPase to generate ATP while driving an influx of protons through the cell membrane. The down-regulation of this pyrophosphatase activity would limit the availability of inorganic phosphate in the cell and move the equilibrium of the reaction catalyzed by F_0F_1-ATPase towards ATP hydrolysis and consequent use of the energy released to pump protons out of the cell against their thermodynamic gradient. The *citEF* genes encode the beta and alpha chain, respectively, of the three-subunit enzyme citrate lyase that catalyzes the first step of the citrolactic fermentation pathway leading to the intracellular conversion of citrate to lactate. In *L. lactis*, this pathway is known to generate metabolic energy by a secondary mechanism through the creation of a PMF that is sufficiently high to drive ATP synthesis via the F_0F_1-ATPase (Hugenholtz *et al.*, 1993). The citrate enzymes generate a ΔpH across the cell membrane as they consume a cytosolic proton during citrate decarboxylation to lactate, whereas the secondary transporter generates a $\Delta\Psi$ of physiological polarity by coupling the uptake of divalent citrate to the exit of monovalent lactate. Consequently, the net result of the down-regulation of *yshC* and *citEF* genes expression would be a collapse of the PMF in *L. lactis* IL1403 as a result of the abolished generation of the $\Delta\Psi$ and ΔpH generally associated with the F_0F_1-ATPase activity and the citrolactic fermentation pathway. Phage-induced differential regulation of other genes (*ahrC* and *argCJD*) in *L. lactis* IL1403 concurred to confirm this hypothesis. Arginine metabolism in *L. lactis* is usually modulated by

ahrC towards repression of the biosynthesis pathway and activation of its catabolism. This leads to ammonia production which is proposed to maintain pH homeostasis in *L. lactis* probably via neutralization of intracellular low pH values (Larsen *et al.*, 2004, Larsen *et al.*, 2008). In response to the phage stimuli, *L. lactis* IL1403 was observed to repress *ahrC* and induce the biosynthetic genes *argCJD*, potentially leading to accumulation of intracellular arginine. This is likely to be consequent to the F_0F_1-ATPase-mediated export of protons that, by leaving a more alkaline intracellular pH, would generate no need for ammonia neutralization.

Why would the cell affect the PMF and switch to anaerobic growth in response to phage adsorption? In *E. coli*, adsorption of phages T4 and T5 has been shown to induce the opening of a channel in the cell membrane, which mediates the transfer of phage DNA into the host. The open conformation of the channel triggers a strong efflux of potassium and calcium ions that, in turn, induces a partial and transient depolarization of the host membrane (Boulanger and Letellier, 1988, Labedan and Letellier, 1981). The infected bacteria respond by a repolarization that leads to a new steady state of reduced $\Delta\Psi$ (Labedan and Letellier, 1981). In *L. lactis* IL1403, the changes mediated by the repression of *yshC* and *citEF* genes would lead to a hyperpolarization of the membrane, which is consistent with the repolarization phenomenon observed in *E. coli* cells following phage adsorption. This observation prompted us to investigate putative changes in the $\Delta\Psi$ of *L. lactis* IL1403 cells following adsorption of phage c2 by using flow cytometry and the $\Delta\Psi$-sensitive dye rhodamine 123. This fluorochrome tends to accumulate on the inner side of energized membranes (i.e. cells maintaining an active $\Delta\Psi$) and, consequently, its intracellular fluorescence intensity decreases following membrane depolarization. Staining of exponentially growing bacterial cells with rhodamine 123 resulted in active accumulation of the dye at fluorescence levels averaging around 10^4 arbitrary units (Figure 4a). A log decrease in the average intracellular fluorescence was observed 10 min after the addition of phage c2, which was indicative of a depolarization of the membrane and consequent efflux of the dye out of the cells (Figure 4b). A similar collapse of the $\Delta\Psi$ was observed when rhodamine 123-stained cells were incubated in the presence of the uncoupler CCCP for 10 min (data not shown). A gradual reuptake of fluorochrome up to pre-infectious levels was then observed between 15 and 25 min within the infection process (Figure 4c,d), which suggested that bacteria had regained

an energized membrane thus allowing a reuptake of fluorochrome into the cells. These data are indicative of an initial dissipation of the ΔΨ followed by a gradual restoration of its physiological level, which is consistent with previous findings in *E. coli* (Labedan and Letellier, 1981, Letellier and Labedan, 1985). They also suggest that, regardless of the phage-host system, a strong depolarization of the host membrane is a common effect induced by phage adsorption. In this context, our transcriptional data appear to provide insights into the strategy adopted by the cell to obtain a rapid repolarization of the membrane. By regulating the expression of *yshC* and *citEF* genes, *L. lactis* IL1403 may simultaneously modulate both components of the PMF, thus restoring the physiological pre-infectious condition in the shortest timeframe possible. Increased respiratory rate and proton influx were shown to be related to the transient depolarization of the membrane in *E. coli* cells following adsorption of phages T4 and T5 (Letellier and Labedan, 1985). Consequently, the "appropriate" regulation of genes involved in the same pathways seems to be the most logical route for the cell to reverse the phage-induced effect.

L. lactis IL1403 appears to switch to anaerobiosis and to an energy-saving state to sustain the PMF and the overall response to the phage-induced stress

Transcriptional analyses showed that phage attack triggered the down-regulation of bacterial genes involved in the metabolism of lipids (*pflA*, *fabG* and *yebB*), carbohydrates (*enoB* and *adhE*), cofactors (*coaA*), proteins and amino acids (*pepXP*, *yeiG*, *yteB*), and in DNA replication (*ruvB*, *dnaC*, *ligA*), transcription (*rheB*, *nusG*), and translation (*gatA*). Expression of genes (*ysxL* and *yphL*) encoding for GTP-ases was also repressed. The activity of these DNA binding proteins is modulated by the GTP pool in the cell and has been recently demonstrated to link various aspects of the cell cycle and metabolism with translation by playing key roles in the assembly of ribosomes in bacteria (Britton, 2009). The results outlined above depict a scenario where *L. lactis* IL1403 is globally reducing growth- and development-promoting activities, to which it generally commits considerable amounts of energy and that are not needed for coping with the phage-induced stress. The biogenesis of lipid membrane is an example of such energy-consuming cellular functions, and notably *L. lactis* IL1403 repressed three genes (*pflA*, *fabG* and *yebB*) associated with this pathway. The *pflA* gene product is required to activate the pyruvate formate-lyase

enzyme, which catalyzes the non-oxidative cleavage of pyruvate to formate and acetyl-CoA (Conradt *et al.*, 1984). This latter metabolite is a central intermediate of the fatty acid biosynthesis pathway, and consequently repression of *pflA* exerts an overall rate-limiting action on lipid biogenesis. *FabG* is an essential gene belonging to the multi-gene *fab* cluster involved in fatty acid biosynthesis in bacteria. It encodes a 3-ketoacyl-acyl carrier protein reductase that is the first enzyme participating in each cycle of chain elongation, and its essential nature is demonstrated by the fact that fatty acid biosynthesis and cell growth are arrested in *E. coli* following abolishment of *fabG* transcription (Zhang and Cronan, 1998). Conversely, the *yebB* gene is considered nonessential for viability in *E. coli* but encodes a key enzyme in the biosynthesis of isoprenoids. These compounds serve numerous essential functions in eukaryotic and prokaryotic bacteria including structural components of membranes, mediation of cellular redox chemistry, message relay during signal transduction, and sugar transport during glycoprotein biosynthesis (Hahn *et al.*, 1999). In tune with this, *L. lactis* IL1403 also down-regulated other genes (*ywaF* and *pmi*) associated with the synthesis of the cell wall. YwaF belongs to the ubiquitous family of glycosyltransferases (PF00534), which are involved in cell envelope biogenesis and function by transferring activated sugars to an acceptor molecule localized at the outer surface of the cell membrane. Regulation of *ywaF* has been previously reported in *L. lactis* cultures following treatment with lacticin 972 (Martinez *et al.*, 2007). Mannose-6-phosphate isomerase (Pmi) catalyzes the interconversion of mannose 6-phosphate and fructose 6-phosphate, which is the first step in the synthesis of mannose-containing sugar chains. The role of Pmi activity has not been characterized so far in lactic acid bacteria, but studies in other bacterial species would suggest a central regulatory role in both cell wall synthesis and energy production (Lerner *et al.*, 2009).

While repressing energy-consuming functions, *L. lactis* IL1403 was also found to induce expression of the *nrdDG* genes and concomitantly to repress that of *nrdE*. The operons *nrdEF* and *nrdDG* of *L. lactis* encode ribonucleotide reductases that are activated during conditions of aerobiosis and anaerobiosis, respectively. These enzymes catalyze the reduction of ribonucleoside di- or triphosphates, thereby providing the building blocks required for DNA replication and repair. During anaerobic growth, *L. lactis* switches on the *nrdDG* operon and relies on the ability of

NrdG to generate the glycyl radical crucial to the reducing activity of NrdD (Jordan *et al.*, 1996, Torrents *et al.*, 2000). Repression of the aerobic ribonucleotide reductase (*nrdEF*) and induction of the anaerobic enzyme (*nrdDG*) would therefore suggest a switch to growth conditions resembling anaerobic fermentation in *L. lactis* IL1403. When integrated into the overall picture of cell wall D-alanylation, PMF restoration and energy conservation, the switch to anaerobic-like growth conditions may be best explained by the bacterium needing to recover an energetic state favourable to sustain the PMF and the overall response to the phage stress. In this scenario, up-regulation of *ndrH* by *L. lactis* IL1403 is not fully understood. NrdH belongs to the NrdH-redoxin family (cd02976) of proteins and is characterized by a glutaredoxin-like sequence and thioredoxin-like activity profile. It is part of the *nrdEF* operon and usually functions as an electron carrier for the aerobic reductase system, which was found to be repressed in this study. Nonetheless, *ndrH* induction in *L. lactis* IL1403 following phage attack might have a different purpose as glutaredoxins/thioredoxins are also known to be involved as alternative pathways in cellular functions such as signal transduction and defence against oxidative stress (Fernandes and Holmgren, 2004).

L. lactis IL1403 overall response to phage infection is probably regulated by a complex cell-envelope stress regulon.

The overall response of *L. lactis* IL1403 to phage infection is likely to be orchestrated by a complex regulatory network involving the products of the strongly up-regulated genes *ythB* (+7.12), *ythC* (+4.10), *yneH* (+7.50), *kinD* (+4.80) and *llrD* (+5.90) (Tables 2 and 3). YthB (67 aa) is identical to a family of conserved membrane proteins acting as stress-responsive transcriptional regulators (PF04024), which includes the Phage shock protein C (PspC) from *E. coli* and *Yersinia enterocolitica*. Topology analysis predicted YthB to be an integral membrane protein, with the N-terminus inserted into the lipid layer and the C-terminus to form a trans-membrane helix with an in/out orientation relative to the membrane (Figure 5c). The last seven C-terminal residues are predicted to project out of the membrane which suggests that they could be involved in stress signal transduction. YthC (371 aa) is probably a cytoplasmic protein, as no membrane-spanning domains were predicted in its sequence, and shares homology to a family of conserved proteins of unknown

function (COG3595). Sequence analysis of the regions surrounding *ythB* and *ythC* revealed a three gene operon-like structure where *ythC* precedes the genes *ythB* and *ythA*. Similarly to YthB, YthA (154 aa) contains a PspC-like domain that, in this case, entails only the N-terminal region (0 to 75 aa) of the protein and is predicted to be located inside the membrane (Figure 5b). The localization of the C-terminus is uncertain as it is predicted to reside inside the membrane by the TOPCONS model but outside by the TMHMM model. The *ythCBA* genes are likely to be transcribed as a single polycistronic unit as computational analysis retrieved promoter sequences for efficient gene expression only upstream of *ythC* as well as a strong *rho*-independent terminator signal ($\Delta G = -7.1$) downstream of *ythA* (Figure 5a).

The Psp system is thought to protect the cell from a variety of extracytoplasmic stressors affecting membrane integrity and causing dissipation of the PMF and a likely change in the cell's redox state (Darwin, 2005, Joly *et al*., 2010). Interestingly, recent studies in *E. coli* identified the Psp system as being involved in regulating the response to phage-induced stress (Jovanovic *et al*., 2006, Poranen *et al.*, 2006). Temporal gene expression analysis of cells during infection with the lytic phage PRD1 revealed up-regulation of genes involved in anaerobic respiration early in the infection process (i.e. 5 to 15 min) and induction of the *psp* system throughout the whole infection process, with a peak of expression observed coincident with the production of PRD1 holin protein (Poranen *et al*., 2006). A *psp* system response was also shown to be induced in *E. coli* by a homologue of protein IV from filamentous phage and to be directed to adjust and maintain energy usage and PMF by switching cell metabolism to anaerobic respiration and by sustaining $\Delta\Psi$ and ΔpH, respectively (Jovanovic *et al.*, 2006). These results are strikingly similar to those obtained in our study and suggest that both Gram-positive and Gram-negative bacteria use a common mechanism to counteract any stress affecting cell envelope integrity. In *L. lactis*, *psp* homologues have been indeed found to be strongly induced in a nisin-resistant strain (Kramer *et al.*, 2006), and following exposure to lactococcin 972 (Lcn972), a bacteriocin that inhibits cell wall synthesis (Martinez *et al.*, 2007). Perturbations of the host membrane and of the associated PMF, which may be caused by the activity of pore-forming agents (holins, protein IV, or bacteriocins) or simply by the opening of a channel for phage DNA delivery (as in our study), induce up-regulation of the Psp regulon. This system activates a genetic reprogramming of the host cell aimed at

restoring membrane integrity and a physiological PMF by modulating the expression of genes affecting both PMF components and switching to anaerobiosis to save energy and maintain the new steady state.

The *psp* regulon has a diverse organization in different Gram-negative bacteria, but only four genes (*pspFABC*) are always conserved and therefore suggested to constitute the minimal functional response system (Darwin, 2005). The *pspF* gene is transcribed from its own promoter in opposite direction to the *pspABC* operon. A basic working model of the Psp response implies that the stress signal is sensed by the membrane proteins PspB and/or PspC, and then relayed to the PspAF complex that serves an effector role of the Psp response. In *L. lactis* IL1403, three genes are organized in a putative operon (*ythCBA*) somewhat resembling the organization and function of the *pspABC* genes. YthB and YthA are indeed stress-responsive PspC proteins with predicted localization on the membrane, whereas no function is known for the cytoplasmic protein YthC. However, no homologues of the *pspAF* genes are found upstream of the *ythCBA* operon. This suggests that *L. lactis* may have evolved a novel organization of this responsive system, where cell envelope stress is still sensed by a PspC-like protein (YthB), but then relayed to a different effector system than PspAF.

It is tempting to speculate that this effector might correspond to the product of the *spxB* (formerly *yneH*) gene, whose expression was among the most highly induced in *L. lactis* IL1403 following phage infection. SpxB is one of seven paralogs in *L. lactis* with homology to the highly conserved Spx proteins, known to act as global transcriptional regulators in many Gram-positive species (Nakano *et al.*, 2003b). Spx homologues have been shown to fulfil an important role in growth, general stress protection, and biofilm formation in *S. aureus* (Pamp *et al.*, 2006), to regulate the global response to oxidative stress in *Bacillus subtilis* (Nakano *et al.*, 2003a), and to mediate the cell-wall stress response in *L. lactis*. In the latter organism, *SpxB* was found to be essential in mediating a multistep response that increases peptidoglycan resistance to lysozyme hydrolysis (Veiga *et al.*, 2007), and to be strongly up-regulated in response to the cell wall-targeting antimicrobial peptides nisin (Kramer *et al.*, 2006) and Lcn972 (Martinez *et al.*, 2007). Spx proteins possess a bivalent regulatory activity that is suggested to be essential for protection and maintenance of the general well-being of bacterial cells under stressful conditions.

They would act as negative regulators to postpone energy-consuming development and growth-related functions, while using their activator capacities to mobilize the operations necessary to reverse the stress-induced effects (Nakano *et al.*, 2003a, Nakano *et al.*, 2003b). This suggests that SpxB might be involved in coordinating the overall transcriptional response of *L. lactis* IL1403 to the phage stimuli as observed in our study, which would include activities related to cell-wall D-alanylation, PMF maintenance and energy conservation.

SpxB might be therefore the effector protein to which YthB relays the stress signal. This could be achieved via direct interaction of YthB with SpxB or, alternatively, via the two-component system CesSR, whose genes were also found to be highly induced in *L. lactis* IL1403 following phage exposure (Tables 2 and 3). The CesSR system has been associated with cell envelope stress response in *L. lactis* and, interestingly, it seems to control the expression of both *spxB* and *psp* genes via the regulator CesR (Martinez *et al.*, 2007, Veiga *et al.*, 2007). Based on these findings, it may be speculated that the stress sensor YthB, the regulator CesR and the effector SpxB might be part of the same signal transduction pathway leading to activation of a cell-wall stressosome in *L. lactis* IL1403 (Figure 6). If this is the case, it is not clear from our data whether YthB relays the signal directly to CesR or via the histidine kinase CesS.

CONCLUSIONS

In this study, we have used a transcriptomic approach to analyze the holistic response of *L. lactis* IL1403 at the onset of a phage challenge and to determine any putative strategy adopted by the host to avoid phage infection. To our knowledge, our results describe for the first time in *L. lactis* the molecular mechanisms involved in the host response to the membrane perturbations mediated by phage infection. The identity of the regulated genes revealed that *L. lactis* IL1403 engages in a strong cell wall-targeted response to phage attack while concomitantly repressing energy-consuming functions related to development and growth. The phage presence is clearly sensed as an extra-cytoplasmic stress and, based on our findings, we propose a putative working model of the cell envelope stress response consequently mounted by *L. lactis* IL1403 (Figure 6). The phage-mediated stress would be sensed by the PspC-like protein YthB, which then relays the stress signal to the effector protein SpxB presumably via the two-component system CesSR or via direct interaction. Upon induction, SpxB would use its global regulator capacities to increase the D-alanine content of cell-wall TAs, to restore a physiological PMF and to activate an energy-saving programme by postponing growth-related functions and switching cell metabolism to anaerobiosis in order to sustain the PMF and the overall response to the phage stress.

In this model, SpxB would play a leading effector role in the bacterial response, which is deduced from the pleiotropic functions reported for this regulator in various organisms (Martinez *et al.*, 2007, Nakano *et al.*, 2003a, Nakano *et al.*, 2003b, Pamp *et al.*, 2006, Veiga *et al.*, 2007). However, it is interesting to note that in our study phage attack stimulated activation of Psp homologues at levels similar to SpxB, and also that these proteins might be expected to control and regulate specific responses (i.e. D-alanylation of LTAs and energy conservation by SpxB; PMF restoration by Psp proteins). Determining the putative crossover or separation of the regulatory activities of these proteins is of great interest and will be the subject of future investigations. Our knowledge to date shows that CesSR seems to control the expression of both *spxB* and *psp* genes in *L. lactis* (Martinez *et al.*, 2007, Veiga *et al.*, 2007), thus suggesting that SpxB and the Psp proteins may actually interact and be part of a common strategy (the CesSR stimulon) aiming at protecting *L. lactis* from cell envelope stresses. Considering the nature of the different stressors (lysozyme,

antimicrobial peptides, phages) stimulating its activation in *L. lactis*, the CesSR stimulon probably represents a general defence mechanism committed to preserve not only cell wall integrity but, ultimately, cell viability. While much is known about this system in Gram-negative bacteria, very little data is available for Gram-positive species. As such, we anticipate further investigations to improve our understanding of this vital defence system in *L. lactis* and in Gram-positive bacteria, in general.

Another interesting finding of our study is that phage attack could trigger an increased incorporation of D-alanine into the bacterium cell-wall TAs, thus perhaps rendering cells more resistant to phage infection. Although the outcome is still a successful phage infection, we speculate that this strategy is likely to allow the survival of a small percentage of the cell population when facing *in vivo* low titer-phage attacks. The survivors may have retained memory of the strategy adopted during previous attacks and have gradually generated this sophisticated response. Theoretically, the efficiency of this strategy could be enhanced through genetic manipulation by over-expression of the *dlt* operon. However, findings from a recent study seem to negate this hypothesis. In *Lb. delbrueckii*, phage inactivation by D-alanyl-substituted LTAs was found indeed to be less effective when high levels of D-alanine residues were present in the LTA backbone (Raisanen *et al.*, 2007). Considering that this result may be related to the specific phage-host system examined, the application of such a genetic approach in *L. lactis* may still prove successful.

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TABLES

Table 1. Primers used in this study for qRT-PCR validation of microarray results

Gene	Primer	Sequence 5' - 3'	Position	Size (bp)
ddl	forward	GTTTTTGAAGCGGTTGGTGT	400-419	187
	reverse	TTAGCGCAGGTTGAAGGTCT	586-567	
dltA	forward	AAGCGATTAATGCGGTGACTT	198-218	283
	reverse	GGTGATTCGGTAGCCAAAGGT	480-460	
dltB	forward	AGAAGCTTGGGTGTATTGTTGC	555-576	161
	reverse	ACTAAAAGCGCCAGTTGTCAC	715-695	
dltC	forward	GAACGCGTGCTGCTATTTTAT	24-44	163
	reverse	CGTGAAGACTTAGACATGGATTTAT	186-162	
dltD	forward	GGGTGGTGGTATTTTCTTTAGGA	783-805	228
	reverse	GTTTTACGCCATTCTTAGTAGGAC	1010-987	
nrdG	forward	GTGCGCTGCTCACTTTATGTG	97-117	398
	reverse	AATCCGCTGATTTGATGACCC	495-475	
yhbF	forward	CTACAATCGGGCGGTTAGAGC	195-215	394
	reverse	TTTCAAATTGCCATTTTCATCAG	588-566	
yndF	forward	CAGTCGTTGCAAGCCCTAAAGT	1530-1551	383
	reverse	AACAAACAGCACCAACAAGTCAAA	1835-1812	
yneH	forward	GGAGACGCCAAGGAATAAAGG	6-23	154
	reverse	GGAATCAAAAGCACCCAAACT	198-179	
ythB	forward	GTGTGTCATCAATAGACTCGCA	23-46	145
	reverse	GGACTTGGTGAATACTTTGGCT	167-151	
ythC	forward	AATATTTCCGTTTACGAGTGAGACA	286-310	185
	reverse	TTATGAACGGAAATGCTGAAGTG	470-448	
kinD	forward	GGATTACTTGCGCTGATTTTTACCC	178-202	395
	reverse	AAGAGTGCTCGCATTTCATTTTGTG	572-548	
llrD	forward	CCCGATATCGAAGTGGTTGG	79-98	256
	reverse	CAGCGGCTTGTGATGTTTTA	334-315	
yhbE	forward	GAACGTTCAGTAGGCAGTTTCTTA	172-195	129
	reverse	AGGTTCGGCGATTTCTTTTT	300-283	
ywaF	forward	GTGACAGCTGCCCTTTATTTTA	493-514	215
	reverse	GCAGATGACATGGCTCCTTC	707-688	
yveF	forward	TTTTCTCCCTTCTGCTCACTCAAT	17-40	138
	reverse	CCCCGCTCGAAAATGGTATC	154-135	
alaS	forward	AGTAAGCGACGTAAAACATAACCAC	1691-1715	207
	reverse	TCAAGGCGGAAAAACAAACT	1897-1878	

Table 2.

Functional category / Pathway[a]	Gene	Expression ratio (n-fold)	Gene product / Putative function[b]	Subcellular localization[c]
Carbohydrate metabolism				
Citrate (TCA) cycle	*citE*	-2.25	Citrate lyase beta chain (EC:4.1.3.6)	Cytoplasmic
Citrate (TCA) cycle	*citF*	-2.28	Citrate lyase alpha chain (EC:4.1.3.6)	Cytoplasmic
Glycolysis/Gluconeogenesis	*enoB*	-2.26	Phosphopyruvate hydratase (EC:4.2.1.11)	Cytoplasmic
Glycolysis/Gluconeogenesis	*adhE*	-2.35	Acetaldehyde-CoA / alcohol dehydrogenase (EC:1.2.1.10)	Cytoplasmic
Starch and sucrose metabolism	*malQ*	-2.18	4-alpha-glucanotransferase (EC:2.4.1.25)	Cytoplasmic
Amino sugar and nucleotide sugar metabolism	*pmi*	-2.05	Mannose-6-phosphate isomerase (EC:5.3.1.8)	Cytoplasmic
Starch and sucrose metabolism	*pgmB*	2.08	Beta-phosphoglucomutase (EC:5.4.2.6)	Cytoplasmic
Cell envelope				
Surface (lipo-)polysaccharides and antigens	*yndF*	3.16	Hypothetical protein	Cell wall
Surface (lipo-)polysaccharides and antigens	*floL*	-2.72	Flotillin-like protein	Cytoplasmic membrane
Surface (lipo-)polysaccharides and antigens	*ywaF*	-2.34	Glycosyltransferase	Cytoplasmic
Peptidoglycan biosynthesis	*dltA*	2.11	D-alanine-poly(phosphoribitol) ligase (EC:6.1.1.13)	Cytoplasmic
Peptidoglycan biosynthesis	*dltD*	2.11	D-alanine transfer protein	Unknown
Peptidoglycan biosynthesis	*ddl*	2.18	D-alanine-D-alanine ligase (EC:6.3.2.4)	Cytoplasmic
ABC transporters	*optS*	-2.13	Oligopeptide ABC transporter substrate binding protein	Cell wall
Unknown	*yfbG*	2.09	Hypothetical protein	Cytoplasmic membrane
Unknown	*ybfC*	2.08	Hypothetical protein	Cytoplasmic membrane
Unknown	*yghD*	2.54	Hypothetical protein	Cytoplasmic membrane
Unknown	*ythB*	2.46	Putative stress response protein, PspC-like	Cytoplasmic membrane
Unknown	*ythC*	2.83	Hypothetical protein	Cytoplasmic
Energy Metabolism				
Oxidative phosphorylation	*yshC*	-2.51	Putative Mn-dependent inorganic pyrophosphatase (EC:3.6.1.1)	Cytoplasmic
Electron transport	*ndrH*	2.18	Glutaredoxin-like protein NrdH	Cytoplasmic
Lipid Metabolism				
Fatty acid biosynthesis	*fabG1*	-2.23	3-oxoacyl-acyl carrier protein reductase (EC:1.1.1.100)	Cytoplasmic
Fatty acid biosynthesis	*acpD*	2.04	Acyl carrier protein phosphodiesterase [EC:3.1.4.14]	Unknown
Pyruvate metabolism	*pflA*	-2.29	Pyruvate-formate lyase activating enzyme (EC:1.97.1.4)	Cytoplasmic
Terpenoid backbone biosynthesis	*yebB*	-2.00	Isopentenyl-diphosphate delta-isomerase (EC:5.3.3.2)	Cytoplasmic
Metabolism of Cofactors and Vitamins				
Pantothenate and CoA biosynthesis	*coaA*	-2.55	Pantothenate kinase (EC:2.7.1.33)	Cytoplasmic
Riboflavin metabolism	*ribC*	2.29	Riboflavin kinase (EC:2.7.1.26	Cytoplasmic
Protein and Amino Acid metabolism				
Amino acid transport and metabolism	*yteB*	-2.54	Putative D-amino acid oxidase protein	Cytoplasmic
Amino acid transport and metabolism	*yeiG*	-2.67	Aminotransferase (EC:2.6.1.-)	Cytoplasmic
Arginine and proline metabolism	*argC*	2.27	N-acetyl-γ-glutamyl-phosphate reductase (EC:1.2.1.38)	Unknown
Arginine and proline metabolism	*argJ*	2.06	Ornithine acetyltransferase (EC:2.3.1.35)	Cytoplasmic
Arginine and proline metabolism	*argD*	2.22	acetylornithine aminotransferase (EC:2.6.1.11)	Cytoplasmic
Degradation of proteins and (glyco-)peptides	*pepXP*	-2.10	X-prolyl dipeptidyl aminopeptidase (EC:3.4.14.11)	Cytoplasmic
Purines, Pyrimidines, Nucleosides and Nucleotides				
Purine metabolism; Pyrimidine metabolism	*nrdE*	-2.29	Ribonucleoside-diphosphate reductase alpha chain (EC:1.17.4.1)	Unknown
Purine metabolism; Pyrimidine metabolism	*nrdD*	2.65	anaerobic ribonucleoside triphosphate reductase (EC:1.17.4.2)	Cytoplasmic
Purine metabolism; Pyrimidine metabolism	*nrdG*	2.87	Anaerobic ribonucleoside-triphosphate reductase activating protein (EC:1.97.1.4)	Cytoplasmic
Regulatory functions				
Transcription factors	*rliA*	-2.85	Transcription regulator, LacI family	Cytoplasmic
Transcription factors	*yddC*	2.23	Putative transcription regulator	Cytoplasmic
Transcription factors	*yveF*	2.24	Putative PadR family transcriptional regulator	Cytoplasmic
GTP-binding proteins	*ysxL*	-2.17	GTPase EngB	Cytoplasmic
GTP-binding proteins	*yphL*	-2.04	GTP-binding protein EngA	Cytoplasmic membrane
GTP-binding proteins	*yyaL*	2.10	GTP-dependent nucleic acid-binding protein	Cytoplasmic
General	*yeeG*	-2.28	Transcription regulator	Cytoplasmic membrane
General	*ahrC*	-2.04	Transcription regulator of arginine metabolism	Cytoplasmic
General	*yjaI*	2.27	Hypothetical protein	Unknown
Unknown	*yneH*	2.72	Putative transcription regulator	Unknown
Two-component systems	*kinD*	2.11	Sensor protein kinase KinD	Cytoplasmic membrane
Replication and Repair				
DNA replication; Nucleotide excision repair	*ligA*	-2.45	NAD-dependent DNA ligase (EC:6.5.1.2)	Cytoplasmic
DNA replication	*dnaC*	-2.05	Replicative DNA helicase (EC:3.6.1.-)	Cytoplasmic
DNA repair	*ywaC*	2.81	Hypothetical protein	Cytoplasmic
Homologous recombination	*ruvB*	-2.26	DNA helicase RuvB (EC:3.1.22.4)	Cytoplasmic
Transcription				
RNA processing	*rheB*	-2.62	ATP-dependent RNA helicase	Cytoplasmic
RNA synthesis/modification; DNA transcription	*nusG*	-2.32	Transcription antitermination protein	Cytoplasmic
Aminoacyl-tRNA biosynthesis	*gatA*	-2.11	Aspartyl/glutamyl-tRNA amidotransferase subunit A (EC:6.3.5.6 6.3.5.7)	Cytoplasmic
Aminoacyl-tRNA biosynthesis	*argS*	2.04	Arginyl-tRNA synthetase (EC:6.1.1.19)	Cytoplasmic
Unknown				
Unknown	*yhfA*	2.02	Hypothetical protein	Cytoplasmic
Unknown	*yveE*	2.10	Hypothetical protein	Cytoplasmic
Unknown	*ywaH*	2.11	Hypothetical protein	Cytoplasmic
Unknown	*yccL*	2.34	Hypothetical protein	Cytoplasmic
Unknown	*yhbF*	2.34	Hypothetical protein	Cytoplasmic
Unknown	*yhbE*	2.46	Hypothetical protein	Cytoplasmic

Table 2. Transcriptional response of *L. lactis* IL1403 following infection by the lytic phage c2.

Captions:

[a]Pathway assignment is based on the KEGG GENES database (www.genome.jp/kegg/genes.html).

[b]Putative function is based on homology to conserved domains and motifs/signatures by using the InterProScan tool (Zdobnov and Apweiler, 2001).

[c]Subcellular localization is based on predictions retrieved from the PSORTdb v.2.0 database (Rey *et al.*, 2005).

Table 3. qRT-PCR validation of microarray data for selected genes differentially expressed in *L. lactis* IL1403 following infection by phage c2.

Gene	qRT-PCR	microarrays
ddl	2.42	2.18
dltA	2.80	2.11
dltB	2.50	nsd[1]
dltC	3.78	nsd[1]
dltD	2.94	2.11
nrdG	15.88	2.87
yhbF	5.18	2.34
yndF	3.26	3.16
yneH	7.50	2.72
ythB	7.12	2.46
ythC	4.10	2.83
kinD	4.82	2.11
llrD	5.96	nsd[1]
yhbE	11.02	2.46
ywaF	- 2.90	- 2.34
yveF	2.40	2.24

[1]nsd = not significantly different

FIGURES

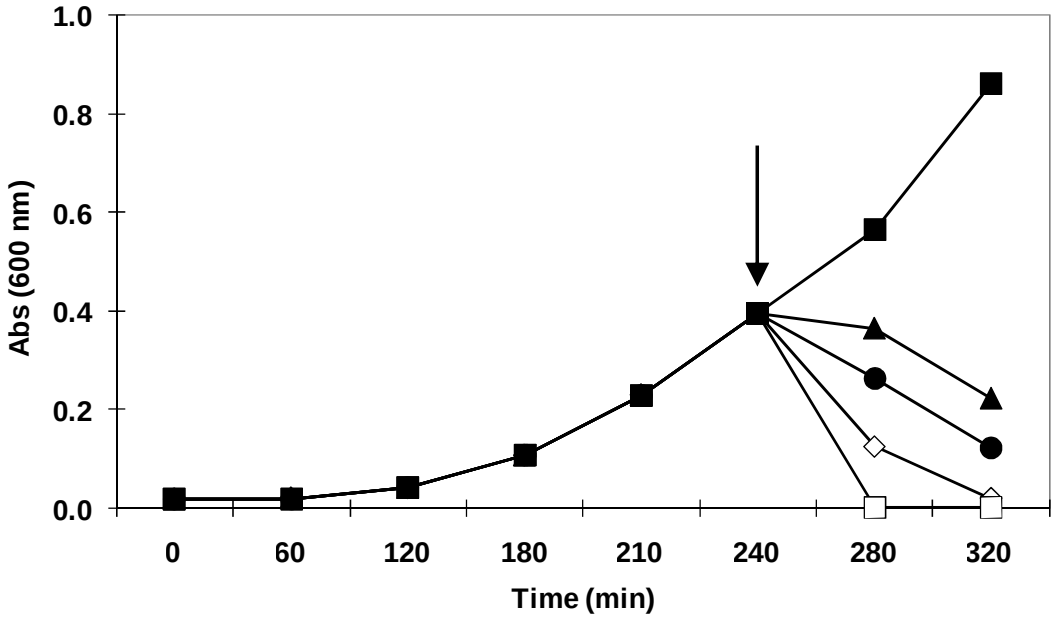


Figure 1. Phage-host challenges to determine the multiplicity of infection (MOI) necessary to lyse completely a bacterial population within 40 min. Effects of the addition of phage c2 (indicated by an arrow) at an MOI of 0 (■), 200 (▲), 400 (●), 600 (◊) and 800 (□) on the viability of *L. lactis* IL1403 cells growing in log phase.

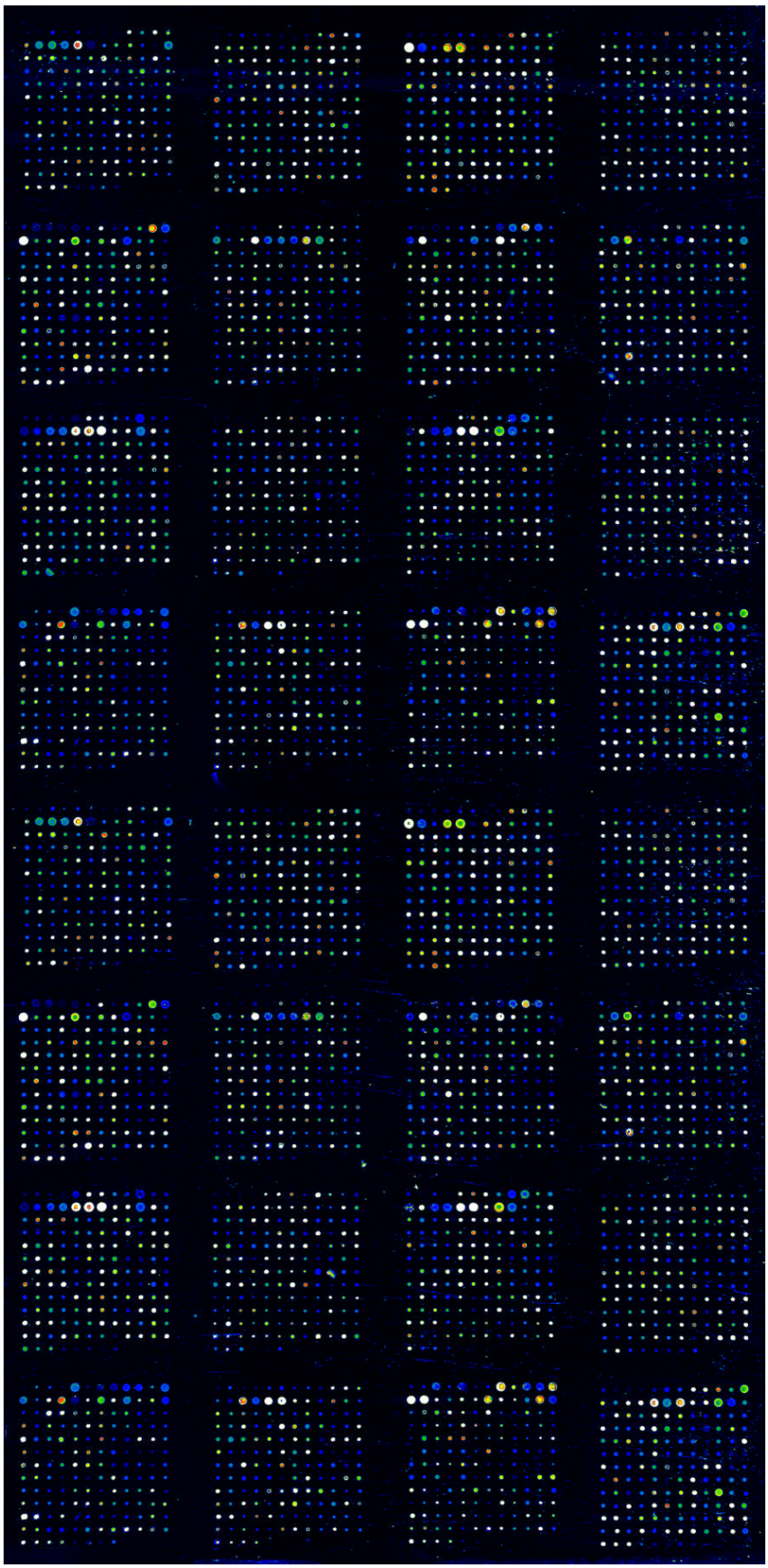


Figure 2. Microarray image of Cy3-labelled cDNA of *L. lactis* IL1403 vs. phage c2

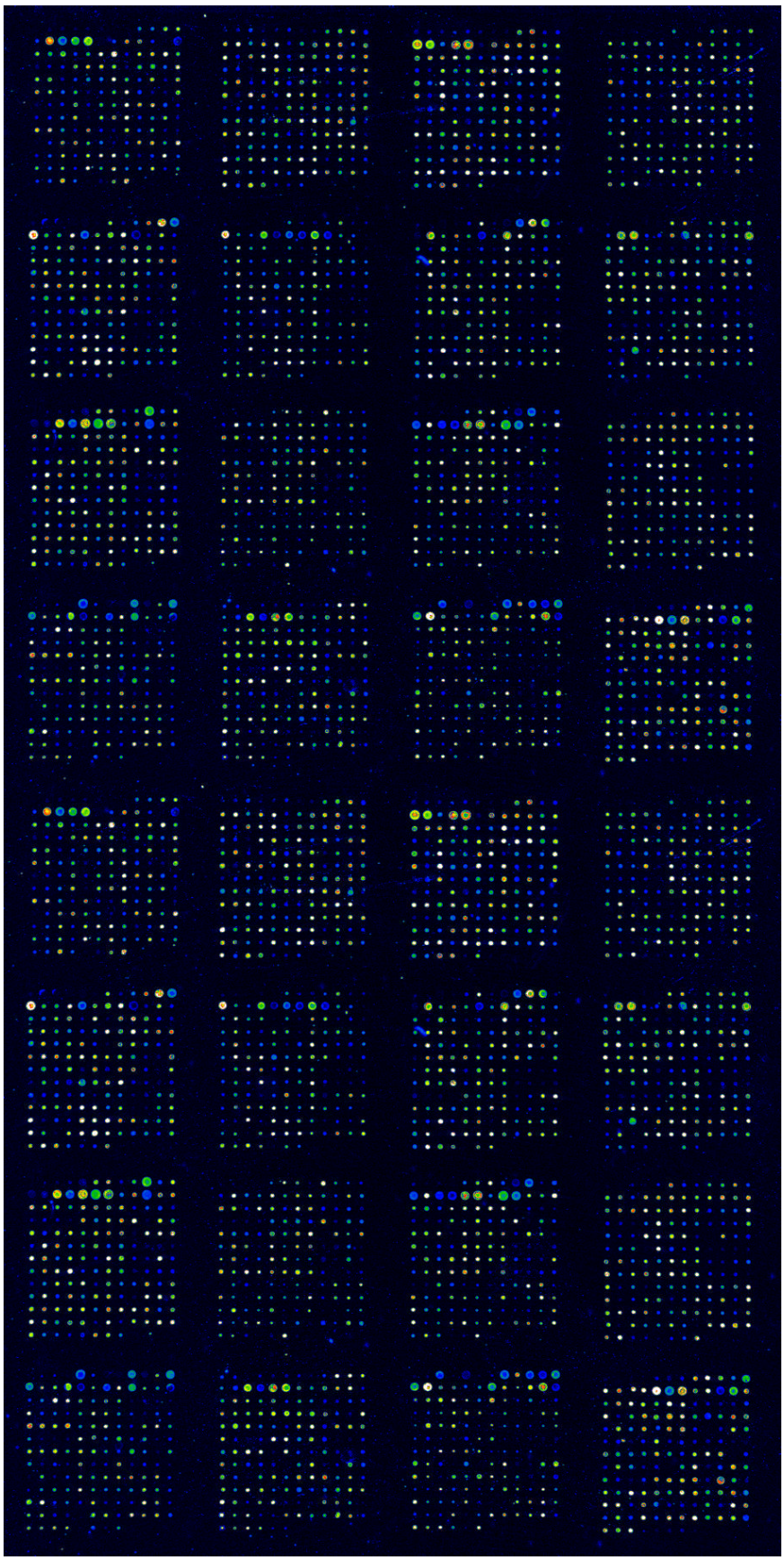


Figure 3. Microarray image of Cy5-labelled cDNA of *L. lactis* IL1403 vs. phage c2

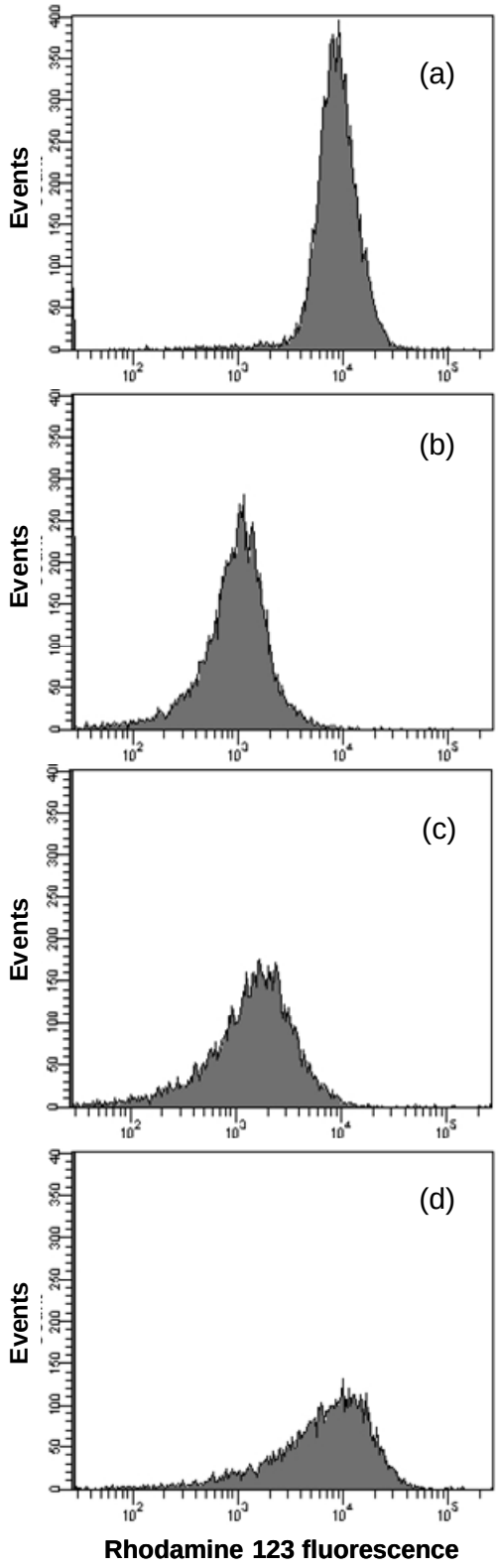
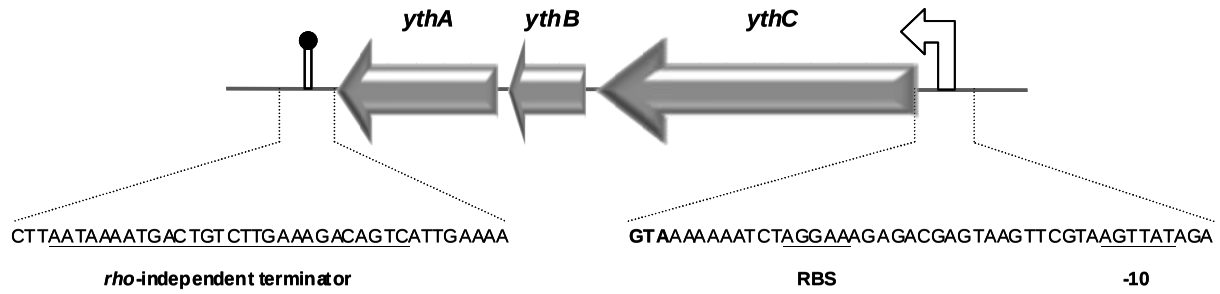


Figure 4. Flow cytometric analysis of membrane potential in rhodamine 123-stained cells of *L. lactis* IL1403 before (a) and after addition of phage c2: (b) 10 min; (c) 15 min; (d) 25 min.

Figure 5. **(a)**



(b)

```
          1                  20                  40                  60                  80
YthA      MSQRQLTKSV TNRRVSGVIA GIAEYFGLGR DVVTILRILF VVLAFGSWGG LIPLYFVASW IIPSARPRNY YDDSEDDYQE
TMHMM     iiiiiiiiii iiiiiiiiii iiiiiiiiii iiiiiiiMM MMMMMMMMM MMMMMMMMM Mooooooooo oooooooooo
TOPCONS   iiiiiiiiii iiiiiMMMMM MMMMMMMMM MMMMMooooo MMMMMMMMM MMMMMMMMM Miiiiiiiii iiiiiiiii

          81                 100                 120                 140                 154
YthA      KWNRKAQHFD EKMDRWSERY SDKMNNWARR YEDKGRQNQQ DSNQWGNPWD EPKSRKTKEA QPVEKEKEDD WSDF
TMHMM     oooooooooo oooooooooo oooooooooo oooooooooo oooooooooo oooooooooo oooooooooo oooo
TOPCONS   iiiiiiiiii iiiiiiiiii iiiiiiiiii iiiiiiiiii iiiiiiiiii iiiiiiiiii iiiiiiiiii iiii
```

(c)

```
          1                  20                  40                  60      67
YthB      MAKKTLKRSS SNRVIAGTVG GLGEYFGLSR TVINVIRVLI VLGTFGSWGT LLIVYVIASL LMTPSQG
TMHMM     iiiiiiiiii iiiiiiiiii iiiiiiiiii iiiiiiiMMM MMMMMMMMM MMMMMMMMM ooooooo
TOPCONS   iiiiiiiiii iiiiiiiiii iiiiiiiiii iiiiiiiMMM MMMMMMMMM MMMMMMMoo ooooooo
```

Figure 5. Sequence analysis of the putative *ythCBA* operon and of the PspC-like products YthA and YthB. (a) Putative regulatory elements of the *ythCBA* operon. The ribosome binding site (RBS) and -10 sequences in the promoter region upstream of *ythC* and the *rho*-independent terminator signal downstream of *ythA* are shown underlined. The start codon of *ythC* is shown in bold. (b, c) Membrane topology prediction for the YthA (b) and YthB (c) proteins by using the TMHMM and TOPCONS models (i = inside ; M = trans-membrane; o = outside).

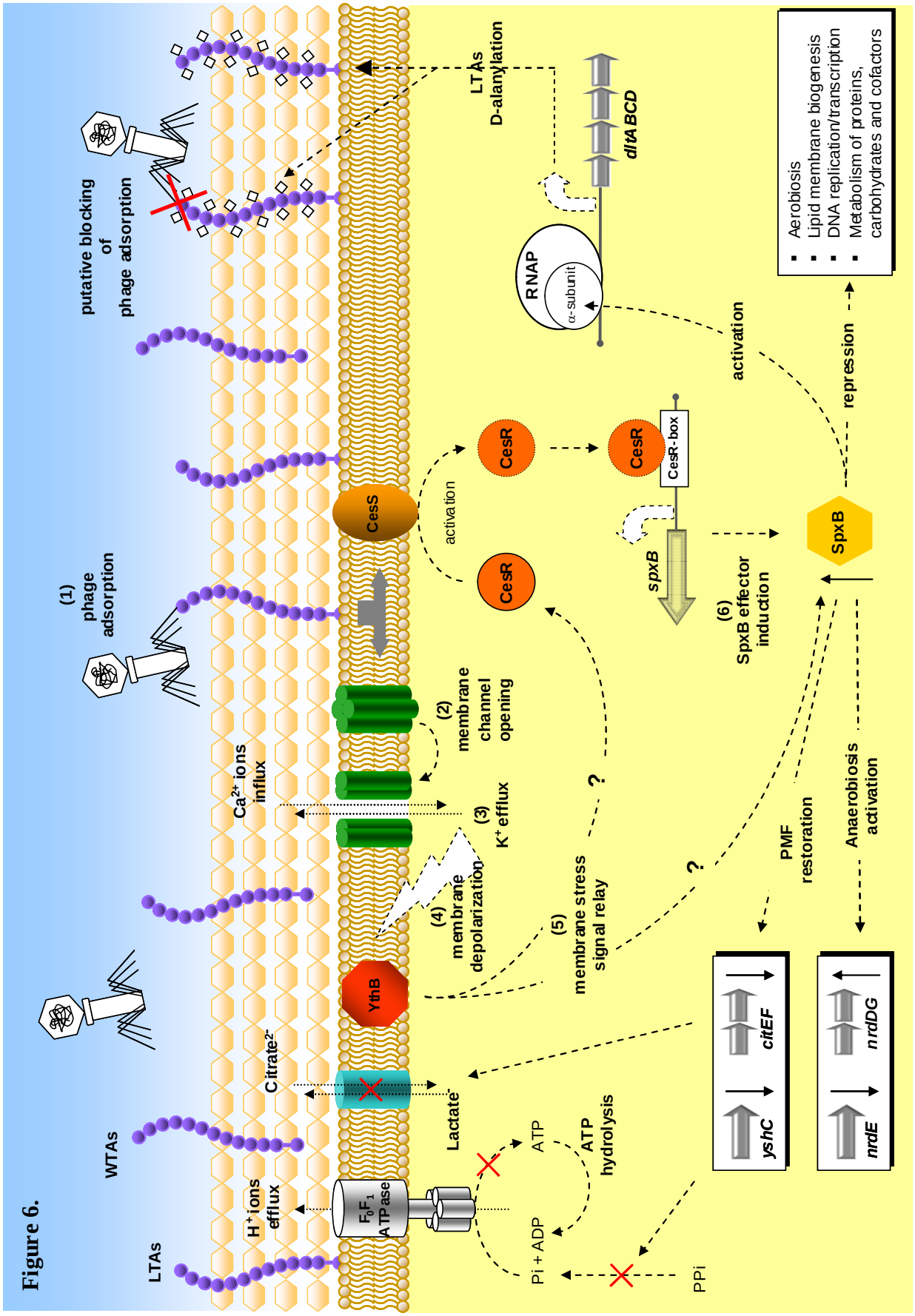

putative blocking of phage adsorption
(1) phage adsorption
WTAs
LTAs
H+ ions efflux
Ca2+ ions influx
Citrate2-
Lactate-
F0F1 ATPase
ATP
Pi + ADP
ATP hydrolysis
PPi
YthB
CesS
activation
CesR
CesR
CesR-box
spxB
(2) membrane channel opening
(3) K+ efflux
(4) membrane depolarization
(5) membrane stress signal relay
?
?
(6) SpxB effector induction
SpxB
activation
repression
LTAs D-alanylation
dltABCD
RNAP
α-subunit
PMF restoration
Anaerobiosis activation
yshC
citEF
nrdE
nrdDG
Aerobiosis
Lipid membrane biogenesis
DNA replication/transcription
Metabolism of proteins, carbohydrates and cofactors

Figure 6.

Figure 6. Putative working model of the cell-wall stressosome of *L. lactis* IL1403 in response to phage infection. The predicted sequence of events in the host cell triggered by phage infection is indicated by broken arrows, and by numbers within brackets as regards the putative steps leading to SpxB induction. D-alanine esters incorporated into LTAs or WTAs are indicated by small squares. Inhibition of metabolic reactions or pathways is indicated by black crosses. Gene up-regulation and down-regulation is indicated by arrows pointing up and down, respectively. Abbreviation are as follows: LTAs, Lipoteichoic acids; WTAs, cell wall teichoic acids; RNAP, RNA Polymerase; ATP, Adenosine Triphosphate; ADP, Adenosine Diphosphate; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; F_0F_1-ATPase, ATP synthase.

Chapter III

Novel conjugative plasmids from the natural isolate *Lactococcus lactis* subsp. *cremoris* DPC3758: a repository of genes for the potential improvement of dairy starters

Submitted to International Journal of Food Microbiology

ABSTRACT

A collection of 20 natural lactococcal isolates from raw milk cheeses were studied in terms of their plasmid distribution, content and diversity. All strains in the collection harboured an abundance of plasmids, including *L. lactis* subsp. *cremoris* DPC3758, whose 8-plasmid complement was selected for sequencing. The complete sequences of pAF22 (22,388 bp), pAF14 (14,419 bp), pAF12 (12,067 bp), pAF07 (7,435 bp) and pAF04 (3,801 bp) were obtained, whereas gene functions of technological interest were mapped to pAF65 (65 kb) and pAF45 (45 kb) by PCR. The plasmids of *L. lactis* DPC3758 were found to encode many genes with the potential to improve the technological properties of dairy starters. These included three anti-phage restriction/modification (R/M) systems (one of type I and two of type II) and genes for immunity/resistance to nisin, lacticin 481, cadmium and copper. Regions encoding conjugative/mobilization functions were present in 6 of the 8 plasmids, including those containing the R/M systems, thus enabling the food-grade transfer of these mechanisms to industrial strains. Using cadmium selection, the sequential stacking of the R/M plasmids into a plasmid-free host provided the recipient with increased protection against 936- and c2-type phages. The association of food-grade selectable markers and mobilization functions on *L. lactis* DPC3758 plasmids will facilitate their exploitation to obtain industrial strains with enhanced phage protection and robustness. These natural plasmids also provide another example of the major role of plasmids in contributing to host fitness and preservation within its ecological niche.

INTRODUCTION

Forty years of studies on plasmid biology have widely shown the crucial contribution of these extra-chromosomal entities to the performance, fitness and genomic evolution of *Lactococcus lactis*. Strains belonging to this genus have become an essential component of modern industrial starters, mostly as a result of plasmid-encoded features promoting efficient growth in milk (i.e. metabolism of lactose, milk proteins, citrate and other complex nutrients), resistance to environmental stresses (e.g. bacteriophages, heavy metals, temperature/osmotic stresses), and competitive advantages (i.e. production of bacteriocins and exopolysaccharides). Plasmids encoding these phenotypes are often mobilizable, facilitating their spread among bacteria occupying the same ecological niche. This feature has greatly contributed to the genome plasticity and evolution of lactococci, enabling them to become adapted and specialized to the dairy environment (Mills *et al.*, 2006).

In addition to being commonly found in milk, *L. lactis* strains are naturally present on plants and thus it is highly likely that dairy strains actually originate from plant-derived strains transferred from forage plants and meadow grasses to milk via cattle (Nomura *et al.*, 2006, Salama *et al.*, 1995). Compared to milk-derived strains, natural lactococcal isolates are thought to possess additional capabilities that enable them to compete and survive to multiple selections deriving from the environment, farm and traditional milk processing. Dairy strains have probably lost these traits in favour of those required for adaptation to the milk environment (Mills *et al.*, 2006, Nomura *et al.*, 2006, Salama *et al.*, 1995). An example is the greater tolerance of stress demonstrated by natural isolates as a result of a variety of protection mechanisms developed to resist the numerous cytotoxic compounds and organisms (antibiotics, heavy metals, bacteriocins and bacteriophages) present in the plant and animal environments (Ballesteros *et al.*, 2006, Leelawatcharamas *et al.*, 1997, Nomura *et al.*, 2006, Schirawski *et al.*, 2002, Tanous *et al.*, 2007). These phenotypes are encoded by genes often carried by plasmids, which, again, reaffirms the role played by these genetic elements in determining the uniqueness, fitness and performance of *L. lactis* strains. Based on these observations, the analysis of plasmid distribution and diversity appears to be an essential tool to evaluate the genetic potential and variability in both dairy and natural lactococcal strains.

Only a relatively limited number of *L. lactis* strains belonging to the subspecies *lactis* and *cremoris* are used in modern large-scale processes due to the need to maintain consistent quality over a vast range of products manufactured worldwide. It is thus arguable that the intensive exploitation of these few cultures has contributed to narrow flavour diversity in dairy products and favoured the emergence of host-specific lytic bacteriophages (phages) (Coffey and Ross, 2002, Cogan *et al.*, 1997, Nomura *et al.*, 2006). There is therefore a renewed interest in the isolation and characterization of new strains in order to replace or complement the starter cultures currently used in dairy industry. Novel genes encoding for phage-defence systems, bacteriocin production/immunity, flavour-forming abilities and good technological properties are particularly sought. These phenotypes are often encoded on plasmids that are easily mobilizable via natural conjugation, thus enabling the use of a food-grade approach to obtain novel starter formulations with enhanced robustness and long-term efficiency or to allow the manufacture of new and safer products (Coffey and Ross, 2002, Forde and Fitzgerald, 1999, Mills *et al.*, 2006).

We have recently focused our attention towards the isolation, identification and characterization of *L. lactis* strains from natural ecological niches and good quality artisanal products, particularly cheese. Artisanal cheeses generally possess unique sensory characteristics compared to large scale industrial products, that may attributed to the activity of the natural milk microflora, left intact by the absence of pasteurization and probably derived from the plant and animal environments (Ballesteros *et al.*, 2006, Cogan *et al.*, 1997, Delgado and Mayo, 2004). In this study, we describe a preliminary analysis of the plasmid complement of 20 lactococcal isolates from artisanal cheeses for traits with potential for the improvement of starter cultures, followed by the sequencing and functional analyses of the 8-plasmid complement of the isolate *L. lactis* subsp. *cremoris* DPC3758. Our results suggest a significant contribution by the plasmid pool in protecting the host from several environmental stresses by means of three anti-phage restriction/modification (R/M) systems, and genes for resistance to bacteriocins and heavy metals. These genetic traits are located on plasmids carrying conjugative/mobilization regions, which is suggestive of a high level of genome plasticity for *L. lactis* DPC3758. We demonstrate that these regions are functional and allow the exploitation of these

natural plasmids to obtain lactococcal strains with enhanced phage protection and robustness.

MATERIALS AND METHODS

Bacterial strains, bacteriophages and media

A collection of 20 natural lactococcal isolates was used in this study (Table 1), which were isolated during a previous screening of artisanal raw milk cheeses from around Europe (Spain, France, Italy and Greece) with a view to identifying strains with the potential as starters for commercial dairy fermentations (Cogan *et al.*, 1997). Bacterial strains were routinely propagated at 30 °C in M17 medium (Oxoid, UK) supplemented with 0.5% (wt/vol) glucose (GM17) or lactose (LM17). Solid media contained 1.0% (wt/vol) bacteriological agar (Oxoid, UK). Lactococcal bacteriophages (Table 1) were propagated on sensitive hosts and phage preparations were titrated at 30 °C according to standard procedures (Terzaghi and Sandine, 1975). Bacterial strains were stocked in M17 containing 40% glycerol at -80 °C. Working cultures were stored at 4 °C and transferred periodically.

Screening for natural lactococcal isolates

A combination of phenotypic and genotypic techniques was used to confirm the preliminary assignment of isolates to the *Lactococcus* genus (Cogan *et al.*, 1997), or to support a new identification. Phenotypic tests were as follows. Gram staining and catalase reaction using 3% (v/v) H_2O_2 were performed on single colonies grown overnight on LM17 agar at 30 °C, while cell morphology was determined from a loopful of culture grown overnight in LM17 broth at 30 °C. Growth of isolates at different temperatures (10 and 45 °C) and in presence of different salt levels (4 and 6.5%) was tested in 10 ml LM17 broth (1% inoculum) over a 5-days period, after which the pH of each culture was measured and a pH difference of 1 units between the test isolate and the uninoculated control was considered indicative of growth. Ability to hydrolize aesculin was assessed by growing isolates on kanamycin aesculin azide (KAA) agar (Oxoid, Hampshire, England) for 24 to 48 h. Acid production from lactose was assessed after streaking isolates onto lactose indicator agar (LIA) containing 0.004% (w/v) bromocresol purple. Genotypic tests involved the use of genus-specific primers, designed within conserved regions of the lactococcal and enterococcal 16S rRNA genes, and PCR conditions as described elsewhere (Deasy *et al.*, 2000). Definitive genus confirmation of presumptive lactococcal isolates was

obtained by using the universal 16S rRNA primers CO1 (5'-AGTTTGATCCTGGCTCAG-3') and CO2 (5'-TACCTTGTTACGACT-3') and the PCR conditions as described elsewhere (Beresford and Condon, 1991). Aliquots (20 μl) of the amplified products were subjected to electrophoresis in 1% agarose (Sigma, St. Louis, USA) gels and stained with ethidium bromide. PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen GmbH, Germany) and sequenced by single run primer extension (Beckman Coulter Genomics, USA). Sequencing data were searched for regions of similarity against DNA sequences available in public databases (GenBank, EMBL, DDBJ, PDB) by using the nucleotide MegaBLAST algorithm of the Basic Local Alignment Search Tool (BLAST) (Zhang *et al.*, 2000).

Classification of natural lactococcal isolates

Isolates identified as belonging to the *Lactococcus* genus were selected for the rest of the study. Differentiation and classification of lactococcal isolates at species and subspecies level was obtained by using traditional phenotypic and genotyping methods in combination with chemometric analyses. The fermentation profiles of 49 sugars and polyalcohols were determined on fully grown lactococcal cultures by using the API 50 CH kit and API 50-CHL medium (bioMerieux SA, France) according to the manufacturer's instructions. *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* biovar. *diacetylactis* DRC3, and *L. lactis* subsp. *cremoris* HP were included in the analysis as internal references. Chemometric analyses of carbohydrate fermentation profiles of lactococcal isolates were performed by using Minitab v.15.1.1 (Minitab Inc., USA). The fermentation profiles typical of the subspecies *lactis*, *cremoris* and *hordniae*, and of *L. raffinolactis*, as reported by the API 50 CH kit's manufacturer, were also included in the analysis as external references. Biochemical tests showing no change in percent of carbohydrate fermentation among all test and reference bacteria were removed from the analysis. Results of the remaining tests were converted to a five-grade score, corresponding to different percents (0, 25, 50, 75 and 100%) of substrate utilization. Hierarchical cluster analysis (HCA) on the converted data was obtained by using the unweighted pair group method with arithmetic averages (UPGMA) and the squared Euclidean

distance. Principal components analysis (PCA) was performed standardizing the variables (mean = 0; SD= 1) (Fallico *et al.*, 2004).

Genomic DNA was extracted from 1 ml-aliquots of overnight cultures (Hoffman and Winston, 1987). Randomly amplified polymorphic DNA (RAPD)-PCR profiles of lactococcal isolates were obtained by using the primer 5'-ATGTAACGCC-3' and the PCR conditions described elsewhere (Jayarao and Oliver, 1994). Aliquots (5 µl) of the amplified products were subjected to electrophoresis in 1% agarose gels. The reproducibility of RAPD-PCR patterns was checked in duplicate assays. HCA analysis of RAPD-PCR profiles was performed by using BioNumerics v.2.00 (Applied Maths, Belgium). Band patterns were normalized before being subjected to HCA by using the Pearson correlation coefficient and the UPGMA method.

Plasmid DNA analysis

Plasmid DNA of the lactococcal isolates was extracted as described elsewhere (O'Sullivan and Klaenhammer, 1993) and analyzed by electrophoresis on horizontal 0.6% agarose gels in TAE buffer (40 mM Tris–HCl, 20 mM Acetic acid, 2 mM EDTA, pH 8.0) for 4 h at 80 V. The large plasmids (65-, 51- and 36-kb) of *L. lactis* DRC3 (McKay and Baldwin, 1984) and the Supercoiled DNA ladder (2 to 16 kb) (Invitrogen, USA) were used as markers of DNA molecular size.

Antimicrobial production and cross-sensitivity assays

Antimicrobial production by the lactococcal isolates was estimated by agar well diffusion assay (Ryan *et al.*, 1996) using *L. lactis* subsp. *cremoris* HP as the indicator strain. The bacteriocin-like nature of the antimicrobials was determined by sensitivity assays to proteases (trypsin, α-chymotrypsin, and proteinase K, 50 mg/ml each) and temperature (50, 70, 90, and 110 °C) as described elsewhere (Ryan *et al.*, 1996). The anti-listerial activity of bacteriocins was tested against *Listeria innocua* DPC3572 in agar well diffusion assays. Cross-sensitivity of antimicrobial producers *L. lactis* DPC3758 and *L. lactis* DPC3694 versus known bacteriocin producers was determined as follows. Agar plates were seeded with 200 µl of *L. lactis* DPC3758 or *L. lactis* DPC3694 overnight cultures, inoculated with 50 µl of neutralized

supernatant from *L. lactis* DPC5558 (nisin producer), *L. lactis* DPC5552 (lacticin 481 producer), *L. lactis* DPC938 (lactococcins A, B and M producer), and *L. lactis* DPC3147 (lacticin 3147 producer) into pre-formed wells, incubated overnight at 30 °C and then examined for zones of growth inhibition.

Phage resistance analyses

Rapid screening of lactococcal strains for phage resistance/sensitivity was performed by using the spot test technique (Sanders and Klaenhammer, 1983). Briefly, GMI7 agar plates were overlaid with top lawns of soft GM17 agar (0.7%, w/v) containing 100 µl of overnight culture, 5 mM $CaCl_2$, and 0.75% (w/v) glycine. Lactococcal lawns were spotted with 20 µl of purified phage lysates at a suitable titre (10^8-10^{10} pfu/ml), incubated overnight at 30 °C and then examined for zones of clearance. Glycine was added during all spot and plaque assay tests to improve visualization of plaques in poor plaque-producing phages (Lillehaug, 1997). Additionally, the plasmid complement of lactococcal strains was screened for the presence of a type I R/M *hsdS* gene by using a degenerate PCR approach. The amino acids sequences of 20 HsdS subunits from lactococcal plasmids were aligned by using T-Coffee (Notredame *et al.*, 2000). Regions of homology within the N-terminal and central conserved domains of the HsdS proteins were used to design the degenerate primers 5'-GGNTTYACNGAYGAYTGGTAR-3' and 5'-GCRAANCGNAGRTCNGGNAC-3' in order to amplify the variable region, or target recognition domain (TRD), located in the middle of these conserved domains. By using total plasmid DNA as template, PCR amplicons of ca. 640 bp were obtained. These were purified by the QIAquick PCR Purification kit (Qiagen GMBH, Germany) and sequenced by double strand primer extension (Beckman Coulter Genomics, USA). Sequencing data were searched for regions of similarity against DNA sequences available in public databases (GenBank, EMBL, DDBJ, PDB) by using the nucleotide MegaBLAST algorithm of the Basic Local Alignment Search Tool (BLAST) (Zhang *et al.*, 2000).

Sequencing and annotation of the plasmid complement of *L. lactis* subsp. *cremoris* DPC3758

Plasmid DNA was prepared as described elsewhere (O'Sullivan and Klaenhammer, 1993). Any residual genomic DNA was removed by treatment with Plasmid-Safe ATP-Dependent DNase (Epicentre, Madison, USA) which selectively hydrolyzes linear double-stranded DNA to deoxynucleotides without affecting closed circular supercoiled or nicked circular dsDNAs. The eight purified plasmids were sequenced as a pool on a 454 Genome Sequencer FLX System, after preparation of a sonication library, barcoding of the library with the Roche multiplex identifier system, and emPCR amplification (Beckman Coulter Genomics, USA). Automatic de-novo assembly of data resulted in 92 large (> 500 bp) contigs (ca. 203 kb) and 115 small (< 500 bp) contigs (ca. 31 kb) providing a total of ca. 234 kb of sequence data. Gap closure was performed by PCR using primers designed with PrimerSelect v.8.0.2 (DNASTAR, USA), followed by sequencing of the PCR products (Beckman Coulter Genomics, USA). Putative open reading frames (ORFs) were automatically predicted and annotated using Glimmer 2.0 (Delcher *et al.*, 1999). Annotated DNA sequences were examined manually using the Artemis viewer (Rutherford *et al.*, 2000) and BLASTX and BLASTP analyses (Altschul *et al.*, 1998) were used to examine intergenic regions for putative ORFs not identified by Glimmer, to identify putative frameshifts, and to refine the start codon assignments. The InterProScan tool (www.ebi.ac.uk/Tools/InterProScan), the PFAM (Finn *et al.*, 2010) and Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2009) were used to predict protein function. InterProScan predicts the occurrence of functional domains and motifs/signatures in a protein by combining 12 different databases and their relative protein signature recognition methods (Zdobnov and Apweiler, 2001).

Association of genetic traits to pAF65 and pAF45 of *L. lactis* DPC3758

A PCR-based approach was used to associate traits of interest (phage resistance, conjugation and stress response) with one of two of the unclosed plasmids, pAF65 (65kb) or pAF45 (45 kb) (Figure 2). The individual plasmids were gel extracted, purified by the Geneclean II kit (QBiogene, USA), and used as template DNA in PCR reactions containing primers specific for each selected genetic trait (Table 1).

Functional analyses of the cadmium and copper resistance operons of *L. lactis* DPC3758

To evaluate the functionality of the cadmium resistance operon, *L. lactis* DPC3758 was grown at 30 °C for 18 h in LM17 containing increasing concentrations (0 to 0.3 mM) of cadmium chloride ($CdCl_2$). Similarly, the functionality of the copper resistance operon was evaluated by growing *L. lactis* DPC3758 at 30 °C for 18 h in LM17 containing increasing concentrations (0 to 6 mM) of cupric sulphate ($CuSO_4$). In both experiments, *L. lactis* MG1363 was used as a reference strain due to its sensitivity to cadmium and copper.

Conjugative studies and phage sensitivity assays

A conjugation study was designed using *L. lactis* DPC3758 (cadmium resistant, Cd^R; rifampicin sensitive, Rif^S), and the plasmid-free *L. lactis* MG1614 (cadmium sensitive, Cd^S; rifampicin resistant, Rif^R) as donor and recipient strains, respectively. Overnight cultures of donor and recipient strains were used to prepare 2% inoculums that were grown at 30 °C for 4 h. Cells from 1-ml aliquot of each culture were harvested (14,000 rpm, 1 min, 4 °C), resuspended in 200 µl of GM17, mixed in a recipient-to-donor ratio of 1:2 and then spotted onto the centre of a non-selective GM17 agar plate. Donor and recipient controls were prepared in a similar manner. Following an overnight incubation at 30 °C, the mating mix was recovered from the plate, resuspended in 10 ml of GM17 broth containing 0.3 mM cadmium chloride ($CdCl_2$) and 100 µg ml^{-1} rifampicin, and then incubated a further overnight at 30 °C to enrich for Cd^R Rif^R transconjugants. Donor and recipient controls were recovered from the plates, resuspended in 1 ml of GM17 broth, and directly plated onto LIA, containing 0.3 mM $CdCl_2$, 100 µg ml^{-1} rifampicin, and 0.004% bromocresol purple. Following overnight incubation, cells from 1-ml aliquot of the mating mix were harvested, resuspended in 1 ml of GM17, and then plated onto selective media. Putative transconjugants, appearing as white colonies, selected and plasmid DNA profile analyzed to verify the acquisition of the plasmids. Confirmed transconjugant strains were examined for their sensitivity to phages c2 and 712 by using the plaque

assay technique (Terzaghi and Sandine, 1975). Efficiency of plaquing (EOP) was determined by dividing the phage titer on the test strains by the titer on the homologous host *L. lactis* MG1614.

RESULTS AND DISCUSSION

Screening for natural *Lactococcus lactis* isolates

All twenty isolates were found to be gram-positive, catalase-negative, coccus-shaped bacteria that existed in pairs or short chains; they were all growing in 4% NaCl and producing acid from lactose. Enterococci are found in a wide range of dairy products, especially those made from raw milk, at a magnitude comparable to lactococci (Cogan *et al*., 1997, Delgado and Mayo, 2004). Consequently, bacterial isolates were subjected to a number of phenotypic tests (growth at 10 °C and in 6.5% NaCl; esculin hydrolysis on KAA medium) commonly used for the identification of the genus *Enterococcus* (Deasy *et al*., 2000, Delgado and Mayo, 2004). Four isolates (DPC3694, DPC3901, DPC3758 and DPC3912) showed a positive response to all tests, which is typical of enterococci, whereas four other isolates (DPC3757, DPC3776, DPC3907 and DPC3749) exhibited negative responses, typical of lactococcal strains (Table 3). The remaining twelve isolates showed undefined intermediate reactions, a finding that confirms the inaccuracy of traditional tests in clearly differentiating lactococci from enterococci. There are indeed numerous reports of lactococcal strains, especially those isolated from natural ecosystems, exhibiting an *Enterococcus*-like phenotype and vice versa. Consequently, researchers now prefer to use molecular techniques for both classification and typing purposes (de la Plaza *et al.*, 2006, Deasy *et al.*, 2000, Delgado and Mayo, 2004, Nomura *et al.*, 2006). A genotyping approach involving genus-specific 16S rRNA gene sequencing was thus used to obtain a reliable and definitive discrimination between lactococci and enterococci in our bacterial collection. Seventeen isolates produced an amplicon only in presence of the *Lactococcus*-specific primers, whereas three isolates gave reaction only with the *Enterococcus*-specific primers (Table 3).

Chemometrics of carbohydrate fermentation profiles is a valid tool for differentiation and classification of lactococcal strains

The 17 isolates identified as belonging to the *Lactococcus* genus were selected for the remaining part of this study. Differentiation and classification of these bacteria at species and subspecies level was attempted by chemometric analysis of carbohydrate fermentation profiles. All lactococcal isolates were able to fully ferment

ribose, galactose, glucose, fructose, mannose, esculin, maltose, lactose and trehalose, but unable to utilize glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl-βD-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, D-mellibiose, inulin, D-melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, and potassium 2- and 5-ketogluconate.

A sub-dataset of 26 tests, showing the actual inter-strains differences, was subjected to cluster analysis by the UPGMA method (Figure 1). Levels of similarity greater than 80% in the homology tree dendrogram were considered indicative of strain homology. With the exceptions of *L. lactis* DPC3749 and DPC3758, the isolates shared high similarity (greater than 90%) and were grouped into three main clusters. The two larger clusters (I and III) were composed of 12 isolates that are likely to belong to the *lactis* subspecies as they grouped together with reference standards *L. lactis* subsp. *lactis* 1 and 2. However, clusters I and III were clearly separated in the dendrogram and shared less than 80% homology, which is indicative of a phenotypic diversity between members of the two groups. PCA results (Figure 2) revealed that these differences consisted in the ability of cluster I members to ferment D-mannitol, D-saccharose, D-xylose, gentibiose, arbutin, salicin, potassium gluconate, amygdalin, N-acetylglucosamine, D-lactose, L-arabinose, and D-cellobiose at a greater extent than strains of cluster III. Within this cluster, *L. lactis* DPC3776, DPC3778 and DPC3926 shared virtually identical fermentation profiles. The smallest cluster (II) included three isolates (*L. lactis* DPC3901, DPC3880 and DPC3911) sharing greater than 90% similarity with the experimentally obtained profile of the reference strain *L. lactis* subsp. *lactis* biovar. *diacetylactis* DRC3. Interestingly, *L. lactis* DPC3749 and DPC3758 were not grouped in any of the above clusters. *L. lactis* DPC3749 shared less than 80% similarity with clusters II and III and with the reference strain *L. lactis* subsp. *lactis* IL1403. This suggested an atypical subspecies *lactis* phenotype for this isolate. Similarly, *L. lactis* DPC3758 is likely to represent an atypical *cremoris* isolate as it was found to share less than 80% similarity to *L. lactis* subsp. *cremoris* 1 but only 40% similarity to *L. lactis* subsp. *cremoris* HP. The 16S rRNA gene of five strains (*L. lactis* DPC3694, DPC3749, DPC3901, DPC3776 and DPC3758), including a representative of each cluster and both outliers, was amplified and sequenced in order to confirm the reliability of this classification.

BLAST analysis confirmed the *cremoris* genotype for *L. lactis* DPC3758 and the *lactis* genotype for the other isolates, with *L. lactis* DPC3694 and DPC3901 exhibiting strong similarity to the 16S rRNA gene of the biovariant *diacetylactis*. Among the 17 natural isolates, *L. lactis* DPC3758 was the only one to belong to the *cremoris* subspecies. This finding is consistent with the observation that most natural isolates of lactococci belong to the *lactis* subspecies, whereas the subspecies *cremoris* is only rarely isolated (Cogan *et al.*, 1997, Salama *et al.*, 1995). Interestingly, *L. lactis* DPC3758 exhibited a *cremoris* genotype but it was found to grow at high temperatures and NaCl concentrations, properties that are typical of the subspecies *lactis*. This is consistent with the reports of strains with a *lactis* phenotype that may fall either into the *lactis* or in the *cremoris* genotype (de la Plaza *et al.*, 2006, Delgado and Mayo, 2004). In this study, data generated by carbohydrates fermentation tests provided a differentiation of the isolates at the subspecies level that was further confirmed by 16S rRNA gene sequencing. In contrast to previous reports (Delgado and Mayo, 2004), our results therefore support the use of chemometric analysis of fermentation profiles as a valid and reliable tool for the classification and typing of novel lactococcal isolates.

Following the phenotypic characterization, the lactococcal isolates were differentiated at the strain level by using HCA of RAPD-PCR profiles (Figure 3). All isolates exhibited different profiles, with the exceptions of *L. lactis* DPC3776, DPC3778, and DPC3926 that were already found to share identical fermentation profiles (Figure 1). These isolates were collected from the same dairy batch and, therefore, they are likely to represent the same strain. HCA of RAPD-PCR profiles separated the lactococcal isolates into three main clusters (Figure 3). The smallest one included three isolates (*L. lactis* DPC3757, DPC3780 and DPC3694) sharing each other low genetic similarity (60 to 70%) but high phenotypic relatedness as they were grouping together (cluster I) in the HCA dendrogram of fermentation profiles (Figure 1). The remaining two clusters included isolates with high genetic homology (greater than 80%) but varied phenotypic relatedness as these isolates were grouping in different fermentation clusters (Figure 1). These results are not surprising as it is widely recognized that phenotypic and genotypic groupings do not necessarily match in *L. lactis* strains (de la Plaza *et al.*, 2006, Delgado and Mayo, 2004, Nomura *et al.*, 2006).

Preliminary characterization of the natural lactococcal strains

Natural *L. lactis* isolates are thought to possess a broader genetic makeup compared to industrial strains, with plasmid-encoded functions generally reflecting host adaptation to different ecological niches (Mills *et al.*, 2006). Based on this rationale, the 17 natural lactococcal strains were characterized for the diversity and genetic content of their plasmids with the aim of identifying novel plasmid-encoded genes for the technological improvement of dairy starters. A rich and heterogeneous content of plasmids was found in all investigated strains, with each one harbouring between 2 and 10 plasmids with sizes ranging from 2 kb to 80 kb (Figure 4a). This latter finding was especially intriguing as large plasmids are expected to contain most of the genetic information providing a competitive advantage to the lactococcal host to flourish in its natural ecosystem (Siezen *et al.*, 2005).

A number of preliminary tests were undertaken to identify genetic traits of potential interest (i.e. antimicrobial production and phage resistance) on the plasmid complement of these isolates. Firstly, the supernatant from an overnight culture of each strain was assayed for antimicrobial activity. Three strains (*L. lactis* DPC3758, DPC3694 and DPC3901) were found to produce narrow-spectrum antimicrobial compounds, which inhibited the growth of the indicator strain *L. lactis* HP (Figure 4b) but not the growth of indicators such as *Listeria innocua*, *Lactobacillus paracasei* and *Pediococcus acidilactici*. The antimicrobials produced by *L. lactis* DPC3758 and DPC3694 were sensitive to proteases and thermal treatments, which is indicative of a bacteriocin-like nature, whereas the compound produced by *L. lactis* DPC3901 was unaffected by such treatments. Results of cross-sensitivity tests (data not shown) suggested that *L. lactis* DPC3758 and DPC3694 were likely to produce a lacticin 481- or a lactococcin-like protein and to harbour immunity to nisin, lacticin 481 and lactococcins A, B and M. Despite these similarities, differences in RAPD-PCR patterns (results not shown), plasmid profiles and bacteriocin potency excluded that *L. lactis* DPC3758 and DPC3694 are different isolates of the same strain.

Secondly, spot assays with a number of c2-type (c2, ml3, eb1) and 936-type (sk1, 712, hp) phages revealed that 3 isolates (*L. lactis* DPC3776, DPC3874, and DPC3778) were exclusively sensitive to c2-type phages, whereas the remaining strains were resistant to all phages tested (Figure 4c). In *L. lactis*, host protection from phage attack is often afforded by its plasmid complement via one or more of four

mechanisms of resistance known to date: (i) inhibition of phage adsorption, (ii) phage DNA injection blocking, (iii) phage DNA restriction/modification (R/M), (iv) abortive phage infection. Of these, R/M systems are the most interesting as their phage inactivation strategy has no deleterious effects on host viability, and, in the case of type I R/M, they can modulate the level of phage restriction by homologous recombination of HsdS specificity subunits (Mills *et al.*, 2006, Schouler *et al.*, 1998b). Consequently, we investigated our collection of natural lactococcal strains for the presence of type I R/M *hsdS* genes by using degenerate primers designed to target the N-terminal TRD (Figure 4d). Thirteen of the 17 isolates produced an amplicon of the expected size (ca. 640 bp), indicating the presence of at least one *hsdS* gene and possibly, of a complete type I R/M system in their plasmid complement.

Sequence analysis of the plasmid complement of *L. lactis* subsp. *cremoris* DPC3758

These initial findings prompted a deeper investigation of the genetic properties of these natural isolates by sequencing the entire plasmid complement of *L. lactis* subsp. *cremoris* DPC3758. This strain was chosen as it exhibited a number of technologically interesting characteristics: (i) complete resistance to c2- and 936-type phages, (ii) the presence of a *hsdS* gene and putatively of an associated type I R/M system, (iii) antimicrobial production, (iv) and a rich extra-chromosomal complement composed of 8 plasmids, with sizes ranging from 2 kb to 80 kb. Additionally, *L. lactis* DPC3758 was the only strain in the investigated collection to belong to the subspecies *cremoris*, which is highly suitable for the manufacture of Cheddar and other cheese varieties (Nomura *et al.*, 2006, Salama *et al.*, 1995).

High-throughput sequencing of *L. lactis* DPC3758 plasmids generated 234 kb of data distributed over 207 contigs. Following initial BLAST analysis and gene annotation, a PCR-mediated gap closure strategy was adopted to complete each of the plasmid sequences. This approach was successful in the case of five plasmids, namely pAF22, pAF14, pAF12, pAF07 and pAF04 (Figure 5; Tables A1-A5 in Appendix). These plasmids displayed a GC content ranging from 32 to 36%, which is consistent with the 31-36% range described for lactococcal plasmids (Siezen *et al.*, 2005). However, the presence of numerous secondary structures and many homologous

insertion sequences (IS) rendered the primer walking strategy unsuccessful in closing the largest plasmids, pAF65 (65 kb) and pAF45 (45 kb). In these cases, gap closure focused on those regions containing traits of technological interest (phage resistance systems, conjugation/mobilization regions, and stress response/selectable markers), which were then associated with either pAF65 or pAF45 using a PCR approach (Figure 5; Tables A6-A12 in Appendix). The sequencing data revealed that the antimicrobial produced by *L. lactis* DPC3758 is a lactococcin-like bacteriocin whose genetic determinants are located on the chromosome.

A battery of three R/M systems protects *L. lactis* DPC3758 against phage attack

The plasmid complement of *L. lactis* DPC3758 harbours a significant array of defence mechanisms against phage attack, including one type I R/M and two type II R/M systems, which confirms suggestions that the latter mechanisms are the most common in lactococci (Mruk *et al.*, 2003). Type I R/M systems are typically made up of three genes (*hsdRMS*), transcribed in the same direction, and coding for three subunits which are responsible for DNA restriction (HsdR), modification (HsdM) and specificity (HsdS), respectively (Murray, 2000). The type I R/M system (R/M-I) of *L. lactis* DPC3758 is located on pAF65 (Figure 5; Table A7 in Appendix). The *hsdR* gene encodes a restriction endonuclease (REase, 995 aa) containing conserved helicase-like (COG0610) domains, while the 515-aa long product of *hsdM* is a typical N^6-adenine β-class methyltrasferase (MTase, pfam02384). Both proteins are 99% identical to the HsdR and HsdM enzymes encoded by homologous genes present on the chromosome of *L. lactis* IL1403 (Schouler *et al.*, 1998a). In contrast, the specificity protein (428 aa) encoded by R/M-I *hsdS* of pAF65 is 61% similar to listerial HsdS subunits and shares only 36% identity with its homolog from *L. lactis* IL1403. Low similarities among HsdS subunits is generally common and reflects the large variability existing within their TRDs, which are responsible for imparting target sequence specificity to both the restriction and modification activities of the type I R/M complex (Murray, 2000). However, amino acid sequence alignments revealed that the HsdS of pAF65 mostly diverges from that of *L. lactis* IL1403 and other lactococci at the C-terminal TRD (aa positions 281-399), where the sequence identity drops to as low as 18% (Figure 6). Indeed, this region is 84% similar to a

TRD shared by some *Listeria, Enterococcus* and *Lactobacillus* organisms, a finding that would suggest a putative shuffling of *hsdS* TRDs. Such an event has been reported to have generated the co-integrated plasmid pAH90 from the native plasmids pAH33 and pAH82 in *L. lactis* DPC721 (O'Sullivan *et al.*, 2000). In pAH90, the recombination event formed two novel hybrid *hsdS* genes that had interchanged the N- and C-terminal TRDs of the parent subunits and sequence analysis revealed that the site of recombination involved a 19-aa sequence (KVPELRFAGFADDWEERKL) located in the conserved central domain of both HsdS proteins (O'Sullivan *et al.*, 2000). Interestingly, this exact sequence is present at the end of the conserved central domain (aa positions 256-276) of the HsdS of *L. lactis* DPC3758, just preceding the C-terminal TRD and exactly marking the start of the divergence of sequence from the HsdS of *L. lactis* IL1403 (Figure 6). These findings support the hypothesis that a homologous recombination event may have promoted the acquisition of a novel TRD, possibly from a *Listeria*, *Enterococcus* or *Lactobacillus* strain, and of a likely novel specificity in the R/M-I HsdS of *L. lactis* DPC3758. Such an event is highly feasible as members of these genera usually coexist in the rich and diverse microflora typical of raw milk cheeses. O'Sullivan et al. (2000) aligned the variable regions of all known HsdS proteins of *L. lactis* and *S. thermophilus* and found evidence of putative domain shuffling events occurring between members of these two genera. This led the authors to speculate that the generation of new specificities via homologous recombination is a widespread strategy used by *Lactococcus* to enhance its natural defences against phages. Indeed, the formation of pAH90 occurred in a background of high-titre phage challenge for *L. lactis* DPC721 (O'Sullivan *et al.*, 2000). In the case of pAF65, it is tempting to speculate that its raw milk origins would favour the establishment of a resilient microflora and, in turn, the likely emergence of a reservoir of homologous phages. Phages are able to overcome the restriction systems of the host by mutating the restriction sites in their genomes (Coffey and Ross, 2002, Forde and Fitzgerald, 1999). Consequently, the acquisition of novel restriction specificities by genetic recombination may represent the evolutionary response of lactococci to the mutational capacity of phages to avoid the host anti-phage mechanisms.

In addition to R/M-I, *L. lactis* DPC3758 also contains the type II R/M systems R/M-IIa and R/M-IIb, located on pAF45 and pAF22 (Figure 5; Tables A1 and A6 in

Appendix), respectively. Type II R/M systems are typically composed of two enzymes: a REase that recognizes and cleaves a specific short DNA sequence (4–8 bp), and a MTase that modifies the same sequence in order to protect the host DNA against the action of the cognate restriction enzyme (Mruk *et al.*, 2003). The upstream gene in R/M-IIa encodes a protein of 296 aa containing typical N^6-adenine *β*-class MTase (pfam01555) and DNA modification methylase (COG0863) domains, while the downstream gene encodes a protein of 335 aa containing an *Hind*III restriction endonuclease domain (pfam09518). These proteins are virtually identical to the MTase (99%) and REase (96%) enzymes of the LlaCI system found on plasmid pAW153 from *L. lactis* subsp. *cremoris* W15 (Madsen and Josephsen, 1998a). Similar to the LlaCI system, the R/M-IIa genes of *L. lactis* DPC3758 are arranged in a convergent fashion and are separated by a 94-bp sequence. The second type II R/M system, R/M-IIb, consists of a 180-aa REase, belonging to the Bsp6I superfamily (pfam09504), and a 317-aa cytosine-C5 specific DNA methylase (cd00315) belonging to the superfamily of S-adenosylmethionine-dependent MTases (cl12011). In this case, both genes are transcribed in the same direction and their gene products are virtually identical to the REase (98%) and MTase (99%) enzymes of the LlaDII system found on the naturally occurring 8.9-kb plasmid pHW393 in *L. lactis* subsp. *cremoris* W39 (Madsen and Josephsen, 1998b). Taken individually, these R/M-II systems have limited restriction efficiency (Madsen and Josephsen, 1998a, b). However, the most likely benefit for *L. lactis* DPC3758 in harbouring these systems is in their cooperative activity with R/M-I, which confers the strain with wider and greater protection against different phage types. Phages attacking this strain will be sequentially challenged by three systems possessing different R/M specificities; those that will be uncut or restricted with low efficiency by one system will still have to face two other systems before being able to proliferate within the cell. The additive restriction phenomenon and enhanced host protection afforded by the stacking of anti-phage mechanisms is well documented (Coffey and Ross, 2002, Josephsen and Klaenhammer, 1990, Madsen and Josephsen, 1998a, Mills *et al*., 2006, O'Sullivan *et al.*, 1998). *L. lactis* DPC3758 may have naturally opted to stack multiple R/M systems to resist phage invasion as this strategy could prove crucial in providing protection against existing and forthcoming phages, thus allowing the strain to survive and become established in the challenging habitat of raw milk cheeses.

The majority of *L. lactis* DPC3758 plasmids contain genes for conjugative/mobilization functions

Conjugative plasmids are commonly found in lactococci and play a fundamental role in their evolution as they allow the transfer of genome-size segments (Mills *et al.*, 2006). In terms of starter improvement, this feature is extremely important as the conjugal transfer of naturally occurring plasmids is the most widely accepted approach for the genetic improvement of dairy starters designed for human consumption (Coffey and Ross, 2002, Forde and Fitzgerald, 1999, Mills *et al.*, 2006). Six of the 8 plasmids in *L. lactis* DPC3758 contain genetic regions associated with predicted conjugative/mobilization functions. Firstly, pAF45 contains a 17,264 bp conjugal transfer region (*tra1*) (Figure A5; Table 8 in Appendix) composed of 18 genes, which is virtually identical (99% at DNA level, and 97 to 100% at protein level) to the *tra* operon of the lactococcal plasmid pMRC01 (Dougherty *et al.*, 1998), a finding that may be indicative of a genetic exchange event between these plasmids. This region renders pMRC01 self-transmissible, a characteristic that has allowed the efficient transfer of the plasmid to more than 30 different lactococcal strains, including derivatives of commercial starters (Coffey and Ross, 2002, Ryan *et al.*, 1996). Similarly, *tra1* could mediate the transfer of pAF45 and of the associated anti-phage mechanism (R/M-IIa). A second large conjugal region, *tra2,* is present on pAF22, whose overall genetic makeup is completed by the R/M-IIb system, a replication region and two genes encoding for putative site-specific resolvase/invertase (cd03768) enzymes (Figure 5; Table A1 in Appendix). *Tra2* consists of ten genes (*traABCDEFGJKL*) encoding proteins with 43 to 78% similarity to homologs present on plasmids plca36 from *Lactobacillus casei* Zhang (Zhang *et al.*, 2008), pLgLA39 from *Lb. gasseri* LA39 (Ito *et al.*, 2009) and pWCFS103 from *Lb. plantarum* WCFS1 (van Kranenburg *et al.*, 2005). The third conjugal region, *tra3*, of *L. lactis* DPC3758 is located on pAF65 (Figure 5; Table A9 in Appendix), also carrying the R/M-I system. Data for seven (*traCDEFJKI*) of the 12 putative genes composing *tra3* were obtained from the original sequencing, but further attempts to close gaps within this region were unsuccessful. *Tra3* genes encode for products with 31 to 64% similarity mainly to conjugation proteins from *Carnobacterium sp. AT7*. Homology to Tra proteins from plca36 (Zhang et al., 2008), pLgLA39 (Ito *et al.*,

2009), pWCFS103 (van Kranenburg *et al.*, 2005), and pMRC01 (Dougherty *et al.*, 1998) is much lower, in the region of 30-40%.

In addition to the conjugative abilities of pAF65, pAF45 and pAF22, a mobilization gene cluster (*mobC$_1$ABC$_2$*) is located on pAF14 (Figure 5; Table A2 in Appendix). This *mob* region is 99% identical at the nucleotide level to the corresponding region in pNZ4000 (van Kranenburg *et al.*, 2000). MobC1 (164 aa) and MobA (410 aa) belong to the relaxase family (pfam05713) of enzymes and are likely to be involved in DNA strand separation and nicking of the *nic* site of the *oriT* sequences, respectively (van Kranenburg and de Vos, 1998). Both proteins are 98% identical to their homologues from lactococcal plasmids pNZ4000 (van Kranenburg *et al.*, 2000) and pCI528 (Lucey *et al.*, 1993). MobB (207 aa) and MobC2 (200 aa) share 89% and 99% identity with MobB and MobC from pNZ4000, respectively. These proteins are also thought to be involved in plasmid mobilization but their role is unclear (van Kranenburg *et al.*, 2000), as no canonical mobilization domains are found in their sequence. BLAST analysis showed that the *mobC$_2$* gene is recurrent among the *Bacillus*, *Streptococcus* and *Enterococcus* genera while returning a single hit (pNZ4000) from the *Lactococcus* genus. This suggests that *mobC$_2$* has been probably acquired during a recent horizontal gene transfer. Finally, genes encoding mobilization proteins were also found on pAF12 (*mobCD$_1$D$_2$*) and on pAF07 (*mobCA*) (Figure 5; Tables A3-A4 in Appendix).

L. lactis DPC3758 harbours various stress response determinants that may be used as food-grade selection markers

L. lactis DPC3758 plasmids were found to contain a number of stress response genes conferring immunity/resistance to the bacteriocins nisin and lacticin 481, and to the heavy metals cadmium and copper. In addition to their primary role of enhancing the robustness and survival capabilities of the host, these genes also represent four well documented markers of selection for transfer of plasmids in lactococci. As indigenous genotypes, they are considered food-grade and are therefore acceptable for use in dairy cultures designated for human consumption. All have been previously exploited to produce phage resistant derivatives of *L. lactis* cultures and/or to construct food-grade cloning vectors that are central to future programs of starter

improvement (Coffey and Ross, 2002, Khunajakr *et al.*, 1999, Liu *et al.*, 2002, Liu *et al.*, 2005, Mills *et al.*, 2002, Trotter *et al.*, 2001).

In *L. lactis* DPC3758, nisin resistance is afforded by a 956-bp gene present on pAF65 that is 99% identical to the resistance gene *nsr* present on the 60-kb conjugative plasmid pNP40 from *L. lactis* DRC3 (O'Driscoll *et al.*, 2006). NSR (318 aa) contains an N-terminal membrane-spanning domain and a C-terminal processing peptidase domain (cd06567) typical of serine proteases. A recent study showed that lactococcal Nisin Resistance Protein (NSR) confers the host with nisin resistance by using a novel mechanism involving proteolytic removal of the C-terminal tail of nisin. The peptidase/protease domain of NSR has been suggested to reside on the extracellular side of the membrane, where it might contact and degrade nisin (Sun *et al.*, 2009). Plasmid pAF65 also carries the classical immunity determinants *lctFEG* conferring resistance to lacticin 481, which are generally organized within an operon also containing genes (*lctAMT*) involved in lacticin 481 biosynthesis and export (Rince *et al.*, 1994). However, these latter genes are missing in *L. lactis* DPC3758. The proteins encoded by *lctFEG* genes have hydrophobicity profiles and sequence similarities that strongly suggest an ABC transporter protecting the lacticin 481 producer strain from its own lantibiotic (Rince *et al.*, 1997). A copper resistance operon, *lcoRSABC*, (Figure 5; Table A12 in Appendix) identical to corresponding homologues on lactococcal plasmids pND306 (Khunajakr *et al.*, 1999, Liu *et al.*, 2002) and pSK11P (Siezen *et al.*, 2005) is also present on pAF65. As observed in pSK11P, the operon is flanked by two copies of IS-LL6 transposase A to form a putative composite transposon (Siezen *et al.*, 2005). The operon confers copper resistance in *L. lactis* by reducing the intracellular accumulation of copper ions via a two-component regulatory system composed of a DNA-binding response regulator (LcoR, 223 aa) and a signal transduction histidine kinase (LcoS, 465 aa), which regulate the copper-inducible transcription of downstream genes *lcoABC.* All LcoABC proteins are required for copper resistance, but none of them contain any putative copper-binding motifs. This suggests that LcoABC proteins play an indirect role in the cell to achieve copper homeostasis (Khunajakr *et al.*, 1999, Liu *et al.*, 2002). LcoA (269 aa) is a putative prolipoprotein diacylglyceryl transferase (cl00478) and contains seven membrane-spanning hydrophobic regions, which suggests an intra-membrane location. LcoB (451 aa) is an abundantly secreted protein of *L. lactis*

but its function remains unknown. The C-terminal cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain (PF05257) of LcoB would suggest a putative role as amidase involved in peptidoglycan metabolism (Siezen *et al.,* 2005). It is probably attached to the cell membrane through the N-terminal spectrin repeat (cd00176) domain, which might also provide binding sites to favour the assembly of large multi-protein regulatory or resistance complexes (Djinovic-Carugo *et al.,* 2002). LcoC (464 aa) is highly homologous to multicopper oxidases (COG2132) and contains a lipoprotein signal motif which suggests a likely translocation across the membrane to the outside of the cell to be covalently anchored by LcoA to membrane lipids (Siezen *et al*., 2005). Analysis of growth in the presence of copper showed the functionality of the *lco* operon in *L. lactis* DPC3758 (Figure 7c, d). While the growth of *L. lactis* MG1363 was strongly inhibited by $CuSO_4$ concentrations above 1 mM, *L. lactis* DPC3758 was able to grow relatively well even at copper concentrations of 6 mM.

Resistance to cadmium in *L. lactis* DPC3758 is conferred by two overlapping genes present on pAF14 (Figure 5; Table A2 in Appendix), which are 99% identical to the cadmium resistance cassette *cadAC* present in lactococcal plasmids pAH82 (O'Sullivan *et al.*, 2001), pGdh442 (Tanous *et al.*, 2007) and pNP40 (O'Driscoll *et al.*, 2006), and in several other gram-positive bacteria (Schirawski *et al.*, 2002). These genes are organized as an operon, and their transcription is cadmium dependent in vivo. The complete cassette has been shown to confer cadmium and zinc resistance to both *S. thermophilus* and *L. lactis*, but expression of *cadA* alone is sufficient to give resistance (Schirawski *et al.*, 2002). CadA (705 aa) is a cation-translocating P-type ATPase (COG2217, COG4087) that allows efflux of cadmium, thus resulting in reduced accumulation of the toxic cation. CadC (119 aa) is highly similar to ArsR-type regulatory proteins and has been shown to act as a repressor of the cadmium-dependent expression of *cadA* by binding to its own promoter region, an interaction that is lost in the presence of cadmium (Schirawski *et al.*, 2002, Yoon *et al.*, 1991). The functionality of the *cadAC* cassette of pAF14 was tested by comparing the growth of DPC3758 to that of the Cd^S strain *L. lactis* MG1363, in presence of increasing concentrations of $CdCl_2$ (Figure 7a, b). *L. lactis* DPC3758 was able to tolerate $CdCl_2$ concentrations of up to 0.3 mM, whereas *L. lactis* MG1363 was inhibited by concentrations as low as 0.05 mM.

In *L. lactis* DPC3758, three markers (nisin, lacticin 481 and copper resistance) are contained on a plasmid (pAF65) also carrying conjugative proteins (Tra3) and a phage resistance mechanism (R/M-I). This represents the ideal situation as it overcomes the known difficulties encountered in delivering natural anti-phage systems to industrial strains (Coffey and Ross, 2002, Forde and Fitzgerald, 1999, Mills *et al.*, 2006).

The R/M systems of *L. lactis* DPC3758 are transmissible and their stacking increases the phage resistance of the host

L. lactis DPC3758 plasmids are of significant interest for programmes of starter improvement as the presence of multiple transfer regions and food-grade selectable markers would facilitate their delivery to industrial strains. Therefore, a series of conjugation experiments were undertaken to evaluate the transmissible nature of these plasmids. In agreement with previous reports (O'Sullivan *et al.*, 2001, Trotter *et al.*, 2001), cadmium resistance proved to be a very effective selectable marker for plasmid transfer to the chosen recipient, *L. lactis* MG1614. Analysis of the plasmid profiles of 20 random transconjugants showed that all had acquired a plasmid of approximately 14 kb, similar in size to pAF14 which contains the cadmium determinants. Interestingly, only few transconjugants contained pAF14 on its own, whereas the majority had acquired one or more additional plasmids, giving rise to a total of seven major plasmid patterns (Figure 8a). Based on plasmid profile analysis, it was deduced that the transconjugants Tc5, Tc6 and Tc7 harboured different combinations of the three *L. lactis* DPC3758 R/M systems, a finding that was confirmed using primers specific to each of these mechanisms (Figure 8b). This allowed the evaluation of the restriction efficiency afforded by each R/M system against phages c2 and 712, representatives of the predominant phage types found in dairy industry, in a plasmid-free background (Figure 8c).

R/M-IIa and R/M-IIb were shown to restrict the 936 phages more efficiently than the c2 phages, presumably due to the larger genome and correspondingly higher number of recognition sites in the former. The phage EOP is known to decrease exponentially as the number of sites in the viral genome increases (Forde and Fitzgerald, 1999). The presence of R/M-IIa in MG1614-Tc6 resulted in a 2-log

decrease of the EOP of phage 712 whereas it proved almost ineffective against phage c2. These results are in agreement with the restriction efficiency reported for the LlaCI system, which is homologous to R/M-IIa and was shown to confer *L. lactis* strains with decreased sensitivity to phages of the 936 or P335 but not to the c2 species (Madsen and Josephsen, 1998a). The inefficacy against these latter phages was associated with the lack of *Hind*III/*LlaC*I restriction sites in their genomes (Madsen and Josephsen, 1998a). Superimposition of R/M-IIb on R/M-IIa (MG1614-Tc7) resulted in additive restriction of phage 712 by causing a further 2.5-log EOP reduction and increasing the efficiency of restriction to close to 10^{-5}. Conversely, R/M-IIb stacking did not improve host resistance against phage c2. These results are less than what expected from the additive restriction afforded by a LlaDII homologous, as this mechanism was reported to reduce by 2- and 4-fold the EOPs of phage species c2 and 936, respectively (Madsen and Josephsen, 1998b).

While the chromosomally-encoded type I R/M system of *L. lactis* IL1403 is known to restrict c2-type phages with low efficiency (2 logs) (Schouler *et al.*, 1998b), its homolog in *L. lactis* DPC3758 (R/M-I) was found to possess a broader and greater restriction ability. Indeed, the superimposition of R/M-I on the low efficiency R/M-IIb system (MG1614-Tc5) provided the *L. lactis* MG1614 host with complete protection against both phages c2 and 712. These results confirm that the novel TRD of R/M-I HsdS compared to that of the *L. lactis* IL1403 homolog is able to provide the system with altered specificity and restriction activity. HsdS subunits determine the target sequence specificity of type I R/M systems via both TRDs, each one responsible for recognizing half of a bipartite target site (Murray, 2000). It is therefore obvious that the acquisition of a different TRD, which may occur via recombination of *hsdS* genes as observed in pAH90 (O'Sullivan *et al.*, 2000), is able to generate a new R/M specificity.

Results of the conjugation study reveal that most of *L. lactis* DPC3758 plasmids, including those containing the R/M systems, are self-conjugative and may be co-mobilized alongside other plasmids even in the absence of specific selection. This feature has important commercial implications as it may be exploited to develop industrial cultures with improved phage resistance through targeted delivery of anti-phage mechanisms. Theoretically, all R/M systems of *L. lactis* DPC3758 may be sequentially or concomitantly transferred to phage-sensitive industrial starters, thus

providing the host with enhanced protection against phage attack as a result of the additive resistance mediated by the stacking of three different restriction specificities. Such strategies are generally limited by the lack of a suitable marker on the plasmid, which would facilitate the selection of transconjugants harbouring the newly acquired trait. This limitation should not affect the delivery of *L. lactis* DPC3758 plasmids as they encode four phenotypes with well-documented application as food-grade selectable markers (Liu *et al.*, 2002, Mills *et al.*, 2002, Trotter *et al.*, 2001).

CONCLUSION

In this study, we have investigated the plasmid distribution and diversity of a collection of natural *L. lactis* isolates from raw milk cheeses for the presence of genetic traits with potential application in programmes of dairy starters' improvement. Natural lactococci are expected to possess a heterogeneous range of properties, which are mostly plasmid-encoded and provide the host with a competitive advantage to adapt and become established within its ecological niche (Mills *et al.*, 2006). The results of this study support the assumption and confirm the value of the approach targeting natural isolates. An intriguing abundance and diversity of plasmids was observed in all investigated strains. Moreover, when the 8-plasmid complement of the natural isolate *L. lactis* DPC3758 was sequenced and functionally analysed, a wealth of genetic traits encoding multiple anti-phage systems, resistance genes/selectable markers and functional conjugative/mobilization regions was found. The identification of such natural plasmids is of tremendous benefit for the successful outcome of any programme of starter improvement. Indeed, the association of food-grade selectable markers and mobilization functions on many of these plasmids will facilitate their exploitation to obtain lactococcal industrial strains with enhanced phage protection and robustness.

L. lactis DPC3758 plasmids also provide another example of the major role played by these genetic elements in contributing to host fitness and preservation within its ecological niche. Raw milk is likely to prove an extremely challenging habitat to live in due to the presence of a large and heterogeneous microflora and, putatively, of related phages. Harbouring multiple conjugative plasmids containing three separate R/M systems and various stress resistance determinants clearly provides *L. lactis* DPC3758 with a competitive advantage to survive in this environment. The observation that 6 out of 8 plasmids carry mobilization functions is suggestive of their likely acquisition by horizontal transfer from organisms occupying the same habitat, and also indicative of the high level of genome plasticity of the strain.

In conclusion, our study provides the gene knowledge base to exploit single plasmids from *L. lactis* DPC3758, although the whole strain might be well and safely employed as a component of novel starter formulations. *L. lactis* DPC3758 has been indeed genotyped as a *cremoris* strain, which is highly desirable in the manufacture of

Cheddar and other cheese varieties (Delgado and Mayo, 2004, Salama *et al.*, 1995), and also does not contain any undesired virulence or antibiotic resistance genes. The wealth of potentially useful genetic traits found in *L. lactis* DPC3758 plasmids also reaffirms the importance of ongoing screening aimed at isolating and characterizing new lactococcal strains from natural non-dairy ecosystems. If pursued, this strategy will provide a whole new range of natural starters or properties to develop novel or improved starter formulations with enhanced robustness and long-term efficiency.

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TABLES

Table 1. Bacterial strains and lactococcal bacteriophages used in this study

Strains or bacteriophages	Description	Source[a] or reference
Natural bacterial isolates		
L. lactis DPC3758	Isolated from a French raw milk cheese: contains 8 plasmids	TFRC culture collection
L. lactis DPC3776	Isolated from a Spanish raw milk cheese: contains 10 plasmids	TFRC culture collection
L. lactis DPC3874	Isolated from a French raw milk cheese: contains 6 plasmids	TFRC culture collection
L. lactis DPC3749	Isolated from a Spanish raw milk cheese: contains 9 plasmids	TFRC culture collection
L. lactis DPC3880	Isolated from a French raw milk cheese: contains 6 plasmids	TFRC culture collection
L. lactis DPC3694	Isolated from a Spanish raw milk cheese: contains 6 plasmids	TFRC culture collection
L. lactis DPC3754	Isolated from a French raw milk cheese: contains 4 plasmids	TFRC culture collection
L. lactis DPC3756	Isolated from a French raw milk cheese: contains 5 plasmids	TFRC culture collection
L. lactis DPC3783	Isolated from a Italian raw milk cheese: contains 7 plasmids	TFRC culture collection
L. lactis DPC3901	Isolated from a Greek raw milk cheese: contains 4 plasmids	TFRC culture collection
L. lactis DPC3780	Isolated from a Italian raw milk cheese: contains 2 plasmids	TFRC culture collection
L. lactis DPC3757	Isolated from a French raw milk cheese: contains 4 plasmids	TFRC culture collection
L. lactis DPC3778	Isolated from a Spanish raw milk cheese: contains 10 plasmids	TFRC culture collection
L. lactis DPC3881	Isolated from a French raw milk cheese: contains 7 plasmids	TFRC culture collection
L. lactis DPC3907	Isolated from a Italian raw milk cheese: contains 2 plasmids	TFRC culture collection
L. lactis DPC3911	Isolated from a Italian raw milk cheese: contains 7 plasmids	TFRC culture collection
L. lactis DPC3926	Isolated from a Spanish raw milk cheese: contains 5 plasmids	TFRC culture collection
L. lactis DPC3898	Isolated from a Greek raw milk cheese	TFRC culture collection
L. lactis DPC3904	Isolated from a Spanish raw milk cheese	TFRC culture collection
L. lactis DPC3912	Isolated from a Italian raw milk cheese	TFRC culture collection
Bacterial strains		
L. lactis MG1363	Plasmid-free derivative of *L. lactis* subsp. *cremoris* 712	TFRC culture collection
L. lactis MG1364	Rifampicin and streptomycin-resistant derivative of *L. lactis* MG1363	TFRC culture collection
L. lactis IL1403	Plasmid-free derivative of *L. lactis* IL594; representative of subspecies *lactis*	(Chopin *et al.* , 1984)
L. lactis HP	Cheese starter; indicator strain; representative of subspecies *cremoris*	TFRC culture collection
L. lactis DRC3	DNA size marker strain; contains 8 plasmids (2, 3.4, 4.8, 5.2, 35.8, 51, 65 and 78 kb); representative of subspecies diacetylactis	(Mckay and Baldwin, 1984)
L. lactis DPC3147	Lacticin 3147 producer	(Ryan *et al.* , 1996)
L. lactis DPC5558	Nisin producer	TFRC culture collection
L. lactis DPC5552	Lacticin 481 producer	TFRC culture collection
L. lactis DPC938	Lactococcins A, B and M producer	TFRC culture collection
L. innocua DPC3306	Indicator strain	TFRC culture collection
P. acidilactici DPC5492	Indicator strain	TFRC culture collection
Lactobacillus paracasei 43338	Indicator strain	TFRC culture collection
Bacteriophages		
c2	lytic phage; member of the c2-type phage specie	(Jarvis *et al.* , 1991)
ml3	lytic phage; member of the c2-type phage specie	(Jarvis *et al.* , 1991)
eb1	lytic phage; member of the c2-type phage specie	(Jarvis *et al.* , 1991)
sk1	lytic phage; member of the 936-type phage specie	(Jarvis *et al.* , 1991)
712	lytic phage; member of the 936-type phage specie	(Jarvis *et al.* , 1991)
hp	lytic phage; member of the 936-type phage specie	(Jarvis *et al.* , 1991)

[a] TFRC: Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

Table 2. Primers used in this study to map gene features of technological importance to either plasmid pAF65 or pAF45 of *L. lactis* DPC3758

Primer	Sequence (5'-3')	Size (bp)	Target
R/M-I (F[a])	ACTACTTTAAGGAACTGGGAGTG	850	R/M-I[c]
R/M-I (R[b])	TGCGTAAAACCTTCAACATAATCT		
R/M-IIa (F)	TTGCTAATAAACTAAATAATGTGCTAAG	693	R/M-IIa[d]
R/M-IIa (R)	TTTCTAAATTATATGCTCCTACACCA		
tra1 (F)	AAAAACCTTGAATAAATACCAT	659	tra1[e]
tra1 (R)	TTAAACTTACTCAAAAATCTACACA		
tra3 (F)	CAAGTAGGAGATTAGGTTATAGTCGCAGTT	292	tra3[f]
tra3 (R)	AAATTTTCTAAGGTGGCATATCAAGTCAAG		
lco (F)	GATGTCGGCTTTCTTCTCGGATGATG	660	lco[g]
lco (R)	ATGTAGTTTTGATGGAAGAAGGTAGTAGT		
nsr (F)	GCAGTATGTGCTTTATTTTTAGG	650	nsr[h]
nsr (R)	CTTAAAGCACAAAGCGGTT		
lc481 (F)	AGGTGACATTTTTGGCTTTCT	544	lc481[i]
lc481 (R)	TTCTCCCTTTTTAATAATTGCTACTG		

[a]F = forward primer; [b]R = reverse primer; [c]R/M-I = type I restriction/modification system; [d]R/M-IIa = type II restriction/modification system; [e]tra1 = conjugation region *tra1* ; [f]tra3 = conjugation region *tra3;* [g]lco = copper resistance operon; [h]nsr = nisin resistance gene; [i]lc481 = lacticin 481 immunity genes

Table 3. Selection of phenotypic and genotypic tests showing differences useful to the identification of isolates belonging to the *Lactococcus* genus

Analytical tests	Bacterial isolate (DPC #)																			
	3757	3776	3778	3874	3881	3907	3911	3926	3749	3880	3694	3754	3756	3783	3901	3758	3780	3898	3904	3912
Phenotypic																				
Esculin hydrolysis	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
Growth at 10°C	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–
Growth at 45°C	–	–	+	–	–	–	+	+	–	–	+	+	–	–	+	+	–	–	–	+
Growth in 6.5% NaCl	–	–	–	+	–	–	+	–	–	+	+	–	–	–	+	+	+	+	–	+
Genotypic																				
Enterococcus primers	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
Lactococcus primers	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–

FIGURES

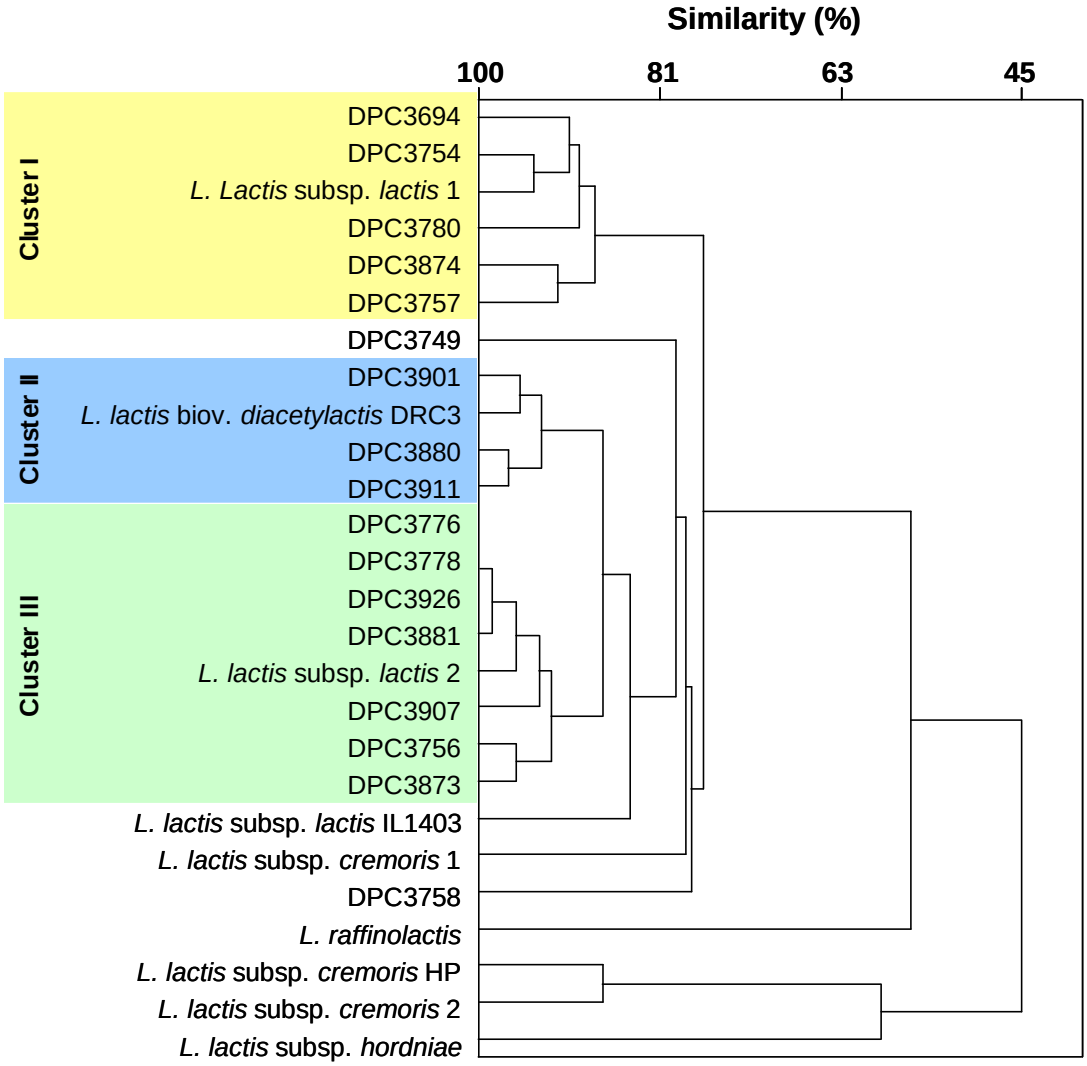


Figure 1. Hierarchical cluster analysis (HCA) of the carbohydrates fermentation profiles of natural *Lactococcus lactis* isolates (DPC #) and of representative strains *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* biovar. *diacetylactis* DRC3, and *L. lactis* subsp. *cremoris* HP. The fermentation profiles reported by the API 50 CH kit's manufacturer to be typical of the subspecies *lactis* (1 and 2), *cremoris* (1 and 2) and *hordniae*, and of *L. raffinolactis* are included in the analysis as external references.

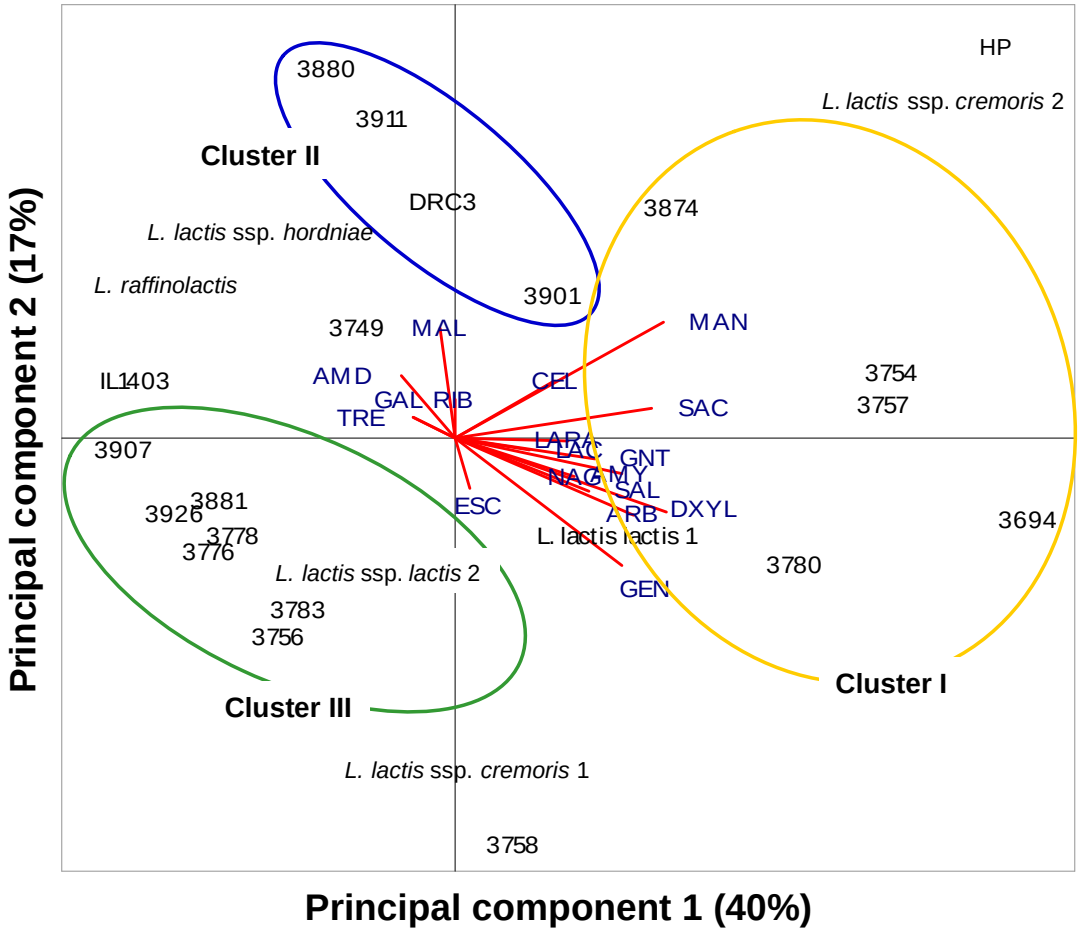


Figure 2. Principal component analysis (PCA) of the carbohydrates fermentation profiles of natural *Lactococcus lactis* isolates (DPC #) and of representative strains *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* biovar. *diacetylactis* DRC3, and *L. lactis* subsp. *cremoris* HP. The fermentation profiles reported by the API 50 CH kit's manufacturer to be typical of the subspecies *lactis* (1 and 2), *cremoris* (1 and 2) and *hordniae*, and of *L. raffinolactis* are included in the analysis as external references.

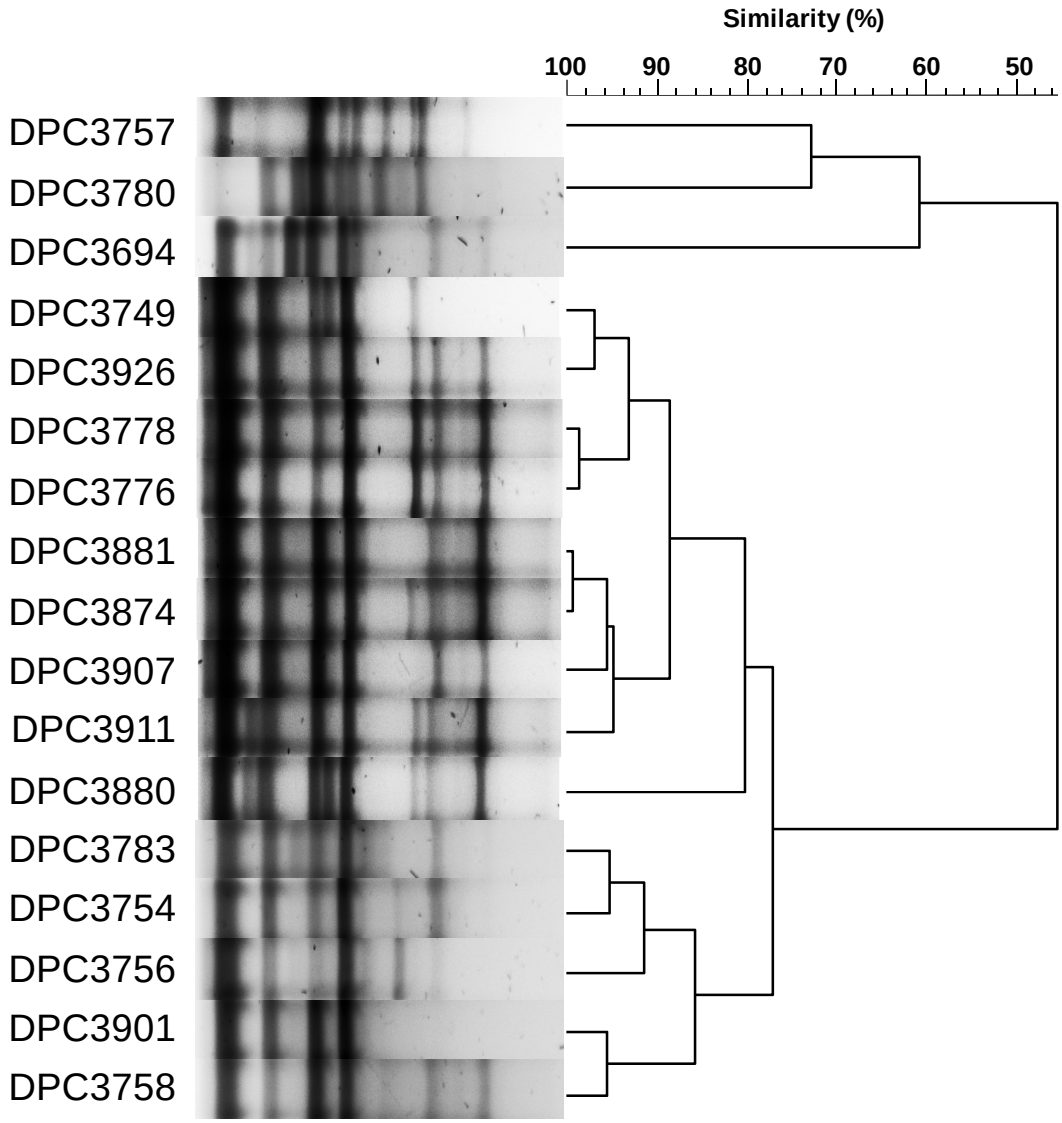


Figure 3. HCA of randomly amplified polymorphic DNA (RAPD)-PCR profiles of natural lactococcal isolates.

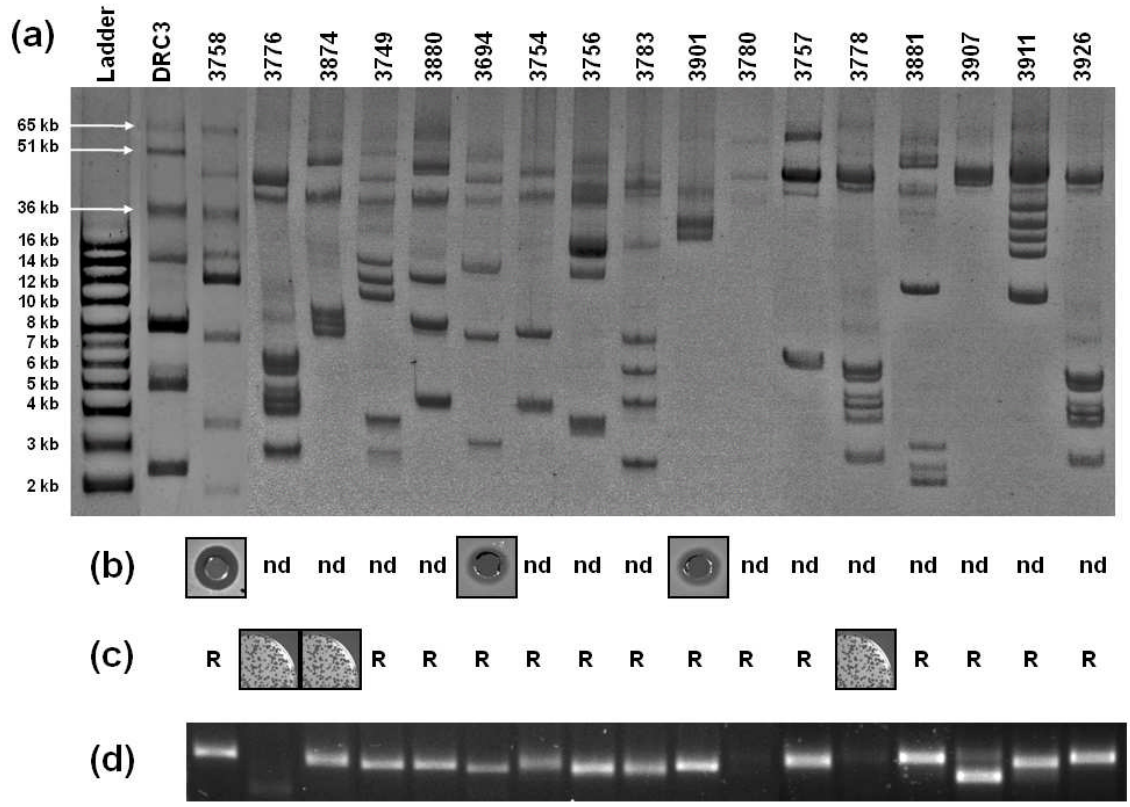
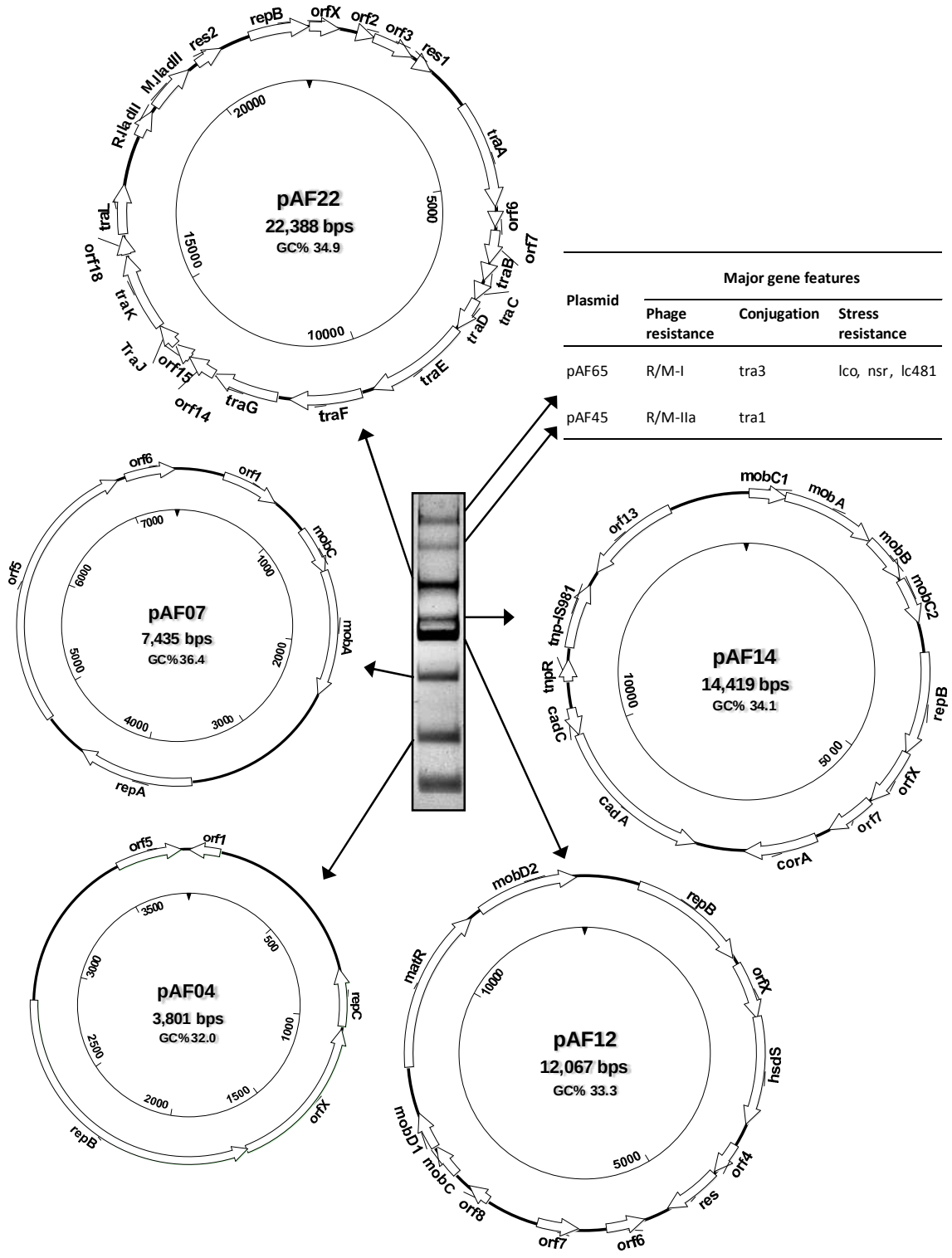


Figure 4. Preliminary characterization of the natural lactococcal isolates (DPC#). (a) Distribution and diversity of the plasmid DNA complement. The plasmid profile of *L. lactis* DRC3 provides reference plasmids of large sizes (36, 51 and 65 kb) as a complement to the 2 to 16 kb molecular size ladder. (b) Antimicrobial production on agar well diffusion assays by using *L. lactis* HP as indicator strain. Icon=positive; nd=not detected. (c) Resistance/sensitivity to c2-type phages (c2, ml3, eb1) by using the spot test technique. R=resistant; Icon=sensitive. (d) PCR detection of type I restriction/modification *hsdS* subunits.

Figure 5.



Plasmid	Major gene features		
	Phage resistance	Conjugation	Stress resistance
pAF65	R/M-I	tra3	lco, nsr, lc481
pAF45	R/M-IIa	tra1	

Figure 5. Physical and genetic maps of plasmids pAF22, pAF14, pAF12, pAF07 and pAF04 of *L. lactis* DPC3758. Position and orientation of genes is indicated by arrows. Inner circles show the nucleotide numbering. The table shows the features of technological interest mapped to plasmids pAF65 and pAF45 by PCR. Features acronyms are as indicated in Table 2.

Figure 6.

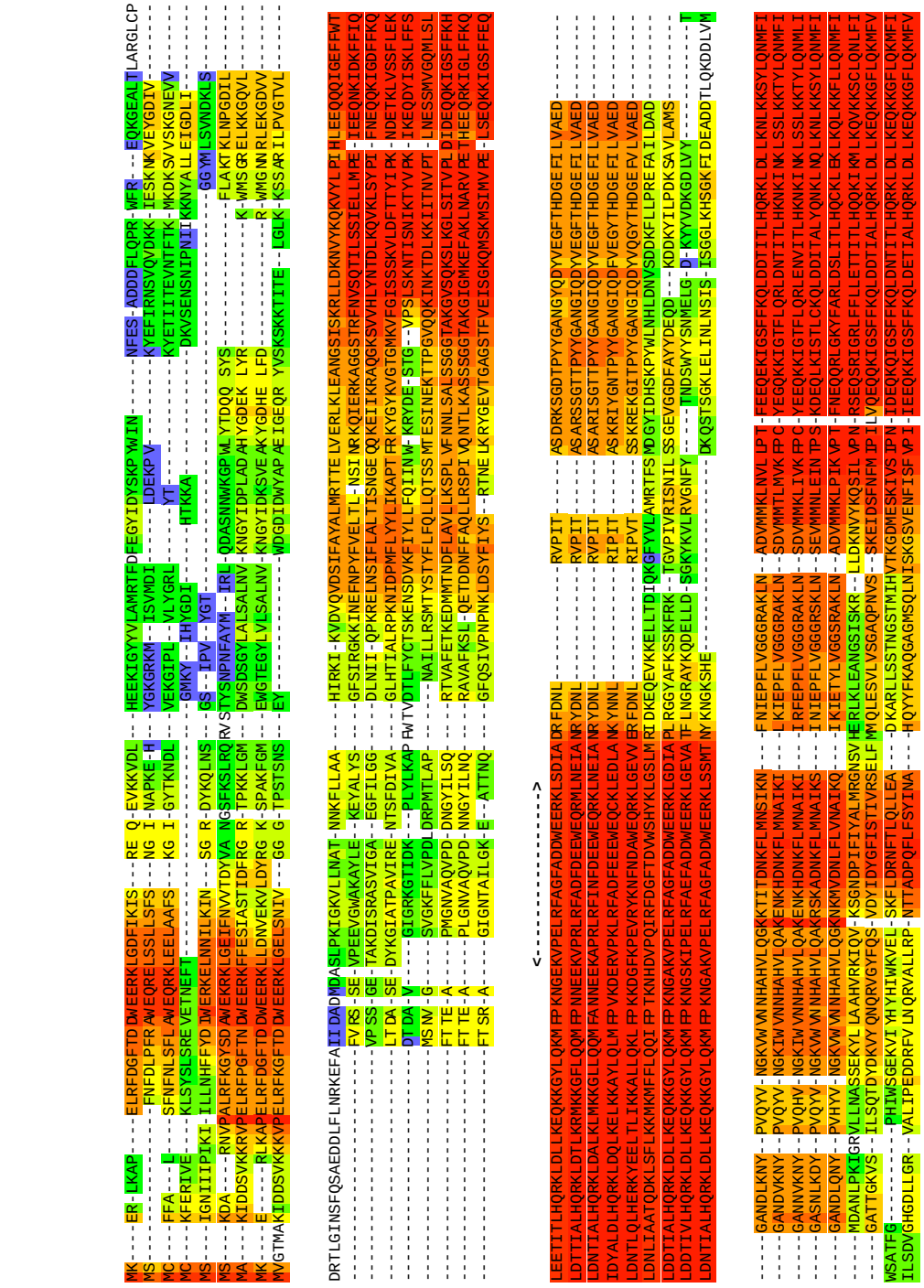

L. lactis DPC3758
L. monocytogenes 5578 (284800800)
L. welshimeri SLCC5334 (116871901)
E. faecalis AR01/DG (257417155)
L. salivarius UCC118 (90961893)
L. casei BL23 (191639032)
L. lactis
L. lactis Il1403 (15672634)
L. lactis diacetylactis (23217024)

Figure 6. Multiple alignment of the amino acid sequence of the type I R/M HsdS of *L. lactis* DPC3758 and top BLAST hits. The GenInfo Identifier for each organism is indicated within brackets. Orange-to-red areas are indicative of high similarity, whereas green-to-yellow-to-blue areas are indicative of low similarity. The 19-aa sequence of the type I R/M HsdS of *L. lactis* DPC3758, in common with the HsdS of pAH90 and potentially involved in TRDs recombination, is indicated by the dotted arrow.

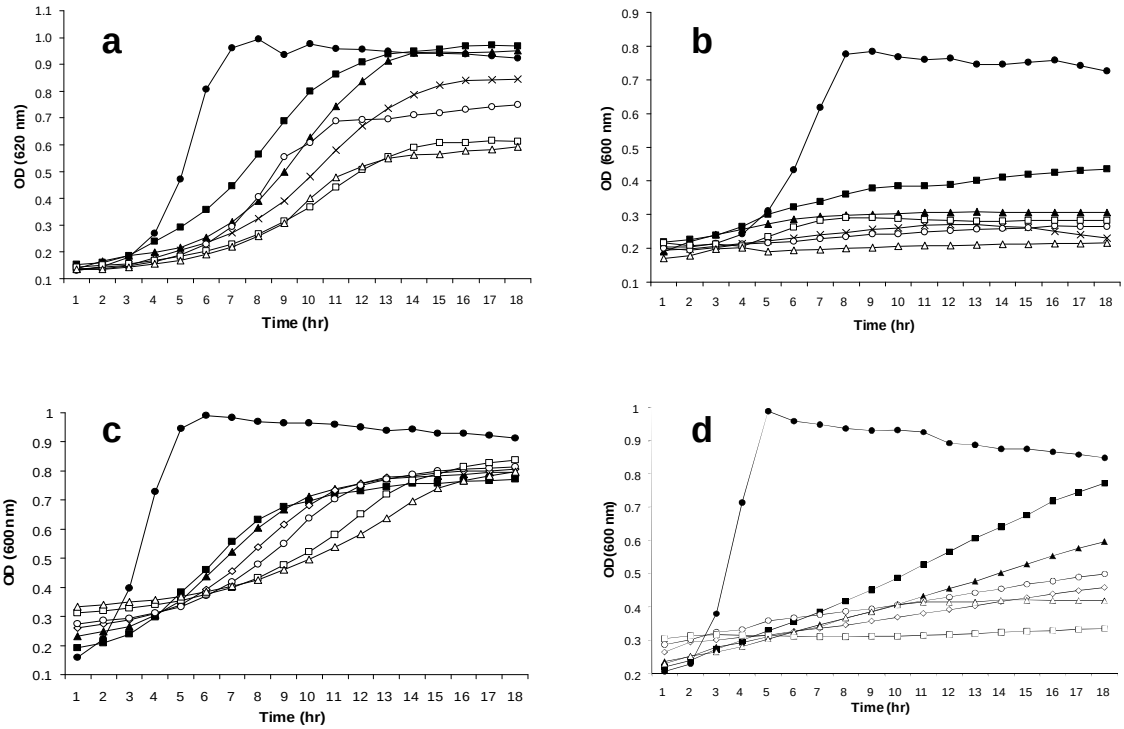


Figure 7. Functional analyses of the cadmium (a, b) and copper (c, d) resistance operons in *L. lactis* DPC3758 compared to the sensitive strain *L. lactis* MG1363. Growth curves of *L. lactis* DPC3758 (a) and MG1363 (b) in media containing 0 (●), 0.05 (■), 0.1 (▲), 0.15 (x), 0.2 (○), 0.25 (□) and 0.3 mM (Δ) cadmium chloride. Growth curves of *L. lactis* DPC3758 (c) and MG1363 (d) in media containing 0 (●), 1 (■), 2 (▲), 3 (◊), 4 (○), 5 (□) and 6 mM (Δ) cupric sulfate.

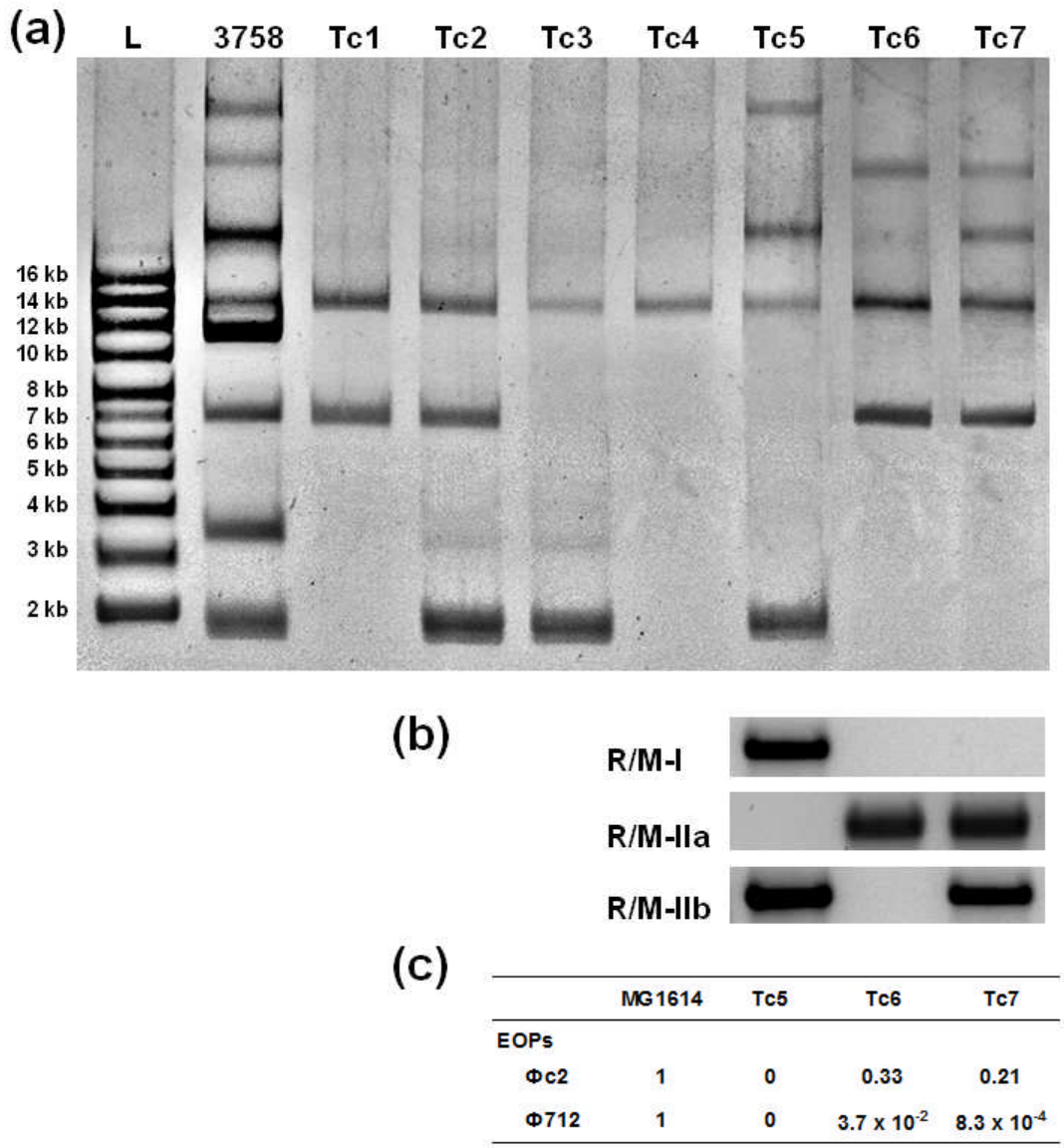


Figure 8. (a) Plasmid profiles of the plasmids donor strain *L. Lactis* DPC3758 and of *L. Lactis* MG1614 transconjugants (Tc1 to Tc7) obtained by using cadmium resistance as selectable marker. (b) PCR detection of R/M systems in selected transconjugants. (c) Efficiency of plaquing (EOP) of phages c2 and 712 on selected transconjugants harbouring different combinations of R/M systems.

Appendix

Table A1. List of ORFs identified in pAF22 (size 22,388 bp; GC content 34.95%)

Gene name	Start (bp)	End (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
orfX	1	576	+	192	33.5	hypothetical protein, OrfX-like	hypothetical protein pSRQ700_02	171/207 (82%)	1e-87	*Lactococcus lactis*, pSRQ700
orf2	918	1259	+	114	29.53	transcriptional regulator, PadR family, HTH_ARSRsuperfamily, cl00088	transcriptional regulator	113/114 (99%)	1e-56	*Enterococcus faecalis* E1Sol
orf3	1252	2070	+	273	26.86	conserved hypothetical protein	conserved hypothetical protein	267/273 (97%)	1e-137	*Enterococcus* sp. 7L76
res1	2179	2532	+	118	38.98	Serine Recombinase family, Resolvase and Invertase subfamily, cd03768	resolvase	118/118 (100%)	2e-61	*Enterococcus faecalis* E1Sol
traA	3407	5473	+	689	40.34	nicking enzyme TraA protein; MobA/MobL family, pfam03389	conserved hypothetical protein	353/693 (50%)	0.0	*Lactobacillus paracasei* subsp. *paracasei* 8700:2
orf6	5562	5861	+	100	35	conserved hypothetical protein, putative transcriptional regulator	unknown	41/83 (49%)	2e-13	*Lactococcus lactis* subsp. *lactis* bv. *diacetylactis*
orf7	5924	6571	+	216	35.8	hypothetical protein	hypothetical protein pWCFS103_36	87/183 (47%)	2e-43	*Lactobacillus plantarum* WCFS1
traB	6581	6913	+	111	41.14	conjugation protein	TraB protein	82/112 (73%)	3e-30	*Lactobacillus buchneri* ATCC 11577
traC	6978	7322	+	115	33.62	conjugation protein	TraC protein	71/115 (61%)	7e-35	*Lactococcus lactis*, pMRC01
traD	7309	7956	+	216	35.03	TraD protein-like	conjugation protein	132/213 (61%)	5e-70	*Lactobacillus plantarum* WCFS1, pWCFS103
traE	7972	9966	+	665	36.94	VirB4_CagE, type IV secretion/conjugal transfer ATPase, VirB4 family, TIGR00929; P-loop containing Nucleoside Triphosphate Hydrolases superfamily, cl09099	protein TrsE	526/670 (78%)	0.0	*Lactobacillus casei* str. Zhang, plca36
traF	10189	11577	+	463	34.41	essB, Predicted membrane protein essB, pfam10140	TraF protein	200/463 (43%)	3e-100	*Lactobacillus buchneri* ATCC 11577
traG	11809	13086	+	426	41.15	conjugation protein, traG-like; glucosaminidase superfamily; NLPC/P60 superfamily	conserved hypothetical protein	231/428 (53%)	8e-121	*Enterococcus faecalis*
orf14	13093	13671	+	193	34.19	hypothetical protein	ORF51	74/192 (38%)	5e-26	*Lactococcus lactis*, pNP40
orf15	13652	14032	+	127	31.75	putative thioredoxin; Thioredoxin-like superfamily domain (cd02947)	ORF52	54/105 (51%)	7e-25	*Lactococcus lactis*, pNP40
traJ	14028	14489	+	154	33.98	conjugation protein, TrsJ-like	protein TrsJ	69/142 (48%)	2e-32	*Lactobacillus casei* str. Zhang, plca36
traK	14489	15976	+	496	36.55	conjugation protein, TrsK-like; P-loop NTPase superfamily (cl09099); TraG/TraD family (pfam02534)	hypothetical protein pLgLA39_p38	344/495 (69%)	0.0	*Lactobacillus gasseri*, pLgLA39
orf18	16002	16370	+	123	32.52	hypothetical protein, contains PS51257 domain	conserved hypothetical protein	48/130 (36%)	2e-12	*Lactobacillus casei* str. Zhang, plca36
traL	16395	17348	+	318	34.48	conjugation protein, TrsL-like protein	conjugation protein	132/284 (48%)	2e-62	*Lactobacillus plantarum* WCFS1, pWCFS103
R.lladII	18303	18842	+	180	32.4	type II restriction endonuclease; RE_Bsp6I superfamily, pfam09504	restriction endonuclease	177/180 (98%)	1e-101	*Lactococcus lactis* subsp. *cremoris*, pHW393
M.lladII	18951	19901	+	317	32.17	type II methylase; DNA cytosine methylase (PRK10458); NADB_Rossmann superfamily (cl09931); Cytosine-C5 specific DNA methylases (cd00315)	methylase	314/317 (99%)	0.0	*Lactococcus lactis* subsp. *cremoris*, pHW393
res2	20093	20650	+	186	40.5	putative resolvase; Serine Recombinase family domain (cd03768); PinR, Site-specific recombinases, DNA invertase Pin homologs (COG1961)	hypothetical protein pSRQ700_09	184/186 (98%)	5e-101	*Lactococcus lactis*, pSRQ700
repB	21222	22385	+	388	36.25	Replication protein, RepB-like (COG5527); Rep_3, Initiator Replication protein (pfam01051); L_lactis_RepB_C, L. lactis RepB C-terminus (pfam06430)	replication initiator protein	365/388 (94%)	0.0	*Lactococcus lactis* subsp. *cremoris* SK11, plasmid 1

Table A2. List of ORFs identified in pAF14 (size 14,419 bp; GC content 34.07%)

Gene name	Start (bp)	End (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity(%)	E-value	Organism, plasmid
mobC1	36	527	+	164	35.16	Mobilization protein, MobC-like (Mob-C superfamily; pfam05713)	Orf1 gene product	162/164 (98%)	8e-89	*L. lactis*
mobA	509	1738	+	410	40.73	Mobilization protein, MobA-like (Relaxase/Mobilisation nuclease domain, pfam03432; phosphodiesterase domain, PRK12704)	Mobilization protein	402/409 (98%)	0.0	*L. lactis*
mobB	1738	2358	+	207	37.03	Mobilization protein, MobB-like	Putative mobilization protein	186/207 (89%)	2e-106	*L. lactis* subsp. *cremoris*
mobC2	2378	2977	+	200	32	Mobilization protein, MobC-like (Fic/DOC superfamily; cl00960)	Putative mobilization protein	199/200 (99%)	2e-111	*L. lactis* subsp. *cremoris*
repB	3361	4683	+	441	36.28	Replication protein, RepB-like; Rep_3, Initiator Replication protein, pfam01051; L_lactis_RepB_C, L. lactis RepBC-terminus, pfam06430; Protein involved in initiation of plasmid replication, COG5527	repB	331/443 (74%)	0.0	*L. lactis*
orfX	4679	5449	+	257	36.7	Replication-associated protein, RepX-like	hypothetical protein pDB0R0p06	254/257 (98%)	5e-141	*L. lactis* subsp. *lactis* bv. *diacetylactis*
orf7	5255	5899	+	215	36.43	hsdR, N-terminus; hsdS, C-terminus; pfam01420, Methylase_S, Type I restriction modification DNA specificity domain	restriction subunit	135/181 (74%)	8e-66	*L. lactis* subsp. *lactis* bv. *diacetylactis*
corA	6088	7035	+	316	31.85	CorA-like Mg2+ transporter protein; CorA super-family, cl00459	CorA like magnesium and cobalt transport protein	310/316 (98%)	1e-177	*L. lactis* subsp. *cremoris* MG1363
cadA	9778	7664	-	705	37.25	COG2217, ZntA, Cation transport ATPase; cd00371, HMA, Heavy-metal-associated domain; COG4087, Soluble P-type ATPase	cadmium efflux ATPase CadA	705/705 (100%)	0.0	*L. lactis* subsp. *lactis*
cadC	9778	10134	-	119	31.37	cd00090, HTH_ARSR, Arsenical Resistance Operon Repressor and similar prokaryotic, metal regulated homodimeric repressors; COG0640, ArsR, Predicted transcriptional regulators	cadmium resistance regulator CadC	119/119 (100%)	1e-61	*L. lactis* subsp. *lactis*
tnpR	10482	10766	+	95	40.35	cl02788, Serine Recombinase superfamily; COG1961, PinR, Site-specific recombinases, DNA invertase Pin homologs; truncated, C-terminus	DNA invertase/resolvase	95/95 (100%)	8e-49	*L. lactis*
tnp-IS981	10917	11768	+	284	36.85	pfam00665, rve, Integrase core domain; PHA02517, putative transposase OrfB; COG2801, Tra5, Transposase and inactivated derivatives	transposase	283/284 (99%)	3e-166	*L. lactis* subsp. *cremoris* SK11
orf13	11947	13200	-	418	24.96	polysaccharide lyase family 9,YwoF	ywof	168/426 (39%)	8e-73	*Bacillus* sp. NRRL B-14911

Table A3. List of ORFs identified in pAF12 (size 12,067 bp; GC content 33.3%)

Gene name	Start (bp)	End (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
repB	604	1926	+	441	36.35	replication initiator protein	repB	384/441 (87%)	0.0	*Lactococcus lactis*
orfX	1922	2638	+	239	33.19	replication-associated protein	hypothetical protein pNZ4000_03	136/239 (56%)	7e-56	*Lactococcus lactis* subsp. *cremoris*, pNZ4000
hsdS	2617	3813	+	399	31.49	Methylase_S domain [pfam01420]; HsdS domain [COG0732]	putative restriction-modification enzyme type I S subunit	257/403 (63%)	1e-112	*Leuconostoc citreum* KM20
orf4	4068	4436	+	123	28.18	hypothetical protein	hypothetical protein pEOC01_p05	123/123 (100%)	4e-62	*Pediococcus acidilactici*, pEOC01
res	4503	5090	+	196	35.88	Serine Recombinase (SR) family, Resolvase and Invertase subfamily, SR_ResInv [cd03768]	putative resolvase	174/197 (88%)	1e-96	*Streptococcus thermophilus*
orf6	5796	5368	-	143	29.37	GCN5-related N-acetyltransferase, NAT_SF [cd04301]	GNAT family acetyltransferase	133/136 (97%)	1e-70	*Weissella paramesenteroides* ATCC 33313
orf7	6558	6103	-	152	38.59	Truncated; C-terminus	putative transposase	126/152 (82%)	3e-72	*Lactococcus lactis* subsp. *cremoris*, pNZ4000
orf8	7151	7411	+	87	30.26	hypothetical protein	Orf8	43/79 (54%)	1e-13	*Lactococcus lactis* subsp. *lactis*, pAH82
mobC	7606	7971	+	122	28.68	Mobilisation protein MobC	Bacterial mobilisation protein MobC	110/118 (93%)	7e-55	*Enterococcus* sp. 7L76
mobD1	7979	8383	+	135	34.81	Relaxase domain [pfam03432]	relaxase/mobilization nuclease domain-containing protein	110/135 (81%)	7e-60	*Lactococcus lactis* subsp. *cremoris* SK11
matR	8924	10720	+	599	34.89	Reverse transcriptases (RTs) with group II intron origin, RT_G2_intron [cd01651]	maturase MatR	596/599 (99%)	0.0	*Lactococcus lactis* subsp. *lactis*
mobD2	10852	11979	+	376	32	Relaxase domain [pfam03432]	Relaxase/Mobilisation nuclease domain	366/376 (97%)	0.0	*Enterococcus* sp. 7L76

Table A4. List of ORFs identified in pAF07 (size 7,435 bp; GC content 36.44%)

Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
orf1	360	800	+	147	34.46	hypothetical protein	hypothetical protein llmg_0481	54/137 (39%)	1e-14	*L. lactis* subsp. *cremoris* MG1363
mobC	1087	1455	+	123	43.36	MobC-like protein	mobilization protein	58/112 (51%)	5e-25	*Staphylococcus epidermidis*
mobA	1440	2399	+	320	41.56	MobA-like protein; Relaxase domain [pfam03432]	relaxase/mobilization nuclease domain-containing protein	217/249 (87%)	8e-127	*L. lactis* subsp. *cremoris* SK11
repA	3609	4496	+	296	33.22	plasmid replication initiator protein, repA-like (Rep_3 domain, pfam01051)	replication initiator protein	243/296 (82%)	3e-143	*L. lactis* subsp. *cremoris* SK11
orf5	4835	6973	+	713	37.44	Phosphoglycerol transferase and related proteins, alkaline phosphotase superfamily (MdoB, COG1368)	hypothetical protein L13927	516/714 (72%)	0.0	*L. lactis* subsp. *lactis* IL1403
orf6	7036	7422	+	129	32.81	hypothetical protein	hypothetical protein L16147	64/124 (51%)	1e-32	*L. lactis* subsp. *lactis* IL1403

Table A5. List of ORFs identified in pAF04 (size 3,801 bp; GC content 32.02%)

Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
orf1	124	2	-	41	26.82	mobilization protein, mobC-like; N-terminal fragment	mobilization protein	39/41 (96%)	2e-13	*Lactococcus lactis*, pNZ4000
repC	1034	807	-	76	33.77	replication-associated protein RepC	RepC	76/76 (100%)	8e-38	*Lactococcus lactis* subsp. *cremoris,* pIL105
orfX	1669	1046	-	208	31.57	replication-associated protein OrfX	OrfX	206/208 (99%)	2e-109	*Lactococcus lactis* subsp. *cremoris,* pHP003
repB	2870	1665	-	402	34.57	Replication protein, RepB-like (Rep_3, Initiator Replication protein, pfam01051; L_lactis_RepB_C, L. lactis RepB C-terminus, pfam06430; Protein involved in initiation of plasmid replication, COG5527)	RepB	399/402 (99%)	0.0	*Lactococcus lactis*
orf5	3513	3773	+	87	26.81	mobilization protein; C-terminal fragment	Orf9	82/86 (96%)	5e-38	*Lactococcus lactis* subsp. *lactis*

Table A6. List of ORFs identified in contig39 (size 3,135 bp) and predicted to be contained in pAF45

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
ctg39.gene1	M.LlaCI	797	1684	+	296	32.88	type II methylase; NADB_Rossmann superfamily, cl09931; N6_N4_Mtase, DNA methylase, pfam01555; DNA modification methylase, COG0863	methylase	294/296 (99%)	3e-173	*Lactococcus lactis*, pAW153
ctg39.gene2	R.LlaCI	1785	2789	-	335	29.05	type II endonuclease; RE_HindIII, HindIII restriction endonuclease, pfam09518	endonuclease	323/335 (96%)	0.0	*Lactococcus lactis*, pAW153

Table A7. List of ORFs identified in contigs9-11-71-62 (size 6,284 bp) and predicted to be contained in pAF65

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
ctgs9-11-71-62.gene1	hsdR	427	3411	+	995	38.65	DEAD-like helicases superfamily, cd00046; P-loop containing Nucleoside Triphosphate Hydrolases superfamily, cl09099; Type I site-specific restriction-modification system, R (restriction) subunit and related helicases, COG0610	type I restriction enzyme R protein	991/995 (99%)	0.0	*Lactococcus lactis* subsp. *lactis* Il1403
ctgs9-11-71-62.gene2	hsdM	3454	4998	+	515	39.8	NADB_Rossmann superfamily, cl09931; Type I restriction-modification system methyltransferase subunit, HsdM, COG0286	type I restriction enzyme M protein	514/515 (99%)	0.0	*Lactococcus lactis* subsp. *lactis* Il1403
ctgs9-11-71-62.gene3	hsdS	4998	6317	+	440	34.46	Type I restriction modification S subunit; Methylase_S superfamily domain [pfam01420]; HsdS Restriction endonuclease S subunit domain COG0732	hypothetical protein LM5578_0548	171/277 (61%)	4e-94	*Listeria monocytogenes* 08-5578

Table A8. List of ORFs identified in the *tra1* region (size 17,264 bp) and predicted to be contained in pAF45

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
tra1.gene1	traA	5	2044	+	680	30.34	nicking enzyme traA; MobA/MobL family, pfam03389; TraA_Ti, Ti-type conjugative transfer relaxase TraA multidomain, TIGR02768	nicking enzyme traA	666/680 (97%)	0.0	*Lactococcus lactis*, pMRC01
tra1.gene2		2135	2473	+	113	34.51	hypothetical protein	hypothetical protein pMRC01_006	109/112 (97%)	8e-44	*Lactococcus lactis*, pMRC01
tra1.gene3		2477	3079	+	201	27.86	hypothetical protein	hypothetical protein pMRC01_007	196/201 (97%)	1e-105	*Lactococcus lactis*, pMRC01
tra1.gene4	traB	3095	3436	+	114	42.69	conjugation protein	protein TraB	114/114 (100%)	5e-57	*Lactococcus lactis*, pMRC01
tra1.gene5	traC	3460	3804	+	115	26.37	conjugation protein	protein TraC	115/115 (100%)	3e-59	*Lactococcus lactis*, pMRC01
tra1.gene6	traD	3791	4441	+	217	28.57	conjugation protein; VirB4_CagE, type IV secretion/conjugal transfer ATPase, VirB4 family, TIGR00929	protein TraD	216/217 (99%)	1e-119	*Lactococcus lactis*, pMRC01
tra1.gene7	traE	4456	6471	+	672	31.25	conjugation protein; CagE, TrbE, VirB family, component of type IV transporter system, pfam03135; P-loop containing Nucleoside Triphosphate Hydrolases superfamily, cl09099	protein TraE	672/672 (100%)	0.0	*Lactococcus lactis*, pMRC01
tra1.gene8	traF	6467	7882	+	472	28.17	conjugation protein	protein TraF	469/472 (99%)	0.0	*Lactococcus lactis*, pMRC01
tra1.gene9		7887	9041	+	385	35.75	NlpC_P60 superfamily, cl11438; Cell wall-associated hydrolases, COG0791; truncated, C-terminus	hypothetical protein pMRC01_013	385/385 (100%)	0.0	*Lactococcus lactis*, pMRC01
tra1.gene10		9057	9671	+	205	26.34	hypothetical protein	hypothetical protein pMRC01_014	205/205 (100%)	5e-112	*Lactococcus lactis*, pMRC01
tra1.gene11	traJ	10029	10484	+	152	29.82	conjugation protein	protein TraJ	152/152 (100%)	8e-82	*Lactococcus lactis*, pMRC01
tra1.gene12	traK	10484	12073	+	530	30.88	conjugation protein; P-loop containing Nucleoside Triphosphate Hydrolases superfamily, cl09099; TraG, TraG/TraD family, pfam02534	protein TraK	530/530 (100%)	0.0	*Lactococcus lactis*, pMRC01
tra1.gene13		12091	12483	+	131	27.48	hypothetical protein	hypothetical protein pMRC01_018	131/131 (100%)	3e-68	*Lactococcus lactis*, pMRC01
tra1.gene14	traL	12501	13334	+	278	31.53	conjugation protein	protein TraL	277/278 (99%)	3e-153	*Lactococcus lactis*, pMRC01
tra1.gene15	traI	13353	15527	+	725	28.27	conjugation protein; DNA topoisomerase III multidomain, PRK07726; N-terminus Topoisomerase-primase domain, cl00718; DNA-binding, ATP-binding and catalytic domain of bacterial DNA topoisomerases I and III, and eubacterial and archael reverse gyrases, cl11986	protein TraI	724/725 (99%)	0.0	*Lactococcus lactis*, pMRC01
tra1.gene16		15533	15739	+	69	29.46	hypothetical protein	hypothetical protein pMRC01_021	68/69 (98%)	7e-31	*Lactococcus lactis*, pMRC01
tra1.gene17	ltrC	15745	16809	+	355	28.63	conserved hypothetical protein; Family of bacterial and viral proteins with undetermined function, DUF955. A conserved H-E-X-X-H motif is suggestive of a catalytic active site and shows similarity to pfam01435	LtrC	352/355 (99%)	0.0	*Lactococcus lactis*, pMRC01
tra1.gene18		16957	17262	+	102	28.75	HTH_XRE, Helix-turn-helix XRE-family like proteins, cd00093	transcriptional regulator, XRE family protein	34/79 (43%)	7e-14	*Lactobacillus reuteri* 100-23

Table A9. List of ORFs identified in various contigs predicted to be part of the *tra3* region contained in pAF45

Code	Gene name	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
ctgs79-45.gene1	traF	-	326	31.59	TraF-like protein; truncated, N-terminus; essB, Predicted membrane protein essB, pfam10140	conjugation protein	117/319 (36%)	2e-52	*Carnobacterium* sp. AT7
ctgs79-45.gene2	traE	-	666	34.23	TraE-like protein; P-loop containing Nucleoside Triphosphate Hydrolases superfamily, cl09099	conjugation protein	412/637 (64%)	0.0	*Carnobacterium* sp. AT7
ctgs79-45.gene3	traD	-	227	32.74	TraD-like protein	conjugation protein	113/224 (50%)	5e-52	*Carnobacterium* sp. AT7
ctgs79-45.gene4	traC	-	114	34.21	TraC-like protein	conjugation protein	46/118 (38%)	3e-16	*Lactococcus lactis*, pMRC01
ctg59.gene1	traJ	-	154	33.11	TraJ-like protein	hypothetical protein CAT7_11190	43/137 (31%)	1e-10	*Carnobacterium* sp. AT7
ctg59.gene2	traK	-	585	37.03	TraK-like protein; P-loop containing Nucleoside Triphosphate Hydrolases superfamily, cl09099; Aspartyl beta-hydroxylase N-terminal region, pfam05279	conjugation protein	322/500 (64%)	0.0	*Carnobacterium* sp. AT7
ctg83.gene1	traI	+	219	34.24	TraI-like protein; stop codon	conjugation protein	60/230 (26%)	1e-15	*Carnobacterium* sp. AT7
ctg83.gene2	traI	+	84	34.52	TraI-like protein; stop codon, frameshift	DNA topoisomerase TopB	36/78 (46%)	2e-12	*Enterococcus faecium* TX1330
ctg83.gene3	traI	+	118	36.15	TraI-like protein; frameshift	topoisomerase IA	66/118 (55%)	7e-30	*Oenococcus oeni* PSU-1

Table A10. List of ORFs identified in contigs78-7 (size 3,534 bp) and predicted to be contained in pAF65

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
ctgs78-7.gene1		624	1	-	208	43.58	aldose 1-epimerase superfamily (cl14648); galactose mutarotase_like (cd09019); truncated, N-terminus	aldose 1-epimerase	85/189 (45%)	2e-39	*Enterococcus faecium* E1039
ctgs78-7.gene2		853	1740	+	296	39.07	Transposase_11 (pfam01609)	transposase for insertion sequence element IS982B	270/296 (92%)	8e-160	*Lactococcus lactis* subsp. *cremoris* MG1363
ctgs78-7.gene3	nsr	1965	2918	+	318	31.65	Peptidase_S41 family (pfam03572)	nisin-resistance protein	315/318 (99%)	0.0	*Lactococcus lactis*, pNP40

Table A11. List of ORFs identified in contig5 (size 4,412 bp) and predicted to be contained in pAF65

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
ctg5.gene1		228	923	+	232	27.72	Abi, CAAX amino terminal protease family, pfam02517	UPF0177 protein in AbiGi 5'region	225/232 (97%)	1e-125	*Lactococcus lactis* subsp. *cremoris*, pCI750
ctg5.gene2	lctT	1151	1822	+	224	26.19	ABCC_MRP_Like domain, cd03228; ABC_tran, pfam00005; C-terminal fragment	Lacticin-481 transport/processing ATP-binding protein lcnDR3	216/218 (99%)	2e-117	*Lactococcus lactis* subsp. *lactis*
ctg5.gene3	lctF	1883	2800	+	306	29.84	ABC_BcrA_bacitracin_resist domain, cd03268; CcmA, ABC-type multidrug transport system, ATPase component, COG1131	LctF	305/306 (99%)	4e-170	*Lactococcus lactis* subsp. *lactis*, pES2
ctg5.gene4	lctT	2803	3552	+	250	26.4	ABC transporter (trans-membrane domain) homolog; involved in immunity to the bacteriocin lacticin 481	LctE	247/250 (99%)	2e-132	*Lactococcus lactis* subsp. *lactis*, pES2
ctg5.gene5	lctG	3549	4283	+	245	25.44	Uncharacterized protein conserved in bacteria, COG4200; proposed to be associated to LctF and LctE to form an ABC transporter involved in immunity to lacticin 481	LctG	242/245 (99%)	3e-129	*Lactococcus lactis* subsp. *lactis*, pES2

Table A12. List of ORFs identified in contigs184-24 (size 15,153 bp) and predicted to be contained in pAF65

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domains	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
ctgs184-24.gene1	mobD	6	1487	+	494	31.17	Relaxase/Mobilisation nuclease domain-containing protein; cl01584	MobD	464/498 (93%)	0.0	*Lactococcus lactis*, pNP40
ctgs184-24.gene2	copB	2345	4447	+	701	37.56	copper-(or silver)-translocating P-type ATPase, TIGR01511; E1-E2 ATPase, pfam00122; Haloacid dehalogenase-like hydrolases, d11391	copper-potassium transporting ATPase B	685/701 (97%)	0.0	*Lactococcus lactis* subsp. *lactis* Il1403
ctgs184-24.gene3	tnp-ISL191	4601	5806	-	402	38.88	ISL191 transposase of IS256 family; MULE transposase domain, cl02997; Transposase and inactivated derivatives, COG3328	ISL191 transposase	392/402 (97%)	0.0	*Streptococcus thermophilus* LMG 18311
ctgs184-24.gene4		5851	6363	-	171	26.9	ATPase involved in DNA repair	ATPase involved in DNA repair	148/159 (93%)	1e-79	*Leuconostoc citreum* KM20, pLCK4
ctgs184-24.gene5	tnpA1-ISLL6	6462	6734	+	91	36.99	transposase A of ISLL6; HTH_Hin_like superfamily, cl01116	transposase A	91/91 (100%)	2e-45	*Lactococcus lactis* subsp. *cremoris* SK11
ctgs184-24.gene6	tnpAB-ISLL6	6641	7600	+	320	35.41	IS3 family transposase, truncated, C-terminus; rve superfamily, Integrase core domain, cl01316; putative transposase OrfB, PHA02517; Transposase and inactivated derivatives, COG2801	transposase AB of ISLL6	305/316 (96%)	2e-179	*Lactococcus lactis*
ctgs184-24.gene7	lcoC	7878	9266	-	463	35.27	Three multicopper oxidase domains, pfam07731; pfam07732; pfam00394; copper-resistance protein, CopA family, TIGR01480	LcoC	463/463 (100%)	0.0	*Lactococcus lactis*, pND306
ctgs184-24.gene8	lcoB	9603	10952	-	450	37.7	Spectrin repeat, SPEC, cd00176; CHAP, PF05257	LcoB	449/470 (95%)	0.0	*Lactococcus lactis*, pND306
ctgs184-24.gene9	lcoA	11431	12234	-	268	34.82	Prolipoprotein diacylglyceryl transferase, LGT, pfam01790	LcoA	266/268 (99%)	6e-147	*Lactococcus lactis*, pND306
ctgs184-24.gene10	lcoS	12421	13812	-	464	33.47	Signal transduction histidine kinase; Histidine kinase, Adenylyl cyclase, Methyl-accepting protein, and Phosphatase, HAMP, cd06225; Histidine Kinase A, cl00080; Histidine kinase-like ATPases, cl00075	LcoS	463/464 (99%)	0.0	*Lactococcus lactis*, pND306
ctgs184-24.gene11	lcoR	13803	14468	-	222	33.03	Response regulator, OmpR, COG0745; Signal receiver domain, REC, cd00156; Effector domain, trans_reg_C, cl00209C	LcoR	222/222 (100%)	3e-126	*Lactococcus lactis*, pND306
ctgs184-24.gene12	tnpA2-ISLL6	14650	14922	+	91	38.82	transposase A of ISLL6	transposase A	91/91 (100%)	8e-44	*Lactococcus lactis* subsp. *cremoris* SK11, pSK11P

Chapter IV

Plasmids of raw milk cheese isolate *L. lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901 suggest a plant-based origin for the strain

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ABSTRACT

The four plasmid-complement of the raw milk cheese isolate *L. lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901 was sequenced and some genetic features functionally analyzed. The complete sequences of pVF18 (18,977 bp), pVF21 (21,739 bp) and pVF22 (22,166 bp) were obtained whereas 49 of the predicted 50 kb of pVF50 were assembled in three contigs. Each plasmid encoded genes not previously described in *Lactococcus*, in addition to genes associated with plant-derived lactococcal strains. Most of the novel genes were found on pVF18 and encoded functions typical of bacteria associated with plants, such as activities of plant cell wall modification (*orf11* and *orf25*). In addition, a predicted high-affinity regulated system for the uptake of cobalt was identified (*orfs19-21)*, which has a single database homolog on a plant-derived *Leuconostoc* plasmid and whose functionality was demonstrated following curing of pVF18. Additional metal transporters are encoded by pVF21 and pVF22. Along with *orfs19-21* of pVF18, these transporters could enhance host ability to uptake growth-limiting amounts of biologically essential ions within the soil. In addition, pVF50 and pVF21 share 35 kb of highly homologous sequence with the plant-derived 68 kb lactococcal plasmid pGdh442, which suggests extensive horizontal transfer between these plasmids and a common plant niche for their hosts. Phenotypes associated with these plasmids include glutamate dehydrogenase activity and Na^+ and K^+ transport. The presence of a wealth of plant-associated markers in *L. lactis* DPC3901 suggests a plant origin for the raw milk cheese isolate and provides for the first time the genetic basis to support the concept of the plant-milk transition for *Lactococcus* strains.

INTRODUCTION

Lactococcus lactis strains belonging to the *lactis* and *cremoris* subspecies are essential components of industrial starters for the manufacture of fermented dairy products and have great biotechnological and economical importance. The success of many *L. lactis* strains in food fermentations is largely due to plasmid-encoded traits promoting efficient growth in milk (i.e. metabolism of lactose, milk proteins, citrate and other complex nutrients), resistance to environmental stresses (e.g. bacteriophages, heavy metals, temperature/osmotic stresses), and selective colonization advantages (i.e. production of bacteriocins and exopolysaccharides). Plasmids encoding these phenotypes are often mobilizable, which has greatly contributed to the genome plasticity of lactococci, enabling them to become adapted and specialized to the dairy environment (Mills *et al.*, 2006).

However, *L. lactis* is also naturally present on plants and on cows' skin (Klijn *et al.*, 1995, Nomura *et al.*, 2006). In particular, plant environments are the second most important ecosystem occupied by *L. lactis*, and, although this has never been demonstrated, it is likely that plant-derived strains are the natural source of dairy strains as they can transfer from forage plants and meadow grasses to milk via cattle (Salama *et al.*, 1995). A recent genotypic and phenotypic diversity analysis of a large collection of dairy and plant lactococcal isolates showed that the latter mostly belong to the subspecies *lactis* and exhibit a molecular diversity not observed within dairy strains (Rademaker *et al.*, 2007). A subsequent genome-scale analysis confirmed that plant strains exhibit an array of metabolic phenotypes exceeding that of dairy strains and including traits associated with the adaptation to grow on diverse plant cell walls substrates, but also genes for defence and stress response (Siezen *et al.*, 2008). Natural isolates often demonstrate greater tolerance of stress as a result of specific protection mechanisms developed to resist the numerous cytotoxic compounds and organisms (i.e. antibiotics, heavy metals, bacteriocins and bacteriophages) present in the plant and animal environments (Rodionov *et al.*, 2006, Siezen *et al.*, 2005, Teuber *et al.*, 1999). They also exhibit phenotypes of industrial interest that can be utilized in dairy and other food fermenting applications such as amino acids conversion to flavor compounds (i.e. α-keto acid decarboxylase and glutamate dehydrogenase) or the production of broad-spectrum bacteriocins (Klijn *et al.*, 1995, Smit *et al.*, 2005, Tanous *et al.*, 2006). As in the case of dairy strains, many of these phenotypes are

often plasmid-encoded in plant-derived strains, a finding that reaffirms the crucial contribution of these extra-chromosomal entities to the performance, fitness and ability of *L. lactis* strains to colonize specific biotopes.

Based on these observations, the analysis of plasmid gene content is therefore essential to evaluate the genetic potential and variability existing in dairy and natural lactococcal strains. Using this approach, novel genotypes useful to the dairy industry can be found, but also, niche-specific traits determining the uniqueness and adaptive capabilities of the strain can be uncovered. Assuming the validity of the plant origin hypothesis for dairy strains, this also implies that *L. lactis* strains recently transferred to the milk, and therefore potentially still carrying plant-specific traits, could theoretically be retrieved in artisanal products such as raw milk cheeses where the natural milk microflora, in turn derived from the grass, remains intact (Florez *et al.*, 2008, Salama *et al.*, 1995).

In this study, we have sequenced and functionally analyzed the four plasmid-complement of the raw milk cheese isolate *L. lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901, which was isolated and phenotypically characterized during previous studies aimed at identifying novel strains or genetic traits to be used in programmes of starter improvement (Cogan *et al*., 1997). These plasmids were found to contain a number of gene functions not previously described in *Lactococcus* and typical of bacteria associated with plants, as well as several other genes displaying homology only to single genes found on the plant-derived lactococcal plasmids pGdh442 or pKF147A (Siezen *et al.*, 2010, Tanous *et al.*, 2007). Additionally, two *L. lactis* DPC3901 plasmids exhibited high levels of homology over many loci with pGdh442, which is indicative of extensive horizontal gene transfer (HGT) and implies that *L. lactis* DPC3901 may have originated from a plant niche.

MATERIALS AND METHODS

Lactococcal strains and media.

The natural strain *L. lactis* DPC3901 was isolated and partially characterized in previous studies (Cogan *et al.*, 1997). Lactococcal strains were routinely propagated at 30 °C in M17 medium (Oxoid, UK) supplemented with 0.5% (wt/vol) glucose (GM17) or lactose (LM17). Solid media contained 1.0% (wt/vol) bacteriological agar (Oxoid, UK). Bacterial strains were stocked in M17 containing 40% glycerol at -80 °C. Working cultures were stored at 4 °C and transferred periodically.

Classification of *L. lactis* DPC3901

L. lactis DPC3901 was genotyped by using the universal 16S rRNA primers CO1 (5'-AGTTTGATCCTGGCTCAG-3') and CO2 (5'-TACCTTGTTACGACT-3') using PCR conditions described elsewhere (Beresford and Condon, 1991). Aliquots (5 µl) of the amplified products were subjected to electrophoresis in 1% agarose (Sigma, USA) gels and stained with ethidium bromide. PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen GmbH, Germany) and sequenced by single run primer extension (Beckman Coulter Genomics, USA). Sequencing data was searched for homology against sequences available in public databases (GenBank, EMBL, DDBJ, PDB) by using the nucleotide MegaBLAST algorithm of the Basic Local Alignment Search Tool (BLAST) (Zhang *et al.*, 2000). Confirmation of the genotypically predicted citrate-fermenting phenotype was obtained by streaking a freshly grown culture of *L. lactis* DPC3901 on KMK medium and checking for the appearance of blue colonies after 48-h incubation at 30 °C (Drici *et al.*, 2010).

Plasmid DNA sequencing, assembly and annotation.

Plasmid DNA was prepared as described elsewhere (O'Sullivan and Klaenhammer, 1993). Any residual genomic DNA was removed by treatment with Plasmid-Safe ATP-Dependent DNase (Epicentre, Madison, USA) which selectively hydrolyzes linear double-stranded DNA to deoxynucleotides without affecting closed circular supercoiled or nicked circular dsDNAs. The purified plasmids were

sequenced as a pool on a 454 Genome Sequencer FLX System, after preparation of a sonication library, barcoding of the library with the Roche multiplex identifier system, and emPCR amplification (Beckman Coulter Genomics, USA). Automatic de-novo assembly of data resulted in 56 large (> 500 bp) contigs providing ca. 135 kb of sequence data. Gap closure was performed by PCR using primers designed with PrimerSelect v.8.0.2 (DNASTAR, USA), followed by sequencing of the PCR products (Beckman Coulter Genomics, USA). Putative open reading frames (ORFs) were automatically predicted and annotated using Glimmer 2.0 (Delcher *et al.*, 1999). Annotated DNA sequences were examined manually using the Artemis viewer (Rutherford *et al.*, 2000) and BLASTX and BLASTP analyses (Altschul *et al.*, 1998) were used to examine intergenic regions for putative ORFs not identified by Glimmer, to identify putative frameshifts, and to refine the start codon assignments. The InterProScan tool (www.ebi.ac.uk/Tools/InterProScan), the PFAM (Finn *et al.*, 2010) and Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2009) were used to predict protein function. InterProScan predicts the occurrence of functional domains and motifs/signatures in a protein by combining 12 different databases and their relative protein signature recognition methods (Zdobnov and Apweiler, 2001). Prediction of topology in putative alpha-helical membrane proteins, including a specification and the in/out orientation of the membrane spanning segments, was obtained by using the TOPCONS hidden Markov model (http://topcons.cbr.su.se/index.php), which uses five different topology prediction algorithms (SCAMPI-seq; SCAMPI-msa; PRODIV-TMHMM; PRO-TMHMM; and OCTOPUS) to generate a consensus prediction for the protein (Bernsel *et al.*, 2009). Putative Ribosome Binding Sites (RBS) and terminator sequences were automatically identified by using the RBSFinder+TransTerm programme (http://nbc11.biologie.uni-kl.de/). Manual identification of terminator sequences was achieved by following three main criteria: (i), a ΔG of less than -6; (ii), a stem loop structure rich in GC; (iii), a T-rich sequence following the stem loop structure.

Growth inhibition assays.

Bacterial sensitivity to cadmium (Cd^{2+}), cobalt (Co^{2+}), tetracycline, streptomycin and kanamycin (Sigma, USA) was assessed by using a micro-plate procedure. Two-hundred microliters of GM17 or LM17 broth containing either $CdCl_2$ (0 to 0.3 mM)

or $CoCl_2$ (0 to 6 mM) or tetracycline (0 to 10 μg/ml) or streptomycin (10 to 500 μg/ml) and kanamycin (10 to 500 μg/ml) were inoculated at 1% with overnight cultures on 96-wells micro-plates (Sarstedt, Newton, USA). Bacterial growth was assessed over 18 h at 30 °C by measuring absorbance at 600 nm with a GENios Plus reader (Tecan, Switzerland).

Determination of glutamate dehydrogenase (GDH) activity.

GDH activity was determined in cell-free extracts (CFEs) as previously described (Kieronczyk *et al*., 2003), with some modifications. Briefly, cell cultures (200 ml) growing in mid-log phase (OD = 0.4) were harvested by centrifugation (4,100 x g, 15 min, 4 °C) and washed twice with 50 mM triethanolamine buffer (TEA, pH 7). Pellets were resuspended in 5 ml of TEA containing lysozyme (1.6 mg/ml) and saccharose (0.2 mg/ml), incubated at 30 °C for 2 h, harvested by centrifugation and resuspended in hypotonic buffer TEA to provoke spheroplast lysis. Cell debris was eliminated by centrifugation at 20,000 x g for 30 min, and the supernatants were filtered through a 0.45 μm filter (Millipore, USA) and used as CFEs. The protein concentration of CFEs was determined by the Bradford method by using bovine serum albumin as the standard (Bradford, 1976). Determination of GDH activity in CFEs was based on a modification of a colorimetric glutamic acid assay (Boehringer Ingelheim GmbH, Germany), where the reduced cofactor (NADH or NADPH), produced by oxidative deamination of glutamate by GDH at pH 9, reacts with iodonitrotetrazolium in the presence of diaphorase to produce a colorimetric product (formazan), which is measured by absorbance at 492 nm. A 300 μl-reaction mixture consisted of 40 μl of 50 mM potassium phosphate–50 mM TEA buffer (pH 9.0) containing 1% of Triton X-100 (Promega Corporation, USA), 20 μl of 100 mM glutamic acid, 20 μl of 2 mM iodonitrotetrazolium, 20 μl of diaphorase (1.76 U/ml), 20 μl of NAD^+ (17.33 mM) or $NADP^+$ (13.8 mM), and 180 μl of water. The colorimetric reaction was started by adding 30 μl of CFEs to the reaction mixture, and the changes in concentration of NADH were measured after incubation for 1 h at 37°C, by measuring the absorbance at 492 nm. A control test lacking glutamic acid was prepared for each strain in order to subtract for non-specific reactions that might produce the reduced cofactors. The

GDH activity was expressed by the increase in absorbance (Abs) at 492 nm, per mg of protein of CFE, and per min of reaction.

Plasmid curing

Plasmid curing experiments were performed by growing strains at 37 °C in LM17 lacking the buffering agent β-glycerophosphate (β-GP) for 50 generations before being transferred to fresh media. The strain was plated on LM17 agar at each transfer in order to analyze plasmid profiles of individual colonies.

Measurement of bacterial growth rate and acidification capacity

The growth rates and acidification capacities of *L. lactis* DPC3901 and of its pVF18/pVF22-cured derivative (*L. lactis* DPC3901-c2) were compared with those of cheese starter strains *L. lactis* subsp. *cremoris* DPC4268 and *L. lactis* subsp. *lactis* SK1 as previously described (Fallico *et al.*, 2009). Briefly, growth rate parameters (max growth rate, μ_{max}, and generation time, G) were determined after growth in LM17 at 30 °C, whereas the acidification capacities (starter activity) were evaluated, based on final pH, after growth in 11% reconstituted skim milk (RSM) over a 5-h incubation time through a simulated Cheddar cheese making temperature profile (32 °C for 70 min; 40°C for 190 min; 32 °C for 40 min).

PCR analysis

Detection of specific genes within the plasmid complement of *L. lactis* DPC3901 and its cured derivatives DPC3901-c1 and DPC3901-c2 was obtained by PCR analysis using the primers listed in Table 2.

Atomic Force Microscopy (AFM) imaging

L. lactis DPC3901 was diluted in distilled water (1:100) prior to imaging. Ten µl of diluted sample were deposited on freshly cleaved mica disc and subsequently dried in a desiccator. Samples were imaged using AC mode (MFP-3D atomic force microscope, Asylum Research, UK). An aluminium reflex coating cantilever with a

tetrahedral tip (AC 240, spring constant: 1.8 N/m, resonant frequency: 79.58 kHz) (Olympus Optical Co., Japan) was used at a scan rate of 1 Hz.

RESULTS AND DISCUSSION

Sequencing of the 16S rRNA gene of the natural isolate *L. lactis* DPC3901 revealed a subsp. *lactis* biovar. *diacetylactis* genotype, thus confirming the phenotypic data obtained from growth on KMK medium and showing the strain's ability to ferment citrate. Biovariant *diacetylactis* strains isolated from raw milk and plant surfaces have been shown to be of significant technological interest due to their abilities to grow rapidly in milk, to ferment a wide range of carbohydrates and to tolerate high pH (9.6) and temperature (50 °C) values, features that are mostly plasmid-encoded (Drici *et al.*, 2010, Nomura *et al.*, 2006). In a previous study, *L. lactis* DPC3901 was shown to harbour four plasmids greater than 10 kb, and to be completely resistant to a number of 936- and c2-type phages (Chapter II, this thesis). In this study, the entire plasmid complement of *L. lactis* DPC3901 was sequenced to investigate the genetic contribution of these extra-chromosomal entities to the technological capacities of the strain. Sequencing of the four plasmids of *L. lactis* DPC3901 returned 135 kb of data organized in 56 large contigs. The complete circular sequences of plasmids pVF22 (22,166 bp), pVF21 (21,728 bp) and pVF18 (18,977 bp) were obtained (Figure 1; Tables 1-3 in Appendix) by using a PCR-based gap closure strategy. The GC content of these plasmids ranged from 33.6 to 35.1%, which is similar to that of other lactococcal plasmids (Siezen *et al.*, 2005). Attempts to obtain the complete sequence of pVF50 were unsuccessful, probably due to the presence of a large number of transposases and secondary structures. However, three large contigs (contig1 of 23 kb; contig2 of 18 kb; and contig3 of 8 kb), accounting for 49 of the predicted ca. 50 kb of pVF50 sequence, were assembled (Tables 4-6 in Appendix). All plasmids appear to replicate according to a theta mechanism as each one is characterized by an origin of replication (*ori*), located upstream of the *rep* gene encoding the replication initiator protein RepB (383 to 441 amino acids (aa); in pVF18, pVF21, pVF22) or RepA (450 aa; in pVF50), containing highly conserved structural motifs typical of theta replicons (Figure 2) (Seegers *et al.*, 1994). The most significant genetic features of *L. lactis* DPC3901 plasmids are discussed below.

The presence of plasmid pVF18 suggests a plant-based origin for *L. lactis* DPC3901

Plasmid pVF18 contains 25 *orfs* encoding predicted proteins with no obvious dairy-associated functions, eight of which (*orf8*, *orf11*, *orfs18-21*, *orf23*, *orf25*) have been rarely if ever described in *Lactococcus* (Figure 1; Table 3 in Appendix). An example is *orf11*, which encodes a putative polysaccharide deacetylase (432 aa) belonging to a family of conserved proteins (PFAM01522) involved in hydrolysis of the plant cell wall. Members of this family include the chitooligosaccharide deacetylase NodB from *Rhizobium* (Freiberg *et al.*, 1997), chitin deacetylases from *Saccharomyces cerevisiae* (Mishra *et al.*, 1997), and microbial endoxylanases (Millwardsadler *et al.*, 1994). These latter enzymes share sequence similarity with the NodB deacetylase (Millwardsadler *et al.*, 1994), whose activity enables *Rhizobium* to associate symbiotically with leguminous plants thus allowing them access to mineral nitrogen necessary for their growth. The *nodB* gene is plasmid-encoded in *Rhizobium* sp. NGR234, and this was suggested to be the result of a recent horizontal gene transfer (HGT) from *Agrobacterium* (Freiberg *et al.*, 1997). Orf11-like proteins are highly conserved in *Enterococcus faecium*, *Staphylococcus aureus* and in some *Ruminococcus* and *Clostridium* members, but no homologues have been found to date in *Lactococcus*.

Similarly, homologues of the product of *orf25* can be found in various environmental subspecies of *Streptococcus*, *Carnobacterium*, *Enterococcus*, *Corynebacterium*, *Bacillus*, *Geobacillus* and *Clostridium*, but not in *Lactococcus* or other milk-derived bacteria. Orf25 is a 140-aa protein containing the conserved barrel domain typical of cupins (PFAM07883). The functions of these proteins range from isomerase and epimerase activities involved in the modification of cell wall carbohydrates in bacteria and plants, to non-enzymatic germins and plant seed storage proteins, which provide the major nitrogen source for the developing plant (Dunwell, 1998).

The genes described above encode products whose functions are irrelevant to bacteria growing in milk but clearly important to those associated or striving to become established within the natural plant ecosystem. In addition, pVF18 lacks any trait favouring colonization of the dairy environment and, interestingly, it was the first

plasmid to be cured from the host during growth at 37 °C in absence of β-GP. Taken together, these observations provide indications that *L. lactis* DPC3901 might have originally occupied a grass plant niche, where pVF18-encoded genes conferred the host with a selective advantage, before transferring to the dairy environment probably via cattle. Such an event is likely to have occurred recently as the bacterium still retains plasmid traits that are dispensable for growth in milk and are indeed immediately lost during growth under stress conditions in synthetic media.

Functional Co^{2+} transport mechanisms – another link to the plant environment

Cobalt (Co^{2+}) is an essential component of many enzymes and must be transported into cells in appropriate amounts when needed. Despite its importance for bacterial metabolism, Co^{2+} uptake must be tightly regulated to avoid toxic effects (Rodionov *et al.*, 2006). Plasmid pVF22 of *L. lactis* DPC3901 is predicted to carry two copies of the *corA* gene (COG0598), *corA1* and *corA2*, which encodes the principal transporter of Mg^{2+} in prokaryotes but also functions in the uptake of Co^{2+} (Niegowski and Eshaghi, 2007). The presence of two CorA transporters is likely to enhance *L. lactis* DPC3901 ability to uptake Co^{2+} in environments (e.g. the soil) where its presence in trace amounts might be growth-limiting for the strain but also increases the strain's sensitivity to the heavy metal. Additionally, pVF18 of *L. lactis* DPC3901 carries a putative polycistronic operon (*orfs19-21*) that may play a role in the high-affinity and regulated uptake of Co^{2+}. Two 4-bp overlaps between *orf20* and its neighbour genes, coupled to the presence of putative promoter sequences upstream of *orf19* and of a strong Rho-independent terminator (ΔG = -7.9 kcal/mol) downstream of *orf21* (Figure 3a), suggest that *orf19, orf20* and *orf21* are transcribed as a single mRNA. This three-gene operon is uncommon to *L. lactis* and can only be found, with high similarity at DNA (99%) and protein (92-99%) level, on the recently sequenced plasmid LkipL4726 from *Leuconostoc kimchii* IMSNU 11154 (Oh *et al.*, 2010). *Orf19* encodes a small protein (75 aa) of unknown function exhibiting 92% identity to its only significant match, a hypothetical protein from LkipL4726 (Oh et al., 2010). *Orf20* and *orf21* encode proteins containing features typical of the ATPase and permease components of ATP-binding cassette (ABC) transporters (Figure 3b,c), which use the energy of ATP hydrolysis to transport specific substrates across the

cellular membrane. Homologs of Orf20 are widespread in *Lactococcus*, whereas Orf21 has a single lactococcal match on pGdh442 (Tanous *et al.*, 2007). A putative role for the Orf20-21 transporter is suggested by the presence in Orf20 of a conserved domain (cd03225) found in the CbiO component of CbiMNQO, which is the most widespread transport system for the high-affinity uptake of Co^{2+} in prokaryotic genomes (Rodionov *et al.*, 2006). Functional analyses of the CbiMNQO system have shown that the integral membrane protein CbiM and the cobalt-binding periplasmic protein CbiN are the minimal requirements for a functional transporter exhibiting significant cobalt uptake activity (Rodionov *et al.*, 2006). In the putative pVF18-encoded Co^{2+} transporter, it is possible that Orf20 may act as the ATPase-like protein CbiO whereas Orf21 functionality might resemble that of CbiM, which also contains seven trans-membrane helices. Orf19 is probably a cytoplasmic protein but lacks any conserved Co^{2+} binding motifs (Thilakaraj *et al.*, 2007), which discards the option that it might be a CbiN homolog. CbiMNQO transporters are generally controlled on the level of translation initiation by B12 riboswitch elements (Rodionov *et al.*, 2006). In pVF18, expression of the *orfs19-21* operon might be controlled by the upstream *orf18*, which encodes a transcriptional regulator belonging to the XRE family (cd00093). Putative promoter sequences but no termination signal could be identified for *orf18* (Figure 3a), suggesting that it might be co-transcribed with *orf19-21* and possibly regulate the activity of this operon. If functional, the putative Co^{2+} transport system encoded by *orfs18-21* should endow *L. lactis* DPC3901 with the ability to uptake Co^{2+} with high-affinity and in a regulated manner, allowing its efflux outside the cell in order to maintain metal homeostasis.

To verify this, the growth of *L. lactis* DPC3901 in presence of increasing concentrations of Co^{2+} was compared to that of *L. lactis* MG1363, which lacks any high-affinity Co^{2+} uptake system but possesses a chromosomally-encoded CorA transporter. *L. lactis* DPC3901 was able to grow relatively well in presence of 4 mM Co^{2+} and to tolerate concentrations as high as 6 mM (Figure 4a). In contrast, the growth of *L. lactis* MG1363 was affected by the presence of 1 mM Co^{2+} and totally inhibited by concentrations of and above 2 mM (Figure 4b). These results are indicative of *L. lactis* DPC3901 ability to regulate Co^{2+} uptake in order to avoid its toxic effects. To further confirm these indications, pVF18 and pVF22 were sequentially cured from *L. lactis* DPC3901 in order to first remove *orfs18-21* and

then the *corA* genes. Growth curves analysis showed that the pVF18-cured derivative, named DPC3901-c1 (*orfs18-21*$^{-}$ *corA*$^{+}$), could not grow at Co^{2+} concentrations higher than 1 mM (Figure 4c), whereas the derivative cured of both pVF18 and pVF22, DPC3901-c2 (*orfs18-21*$^{-}$ *corA*$^{-}$), exhibited an ability to tolerate Co^{2+} very similar to that of the parent strain (Figure 4d). These results confirm the involvement of the *orfs18-21* genes in mediating uptake and homeostasis of the potentially toxic metal Co^{2+} in *L. lactis* DPC3901.

To date, the *orfs19-21* operon has been only found on plasmids from *Leuconostoc kimchii* IMSNU 11154 (Oh *et al.*, 2010) and *L. lactis* DPC3901 (this study). Interestingly, a recent and comprehensive comparative and functional analysis of cobalt transporters in prokaryotic genomes retrieved no homologues of *cbi* genes in both *Leuconostoc* and *Lactococcus* (Rodionov *et al.*, 2006). Thus, bacteria from these genera may have evolved a novel plasmid-encoded uptake system to ensure an efficient homeostasis of the metal. *Leuconostoc kimchii* IMSNU 11154 was isolated from a traditional Korean fermented food made from cabbage (Oh *et al.*, 2010), whereas *L. lactis* DPC3901 derives from a raw milk cheese where the absence of pasteurization preserves the natural milk microflora, mostly derived from grass plants (Salama *et al.*, 1995). It is thus likely that LkipL4726 and pVF18 hosts have inhabited natural environments where perhaps the presence of trace amounts of Co^{2+} may have promoted the development of an appropriate high-affinity uptake system (Rodionov *et al.*, 2006). The observation that lactococcal homologs of the Orfs20-21 transporter can only be found on pGdh442, a plasmid recently isolated from a plant *L. lactis* strain (Tanous *et al.*, 2007), further supports the link between this Co^{2+} uptake system and the plant environment.

Plasmid pVF18 also contains genes (*orf8* and *orf23*) that have been rarely found in *Lactococcus*. Both encode putative GCN5-related *N*-acetyltransferases (GNAT; cd04301) that are much conserved among *Streptococcus*, *Clostridium*, *Enterococcus* and *Staphylococcus*, but have a single lactococcal homolog on the chromosome of *L. lactis* MG1363 (Wegmann *et al.*, 2007). GNAT enzymes catalyze the transfer of an acetyl group to the cognate substrate and are thought to be implicated in a variety of functions, ranging from antibiotic (aminoglycosides) resistance to regulation of cell growth and development (Vetting *et al.*, 2005). To test the possible involvement in antibiotic resistance of Orf8 and Orf23, *L. lactis* DPC3901 and the sensitive strain *L.*

lactis MG1363 were grown in presence of increasing concentrations of streptomycin and kanamycin (data not shown). Both strains were similarly sensitive to both aminoglycosides, which suggests that the GNAT-like proteins encoded by pVF18 are not involved in resistance to these antibiotics.

Plasmid pVF21 confers phage resistance and potential flavour-forming ability to *L. lactis* DPC3901

Traces of a putative plant origin can also be linked to pVF21 (Figure 1; Table 2 in Appendix), which includes *orf15* encoding a 105-aa hypothetical protein identical to its only database match encoded by the plant-derived lactococcal plasmid pKF147A (Siezen *et al.*, 2010), and a 5.6 kb region sharing 97% identity to its only homologue found on the plant-derived pGdh442 (Tanous *et al.*, 2007). In addition to containing genes responsible for cadmium resistance and transposition, this region encodes a glutamate dehydrogenase (GDH) enzyme, an activity that is rare in *L. lactis* and thus far only identified in strains of plant and animal origin (Tanous *et al.*, 2007, Tanous *et al.*, 2002). In pGdh442, this region is part of a remnant *Tn*3-like transposon, also containing a resolvase gene and delimited by two 28-bp IR (Tanous *et al.*, 2005). These latter features are missing in pVF21, which might imply that the transposon is not functional and is therefore stably maintained in this plasmid. The *gdh* gene of pVF21 encodes a putative 448-aa protein identical to the NAD(P)-dependent GDH of pGdh442 (Tanous *et al.*, 2005). GDH enzymes catalyze the reversible oxidative deamination of glutamate to α-ketoglutarate and ammonium by utilising NAD, NADP or both as a cofactor. GDH of pGdh442 has been shown to function mainly in glutamate biosynthesis, but also to be able to stimulate its conversion to aroma compounds in *L. lactis* strains that had acquired the plasmid by conjugation (Tanous *et al.*, 2006). We tested the functionality of the *gdh* gene of pVF21 by using *L. lactis* IL1403 as a GDH-negative reference strain. No GDH activity was registered for *L. lactis* IL1403, whereas *L. lactis* DPC3901 exhibited NAD^+- and $NADP^+$-dependent specific activities of 0.028 and 0.01, respectively, which are similar to those reported for a *cremoris* strain used in the manufacture of Gouda and Cheddar type cheeses (Kieronczyk *et al.*, 2003). These results suggest that pVF21 encodes a functional GDH enzyme providing *L. lactis* DPC3901 with the potential to produce aroma compounds from glutamate catabolism and/or to

synthesize glutamate from α-ketoglutarate and ammonia. This latter capacity has been suggested to be important for bacteria living on plants as it allows more efficient elimination of the high levels of ammonia present in this environment (Tanous *et al.*, 2005). In pVF21, the *gdh* gene is followed downstream by a gene encoding a truncated IS1216-like transposase and by the *cadAC* operon, which is identical to those found on lactococcal plasmids pAH82 (O'Sullivan *et al.*, 2001), pGdh442 (Tanous *et al.*, 2007) and pNP40 (O'Driscoll *et al.*, 2006), and in several other gram-positive bacteria (Schirawski *et al.*, 2002). This operon encodes a two-component system composed of an efflux ATPase (CadA) and a resistance regulator (CadC), which has been shown to confer resistance to both cadmium and zinc (Schirawski *et al.*, 2002).

Upstream of the *Tn*3-like region, pVF21 carries genes encoding a restriction-modification (R/M) system of type I, which is one of the many molecular mechanisms developed by *L. lactis* strains to defend themselves against bacteriophage (phage) attack. Susceptibility to infection by lytic phages is indeed common in lactococci and a major concern for the dairy industry as it affects strains ability to perform efficiently in large-scale fermentations. Type I R/M systems are typically made up of three genes (*hsdRMS*), overlapping and transcribed in the same direction, which encode subunits responsible for DNA restriction (HsdR), modification (HsdM) and specificity (HsdS), respectively (Mills *et al.*, 2006). In pVF21, *hsdR* encodes a restriction enzyme (1025 aa) containing conserved helicases-related domains (cd00046; COG0610), while the 537-aa product of *hsdM* is a typical N^6-adenine methyltransferase (pfam02384). Both proteins are virtually identical (99%) to the HsdR and HsdM enzymes encoded by genes on the lactococcal plasmid pAH82 (O'Sullivan *et al.*, 2001). In contrast, the HsdS specificity protein (414 aa) of pVF21 shares only 38% identity with the homolog encoded by pAH82. Low similarities among HsdS subunits are common and reflect the large variability existing within their target recognition domains (TRDs), which impart target sequence specificity to both restriction and modification activities of the type I R/M complex (Mills *et al.*, 2006). Novel specificities may be generated by recombinational shuffling of TRDs between different HsdS subunits, which confers the host with the ability to alter its phage restriction level (O'Sullivan *et al.*, 2000, Schouler *et al.*,

1998). This makes type I R/M systems one of the most effective mechanisms of defence against phages.

Other interesting genes present on pVF21 are *orf5* and *orf16*. *Orf5* encodes a protein of 365 aa containing the pentapeptide DGQHR motif, the QR pair and the FxxxN motif, which are typical conserved residues of the highly divergent and uncharacterized DGQHR domain (TIGR03187). Orf5 has no homologues in *Lactococcus* or other lactic acid bacteria. The most similar matches (27 to 32%) are proteins from two *Bacillus cereus* strains, whereas lower similarity is shared with proteins from several environmental species. While the function of proteins containing the DGQHR domain is unknown, members of this family occur in contexts that suggest extensive HGT and, interestingly, several of them have been annotated as putative bacteriophage proteins. The 111-aa product of *orf16* contains an Enterocin A immunity domain (pfam08951) and is 90% identical to hypothetical proteins of pNP40 (O'Driscoll *et al.*, 2006) and pGdh442 (Tanous *et al.*, 2007). This domain is found in immunity proteins protecting food-associated LAB strains from the bactericidal activity of their own class IIa or pediocin-like bacteriocins. Immunity genes belonging to this family, which are plasmid-encoded and not associated with bacteriocin genes, have been already described (Chikindas *et al.*, 1993).

Plasmid pVF22 encodes antibiotic resistance

Plasmid pVF22 (Figure 1; Table 1 in Appendix) is predicted to carry a gene (*orf14*) encoding a Tet(S)-like tetracycline resistance protein (cd04168) identical to those found on pKL0018 from the fish pathogen *L. garvieae* (Maki *et al.*, 2008) and on pK214 from a *L. lactis* isolate from raw milk soft cheese (Teuber *et al.*, 1999). This protein functions through ribosomal protection and its gene is typically found on mobile genetic elements, such as transposons or plasmids. In pVF22, *tet*(S) is inserted within a genetic context resembling a transposon-like structure as it is flanked by two IS1216 transposases and by genes responsible for site-specific recombination (pinR and *orf17*) and for conjugal transfer (*mobC*$_1$*ABC*$_2$). However, the genetic organization of this region is different from that of transposon Tn*916*, which usually carries *tet*(S) in a broad range of gram-positive and gram-negative bacteria (Celli and Trieu-Cuot, 1998). *Lactococcus* strains are known to be susceptible to most antimicrobial agents

but, recently, many isolates resistant to chloramphenicol, erythromycin, streptomycin and tetracycline have been reported (Delgado and Mayo, 2004, Florez *et al.*, 2008, Teuber *et al.*, 1999). This has been associated with the fact that lactococci can be naturally present as saprophytes in cow's membrane surfaces, where they could acquire resistance during antibiotic treatments to cure or prevent mastitis (Florez *et al.*, 2008). The absence of pasteurization in the manufacture of raw milk cheeses allows then these bacteria to be transferred from raw milk into the final product.

Plasmid pVF50 contributes to *L. lactis* DPC3901 adaptation to the dairy environment

Annotation of genes contained in the assembled contig1, contig2, and contig3 (Tables 4-6 in Appendix) reveal that pVF50 is largely similar to the plant-derived plasmid pGdh442 (Tanous *et al.*, 2007), with whom it shares five gene modules spanning over 30 kb of sequence and exhibiting 97 to 100% homology at both DNA and protein levels. Plasmid pGdh442 contains 20 IS elements that are likely to have mediated the acquisition of its numerous gene modules (Tanous *et al.*, 2007), and can therefore promote their transfer to plasmids of a different organism. A 20-kb sequence of the 23-kb contig1 of pVF50 is identical to the adjacent modules *orfs55-64* and *orfs1-3* of pGdh442, whereas a 10-kb region of the 18-kb contig2 is highly homologous to three flanking modules in pGdh442 spanning *orf15* to *orf23*. Adjacent modules in pVF50 exhibit a swapped organization compared to their arrangement in pGdh442, which would confirm previous suggestions regarding the ability of these gene blocks to be mobilized by the flanking IS elements (Tanous *et al.*, 2007). The observation that one of these shared gene blocks is to date exclusive to pGdh442 in *L. lactis* further supports the hypotheses of large-scale HGT occurring between the two plasmids and, consequently, of a plant origin for the pVF50 host. This region is composed of three genes encoding membrane components of Na^+ and K^+ transport systems, which are virtually identical in pVF50 (contig2.gene1-3) and in pGdh442 (*orfs15-17*) but share only 41 to 45% identity to other homologues found in *Clostridium* (Tanous *et al.*, 2007).

Genes on pVF50 related to growth on milk components have certainly facilitated the adaptation of *L. lactis* DPC3901 to the dairy environment. The ability of the strain

to grow well in milk and to utilize lactose is encoded by the *lacXGEFDCBA* operon, or the lactose phosphotransferase system (contig3.gene1-8), which is negatively regulated by the product of the divergently transcribed *lacR* gene (contig3.gene9) (Vanrooijen and Devos, 1990, Vanrooijen *et al*., 1991). The *lacXGEFDCBAR* region of pVF50 is 98 to 100% identical at both DNA and protein level to that present on pSK11L (Siezen *et al*., 2005). A further contribution to lactose utilization in *L. lactis* DPC3901 might also be afforded by the *dld* gene (contig2.gene14), which is predicted to encode a D-lactate dehydrogenase (D-LDH) of 559 aa and might function in D-lactate utilization under aerobic conditions (Siezen *et al.*, 2005). However, experiments by Tanous *et al*. showed that *L. lactis* strains containing a pGdh442-encoded *dld* gene, identical to that on pVF50, were unable to grow in M17 containing D-lactate as carbon source, and also that the gene was not over-expressed when the same strains were grown in GM17 (Tanous *et al.*, 2007).

Plasmid pVF50 also encodes a neutral oligoendopeptidase PepO and the complete oligopeptide permease (Opp) system OppDFBCA, which endow *L. lactis* DPC3901 with the ability to utilize proteins as carbon source. The corresponding genes are part of a gene block (contig1.gene7-19), containing the *flp* operon and flanked by transposases of the IS3 (*tnp-IS3*) and IS6 (*tnp-IS946*) families, that is 98% identical to the homologous region on pGdh442 (*orfs*55-64) (Tanous *et al.*, 2007). The Opp system allows the efficient uptake of oligopeptides generated from casein degradation by the cell wall proteinase (Tynkkynen *et al*., 1993), whereas PepO is reported to hydrolyze oligopeptides ranging in length from 5 to 35 residues (Christensen *et al.*, 1999). To date, the combination of *pepO* and *Opp* system has been found only on plasmids pSK11L (Siezen *et al.*, 2005) and pGdh442 (Tanous *et al*., 2007), and has been suggested to be beneficial for the optimal utilization of milk caseins as a nitrogen source, thus resulting in a fast milk coagulation phenotype (Siezen *et al.*, 2005). A further contribution to this process in *L. lactis* DPC3901 may also derive from the oligoendopeptidase F encoded by *pepF* (contig2.gene20), which is known to be part of the complex proteolytic system essential for growth of *L. lactis* in milk. This enzyme hydrolyzes oligopeptides of 8-9 residues and plays a potential role in protein turnover under nitrogen limiting conditions (Christensen *et al*., 1999). The presence of these determinants led us to evaluate the strain's ability to grow and acidify milk under conditions resembling the manufacture of Cheddar cheese. Growth

rates and acidification capacity of *L. lactis* DPC3901 were slightly lower than those of the widely used cheese starters *L. lactis* DPC4268 and *L. lactis* SK1, but still sufficient for cheese manufacturing (Table 3).

Plasmid pVF50 is predicted to carry various stress response genes that may have enhanced the robustness of *L. lactis* DPC3901 within the dairy environment. These include a number of genes encoding putative cold shock proteins including CspA, CspC and CspB homologues. These proteins are identical to the only lactococcal Csps found to date on the plant-associated plasmid pKF147A (Siezen *et al.*, 2010), on pSK11P (Siezen *et al.*, 2005) and on *L. lactis* MG1363 chromosome (Wegmann *et al.*, 2007). It has been suggested that the presence of multiple plasmid-encoded Csps might enhance the survival capability of the *L. lactis* host at low temperatures (Siezen *et al.*, 2005).

pVF50 is the only *L. lactis* DPC3901 plasmid to contain a replication and maintenance region composed of a typical lactococcal theta-type *ori* site (Seegers *et al.*, 1994), a *repA* gene (contig1.gene2) encoding the replication initiator, and two genes (contig1.gene3-4) encoding the partition proteins ParA and ParB. This region is identical to homologous regions present on plant-derived lactococcal plasmids pKF147A (Siezen *et al.*, 2010) and pGdh442 (Tanous *et al.*, 2007), and on pSK08 from *L. lactis* ML3 (accession no. AF300944). The similarity of the *ori* regions of pGdh442 and pSK08-like plasmids, which include the lactose/protease plasmid pLP712 (Gasson, 1983), has been associated with the instability of pGdh442 within a strain containing pLP712 (Tanous *et al.*, 2007). Plasmid pVF50 carries an *ori* region identical to that of pGdh442 and is predicted to contain determinants for lactose fermentation but no protease-associated genes. This suggests that pGdh442-like plasmids are probably incompatible with protease genes in co-resident plasmids, and also that the transfer of pVF50 to strains capable of growing in milk cannot be ruled out as suggested for pGdh442 (Tanous *et al.*, 2007). The partition genes *parA* and *parB* are likely to provide pVF50 with a maintenance system counteracting plasmid loss at cell division. ParA is a DNA-binding protein similar to a conserved family of ATPases (cd02042), which includes the RepA protein of the *repABC* operon involved in replication and partition of the *Rhizobium etli* CFN42 symbiotic plasmid (Cevallos *et al.*, 2002). ParB (242 aa) contains a nuclease-like domain (PF02195) belonging to a highly conserved set of chromosomal and plasmid partition proteins. Shortly

following the initiation of DNA replication, ParB localizes to opposite cell poles where it binds to DNA sequences adjacent to *ori* and regulates the ParA ATPase activity by promoting nucleotide exchange (Easter and Gober, 2002). During plasmid partitioning, ParA polymerizes into filaments forming a mitotic-like apparatus, which is initially located at the centre of the nucleoid and then gradually migrates towards the cell quarters. Plasmid DNA is constantly co-localized with the ParA spot, which therefore acts as a pulling motor determining the destination of the partitioning plasmid (Hatano and Hironori, 2010). In *L. lactis*, the ParAB maintenance system has only been found on pSK08, pCI2000, pGdh442 and pKF147A to date (Kearney *et al.*, 2000, Siezen *et al.*, 2010, Tanous *et al.*, 2007). These plasmids share with pVF50 a large size (over 50 kb) and a low copy number, which make them more at risk of being lost during cell division. The ParAB maintenance system presumably increases the stability of these plasmids within the host by ensuring their faithful segregation at each cell division. In the case of pVF50, this would allow the stable maintenance of lactose and peptide utilization abilities that are essential to *L. lactis* DPC3901 for a successful adaptation to the dairy environment.

A pVF18/pVF22-cured derivative of *L. lactis* DPC3901 retains phage-resistance and potential starter and flavour-forming capacities

The plasmid-encoded capacities of resistance to phages and various stresses, coupled to the starter and flavour-forming potential, render *L. lactis* DPC3901 an all-round candidate for the preparation of starter formulations with enhanced robustness and novel technological attributes. However, the presence of *tet*(M) on the conjugative pVF22 would impede the potential use of *L. lactis* DPC3901 in foods for human consumption due to concerns regarding the spread of antibiotic resistance genes from food-borne LAB to pathogenic organisms (Teuber *et al.*, 1999). In the past, antibiotic resistance genes have been removed from probiotic strains by using methods that did not genetically modify the organism and retained the original probiotic characteristics (Rosander *et al.*, 2008). We took a similar approach and cured pVF22 from the pVF18-cured derivative of *L. lactis* DPC3901 (*L. lactis* DPC3901-c1) (Figure 5a). The strain lost pVF22 after ca. 100 generations but retained pVF50 and pVF21, as also confirmed by PCR (Figure 5b). Compared to the parent strain, the new derivative (*L. lactis* DPC3901-c2) exhibited unaltered

acidification capacities and slightly better growth rates (Table 3), probably as a result of the loss of two metabolically-burdening plasmids. Such a strain might be safely employed to generate novel starter formulations with improved protection against phage attack and ability to diversify and widen the flavour profile of the final product.

CONCLUSION

It is thought that plant-associated strains are the original source of many dairy strains as they can transfer from forage plants and meadow grasses to milk via cattle (Salama *et al.*, 1995). However, to the best of our knowledge, no clear evidence of such an origin has ever been found for any of the lactococcal genomes or plasmids sequenced to date. Plasmids are of great interest as these mobile elements often mediate extensive HGT, thus enabling strains to acquire traits conferring a selective advantage to colonize specific biotopes (Mills *et al.*, 2006). Some of these traits can be as specific to a particular niche as to be reasonably considered equivalent to traceability markers, thus enabling the plasmid complement to be used as a sort of bar-coding system of the organisms' origin. Retrieval of strains containing such traceable traits is feasible within raw milk cheeses where the absence of pasteurization allows the transfer of plant-derived bacteria to cheese via the cow-milk network.

In this study, we found that the four-plasmid complement of the raw milk cheese isolate *L. lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901 encodes a number of gene functions that have not been previously described in *Lactococcus* and are generally typical of bacteria associated with the plant ecosystem (Table 4). These include activities of modification of the plant cell wall and a functional system for the high-affinity and regulated uptake of Co^{2+}. Additionally, these plasmids carry several other genes, encoding transporters with a confirmed or predicted role in the uptake of a variety of metal ions, which have single lactococcal homologues on plant-derived plasmids (Table 4). Considering that none of these traits are required for growth in milk, their presence in a dairy strain may be inferred as genetic traces of the previous niche inhabited by the organism where they conferred the host with a real colonization advantage.

The plant-origin hypothesis is further supported by the observation that vast regions of pVF50 and pVF21 share high degrees of homology to regions of the plant-derived lactococcal plasmid pGdh442 (Tanous *et al.*, 2007), and that these regions encode phenotypes (i.e. glutamate dehydrogenase, Na^+ and K^+ transporters) so far unique to pGdh442 in *L. lactis* (Tanous *et al.*, 2007, Tanous *et al.*, 2002). This is indicative of large HGT occurred between pVF65/pVF21 and pGdh442, which again links *L. lactis* DPC3901 with the plant biotope. Based on these considerations, we

have found plasmid-encoded markers that potentially trace *L. lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901 back to a plant origin and provide for the first time the genetic basis to support the concept of the plant-milk transition for *L. lactis* strains.

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TABLES

Table 1. Bacterial strains used in this study

Bacterial strain	Relevant characteristic(s)	Source[a] or reference
L. lactis subsp. *lactis* biovar. diacetylactis DPC3901	Raw milk cheese isolate; contains four plasmids of 18, 21, 22 and 65 kb	TFRC culture collection
L. lactis subsp. *lactis* biovar. diacetylactis DPC3901-c1	pVF18-cured derivative of *L. lactis* DPC3901	This study
L. lactis subsp. *lactis* biovar. diacetylactis DPC3901-c2	pVF22-cured derivative of *L. lactis* DPC3901-c1	This study
L. lactis subsp. *cremoris* MG1363	Plasmid-free derivative of *L. lactis* subsp. *cremoris* 712	Gasson (1983)
L. lactis subsp. *lactis* biovar. diacetylactis DRC3	DNA size marker strain; contains eight plasmids with sizes from 2 to 78 kb	McKay and Baldwin (1984)
L. lactis subsp. *lactis* SK1	Cheese starter	TFRC culture collection
L. lactis subsp. *cremoris* DPC4268	Cheese starter	TFRC culture collection

[a]TFRC, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

Table 2. Primers used in this study to map gene features of *L. lactis* DPC3901 plasmids

Primer	Sequence (5'-3')	Product size (bp)	Target	Plasmid
orfs18-21 (F[a])	GAAAACATCAAGAAGAACGCAGAGT	1,858	*orfs18-21*	pVF18
orfs18-21 (R[b])	TGGCAATCGAAGCATCAAGTAGAC			
gdh (F)	GTGGGTCGACGATGCTGGTAAAGTT	822	*gdh*	pVF21
gdh (R)	TGGACGCCGCTGGAAATCAATA			
corA (F)	TTTATGTGTATGGTGACGAACGAGAAT	812	*corA*	pVF22
corA (R)	CAGCACAGCAAAAACACCATAAGAAGAT			
ldh (F)	GTGCTGCCAAATATAGATGCCCAACA	484	*ldh*	pVF50
ldh (R)	ATGCCAAAGCGGTTAGATGATTATTACGA			

[a]F = forward primer; [b]R = reverse primer. Table columns indicate primer's name and sequence, expected size of the PCR product, gene(s) target and its plasmid location.

Table 3. Comparison of the growth rate parameters and acidification capacities of *L. lactis* DPC3901 and its pVF18/pVF22-cured derivative (DPC3901-c2) with those of selected lactococcal cheese starters

L. lactis strain	Growth rate		Starter activity (pH)	
	μ_{max}[a] (h^{-1})	Generation time (h^{-1})	11% RSM	GM17
DPC3901	0.90 (± 0.08)	1.17 (± 0.10)	5.37 (± 0.18)	6.06 (± 0.04)
DPC3901-c2	0.99 (± 0.10)	1.09 (± 0.12)	5.25 (± 0.01)	5.90 (± 0.05)
SK1	1.18 (± 0.01)	0.85 (± 0.01)	4.91 (± 0.24)	5.67 (± 0.03)
DPC4268	1.10 (± 0.13)	0.92 (± 0.11)	4.78 (± 0.13)	5.41 (± 0.01)

[a] μ_{max} = maximum growth rate.

Table 4. Novel genes not found before in *L. lactis* and rare genes with homologues on plant-associated lactococcal strains

Plasmid	Gene(s)	Product(s)	Predicted function/role(s)	Best homolog found in	Identity
Novel in *L. lactis*					
pVF18	*orf11*	Polysaccharide deacetylase	Hydrolysis of cell wall carbohydrates in plants; utilization of mineral nitrogen	*Enterococcus faecium* PC4.1	99%
pVF18	*orf25*	Cupin-domain containing isomerase/epimerase or germin/plant storage protein	Modification of cell wall carbohydrates in plants; utilization of mineral nitrogen	*Streptococcus uberis* 0140J	92%
pVF18	*orfs19-20-21*	XRE family transcriptional regulator; Hypothetical protein; ABC transporter, ATPase	Putative high-affinity Co^{2+} transporter; heavy metals homeostasis	*Leuconostoc kimchii* IMSNU 11154, LkipL4726	92-99%
pVF21	*orf5*	DGQHR domain-containing hypothetical protein	putative bacteriophage protein	*Bacillus cereus* G9842	32%
pVF22	*orf1*	Primosomal protein N'	DNA replication factor	*Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293	37%
pVF50	*ctg1.gene5*	Transposase, IS110 family	DNA integration/recombination	*Streptococcus pneumoniae* SP19-BS75	75%
Rare in *L. lactis* (single hit)					
pVF18	*orf4; orf23*	GCN5-related N-acetyltransferase	Regulation of cell growth and development	*Lactococcus lactis* MG1363; *Weissella paramesenteroides* ATCC33313	98%; 84%
pVF18	*orf24*	Transposase, IS30 family	DNA integration/recombination	*Enterococcus faecalis* TUSoD Ef11	100%
pVF21	*gdh*	Glutamate dehydrogenase	Reversible oxidative deamination of glutamate	*Lactococcus lactis*, pGdh442	99%
pVF21	*orf15*	Hypothetical protein LLKF_p0013	Putative cation efflux system protein	*Lactococcus lactis* KF147, pKF147A	99%
pVF22	*orf9*	Hypothetical protein	Putative glycopeptide antibiotics resistance protein	*Streptococcus thermophilus*	98%
pVF22	*orf13*	Hypothetical protein		*Lactococcus garvieae*, pLK0018	99%
pVF50	*contig2.gene1*	Na+/H+ antiporter	Exchange of sodium/hydrogen ions	*Lactococcus lactis*, pGdh442	100%
pVF50	*contig2.gene2*	Putative K+ transporter	Uptake of potassium	*Lactococcus lactis*, pGdh442	99%
pVF50	*contig2.gene3*	Putative K+ transport system TrkA	Uptake of potassium	*Lactococcus lactis*, pGdh442	99%

FIGURES

Figure 1.

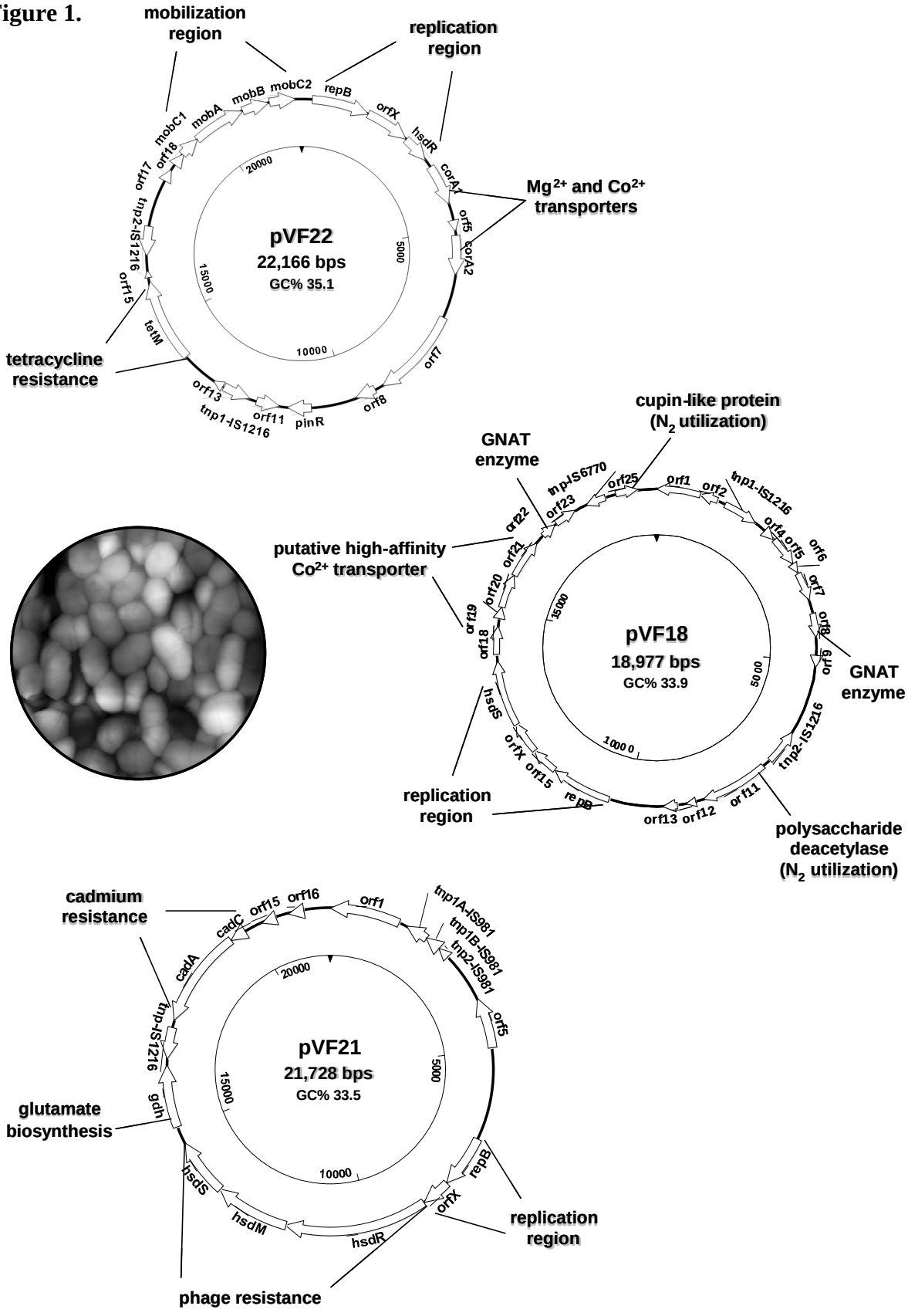


Figure 1. Physical and genetic maps of plasmids pVF18, pVF21 and pVF22 of *L. lactis* DPC3901. Position and orientation of genes is indicated by arrows. Inner circles show the nucleotide numbering. An atomic force microscopy image of *L. lactis* DPC3901 is shown within the black circle.

Figure 2.

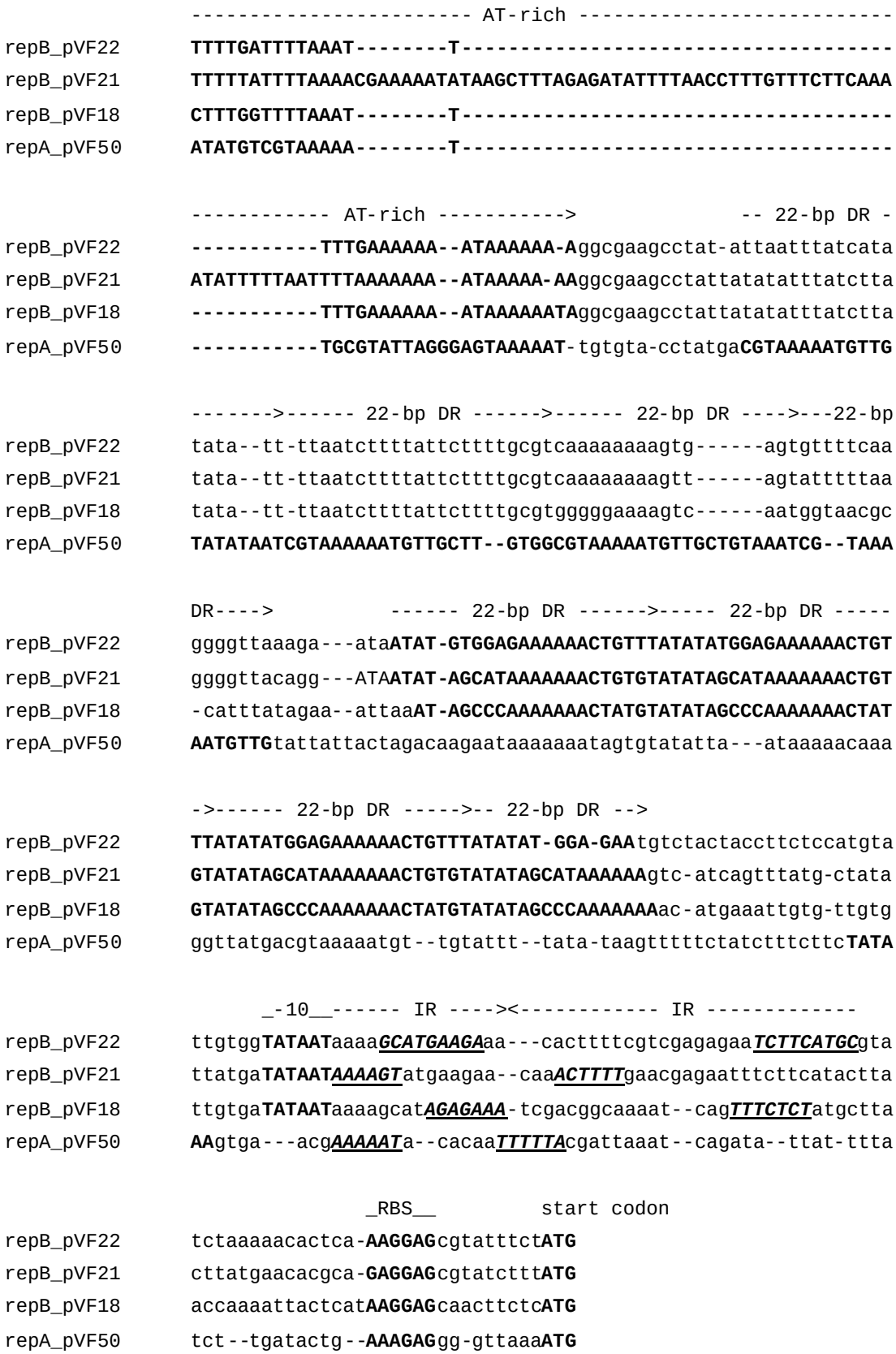
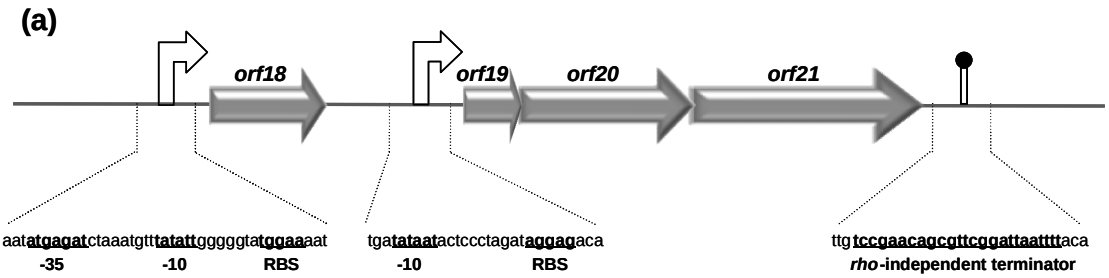

---------- AT-rich ----------
repB_pVF22 TTTTGATTTTAAAT--------T----------
repB_pVF21 TTTTTATTTTAAAACGAAAAATATAAGCTTTAGAGATATTTTAACCTTTGTTTCTTCAAA
repB_pVF18 CTTTGGTTTTAAAT--------T----------
repA_pVF50 ATATGTCGTAAAAA--------T----------
---------- AT-rich ----------> -- 22-bp DR -
repB_pVF22 ----------TTTGAAAAAA--ATAAAAAA-Aggcgaagcctat-attaatttatcata
repB_pVF21 ATATTTTTAATTTTAAAAAAA--ATAAAAA-AAggcgaagcctattatatatttatctta
repB_pVF18 ----------TTTGAAAAAA--ATAAAAAATAggcgaagcctattatatatttatctta
repA_pVF50 ----------TGCGTATTAGGGAGTAAAAAT-tgtgta-cctatgaCGTAAAAATGTTG
------->------ 22-bp DR ------>------ 22-bp DR ---->---22-bp
repB_pVF22 tata--tt-ttaatctttattcttttgcgtcaaaaaaaagtg------agtgttttcaa
repB_pVF21 tata--tt-ttaatctttattcttttgcgtcaaaaaaaagtt------agtatttttaa
repB_pVF18 tata--tt-ttaatctttattcttttgcgtgggggaaaagtc------aatggtaacgc
repA_pVF50 TATATAATCGTAAAAAATGTTGCTT--GTGGCGTAAAAATGTTGCTGTAAATCG--TAAA
DR----> ------ 22-bp DR ------>----- 22-bp DR -----
repB_pVF22 ggggttaaaga---ataATAT-GTGGAGAAAAAACTGTTTATATATGGAGAAAAAACTGT
repB_pVF21 ggggttacagg---ATAATAT-AGCATAAAAAAACTGTGTATATAGCATAAAAAAACTGT
repB_pVF18 -catttatagaa--attaaAT-AGCCCAAAAAAACTATGTATATAGCCCAAAAAAACTAT
repA_pVF50 AATGTTGtattattactagacaagaataaaaaaatagtgtatatta---ataaaaacaaa
->------ 22-bp DR ----->-- 22-bp DR -->
repB_pVF22 TTATATATGGAGAAAAAACTGTTTATATAT-GGA-GAAtgtctactaccttctccatgta
repB_pVF21 GTATATAGCATAAAAAAACTGTGTATATAGCATAAAAAAgtc-atcagtttatg-ctata
repB_pVF18 GTATATAGCCCAAAAAAACTATGTATATAGCCCAAAAAAAac-atgaaattgtg-ttgtg
repA_pVF50 ggttatgacgtaaaaatgt--tgtattt--tata-taagtttttctatctttcttcTATA
_-10__------ IR ----><------------ IR -------------
repB_pVF22 ttgtggTATAATaaaaGCATGAAGAaa---cacttttcgtcgagagaaTCTTCATGCgta
repB_pVF21 ttatgaTATAATAAAAGTatgaagaa--caaACTTTTgaacgagaatttcttcatactta
repB_pVF18 ttgtgaTATAATaaaagcatAGAGAAA-tcgacggcaaaat--cagTTTCTCTatgctta
repA_pVF50 AAgtga---acgAAAAATa--cacaaTTTTTAcgattaaat--cagata--ttat-ttta
_RBS__ start codon
repB_pVF22 tctaaaaacactca-AAGGAGcgtatttctATG
repB_pVF21 cttatgaacacgca-GAGGAGcgtatctttATG
repB_pVF18 accaaaattactcatAAGGAGcaacttctcATG
repA_pVF50 tct--tgatactg--AAAGAGgg-gttaaaATG

Figure 2. Multiple sequence alignment of the origin of replication (*ori*) region upstream the replication initiator (*rep*) gene of *L. lactis* DPC3901 plasmids. The putative AT-rich region, the 22-bp direct repeats (22-bp DR), the 6-9 bp inverted repeats (IR), the promoter -10 signal (consensus sequence TATAAT) and ribosome binding site (RBS) (consensus sequence AAGGAG), and the ATG start codon of the *rep* gene are indicated in boldface and by dashed or solid arrows above the sequence.

Figure 3.



(b)

```
          1                   21                  41                  61
MNSLISLEKVNYQIADQHIL HDVDWQIPAGAHITLTGPSG GGKSTLLRIIAAMISKTSGT LIFDGQPIESYDPIMYRRQV
                                        Walker A
          81                  101                 121                 141
SYCFQQPTLFGETVADNLAF PYQIRKQVMDTKRVVTALNN VGLSERTLHQPIIELSGGER QRVALIRNILFLPKVLLLDE
                                                      Walker C          Walker B
         161                  181                 201            216
VTAGLDENNKQIVHAWLRQL NEQDHVTTIMITHDATEIAA ADQLAKVVAGRLEVHA
```

(c)

```
          1                   21                  41                  61
Orf21     MNLAVNNTSLFLAAMLVLVA LGISLWQKLGLDRDIVIGVV RAVVQLFIVGYLLKYIFRVN NLWLTLAMIGFIIFNAAWNA
TOPCONS   ooooMMMMMMMMMMMMMMMM MMMMMiiiiiiiiiiMMMMM MMMMMMMMMMMMMMMMooooM MMMMMMMMMMMMMMMMMMMM
          81                  101                 121                 141
Orf21     KKRGPGIDHALAISLLAIFV STGVTLGVLVLSGAIKFVPS QMIPISGMIASNSMVAIGLA YRSLNSQFHDQRQAVLERLA
TOPCONS   iiiiiiiiiiMMMMMMMMMM MMMMMMMMMMMooooooooo MMMMMMMMMMMMMMMMMMMM Miiiiiiiiiiiiiiiiiii
          161                 181                 201                 221
Orf21     LGAGLLDASIAIVREAIRTG MSPTIDSAKTVGLVSLPGMM SGLIFAGVDPVRAIRYQIMV TFMLLSATSLGSIIACYLAY
TOPCONS   iiiiiiiiiiiiiiiiiii iiiiMMMMMMMMMMMMMMMM MMMMMoooooooooooooMM MMMMMMMMMMMMMMMMMMMi
          241       251
Orf21     RNFYNEQKQL K
TOPCONS   iiiiiiiiii i
```

Figure 3. Sequence analysis of the putative polycistronic operon *orfs18-21*. (a) Putative regulatory elements of the *orfs18-21* operon. The ribosome binding site (RBS), -10 and -35 promoter sequences and the *rho*-independent terminator signal are shown in bold and underlined. (b) Location of the conserved domains typical of ABC-transporter ATPases in Orf20: the Walker A (consensus GxxGxGKS/T, where x is any residue) and Walker B (consensus hhhhDE, where h is a hydrophobic residue) motifs involved in ATP binding, and the Walker C (consensus LSGGQQ/R/KQR) motif involved in ATP hydrolysis are shown underlined. (c) TOPCONS consensus prediction of membrane topology for Orf21 showing seven trans-membrane motifs (i = inside ; M = trans-membrane; o = outside) typical of ABC-transporter permeases.

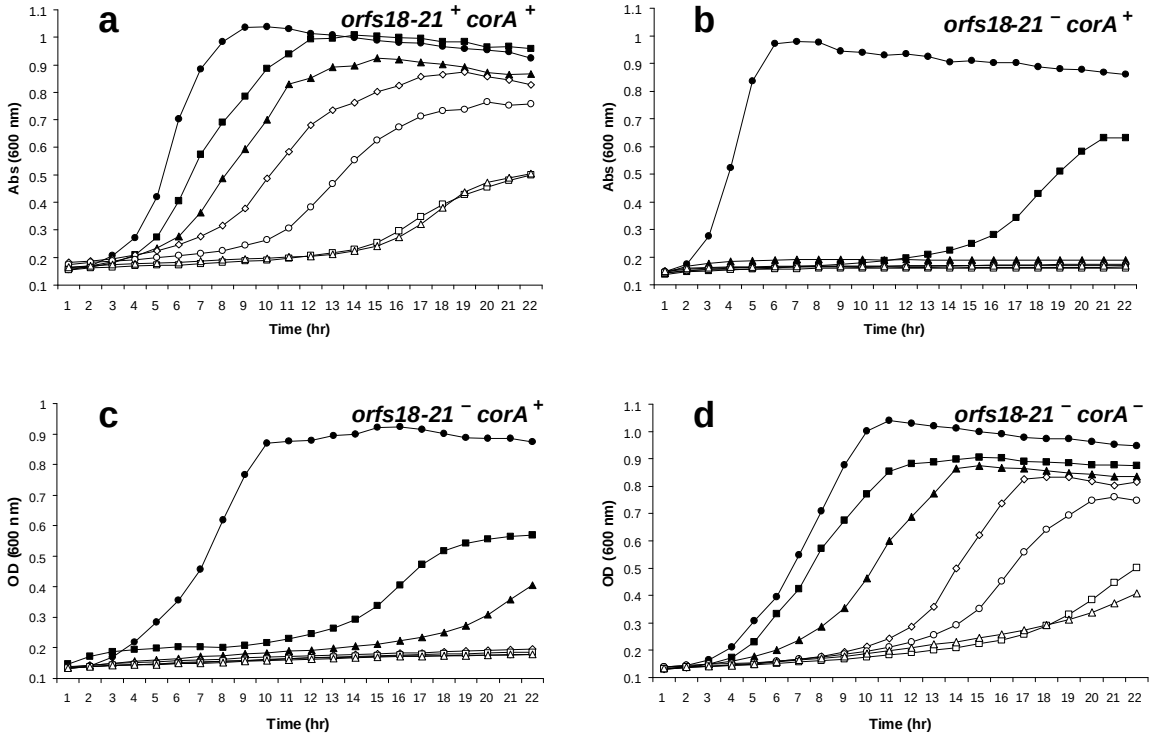


Figure 4. Growth curves of *L. lactis* DPC3901 (a), MG1363 (b), DPC3901-c1 (c) and DPC3901-c2 (d) in media containing 0 (●), 1 (■), 2 (▲), 3 (◊), 4 (○), 5 (□) and 6 mM (Δ) cobalt chloride. The "minus" or "plus" superscripts are indicative of absence or presence, respectively, of the *orfs18-21* and *corA* genotypes in each strain or derivative.

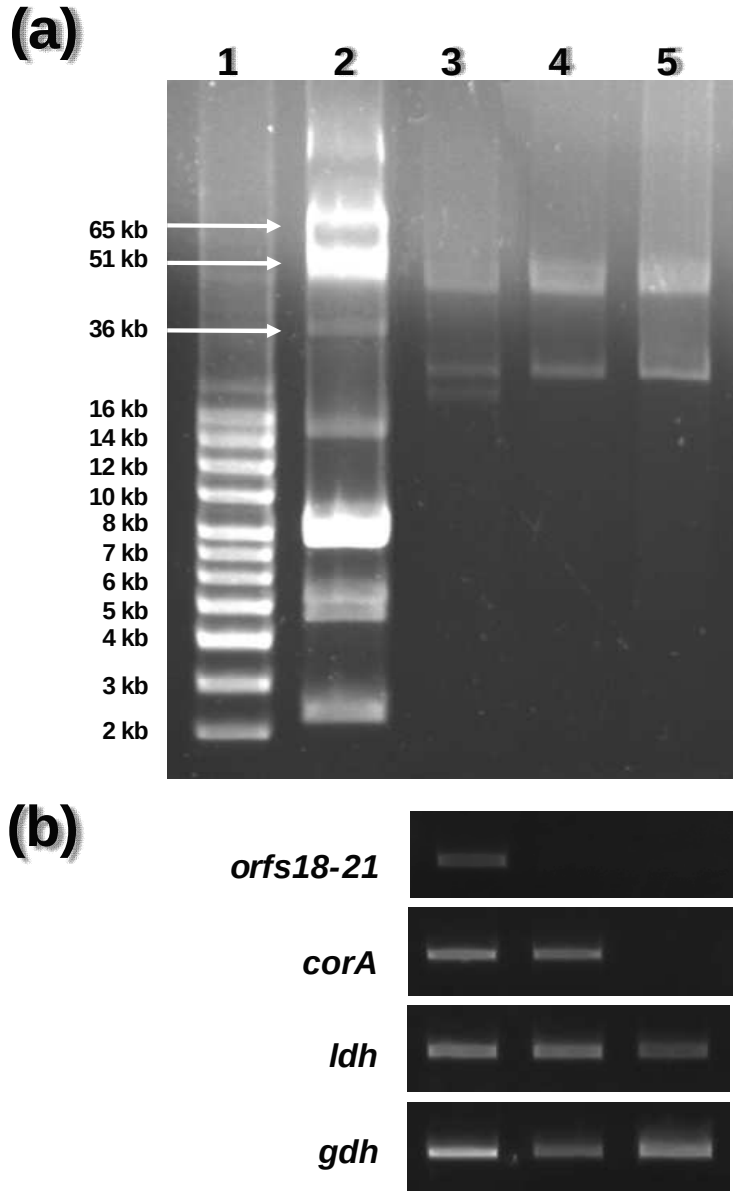


Figure 5. (a) Plasmid DNA profiles of *L. lactis* DPC3901 (lane 3) and its cured derivatives DPC3901-c1 (pVF18$^-$, lane 4) and DPC3901-c2 (pVF18$^-$ pVF22$^-$, lane 5). The plasmid profile of *L. lactis* DRC3 (lane 2) provides reference plasmids of large sizes (36, 51 and 65 kb) as a complement to the 2 to 16 kb molecular size ladder (lane 1). (b) PCR-based detection of specific gene features in the plasmid complement of *L. lactis* DPC3901, DPC3901-c1 and DPC3901-c2.

Appendix

Table A1. List of ORFs identified in pVF22 (size 22,166 bp; GC content 35.14%)

Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domain(s)	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
repB	241	1563	+	441	36.58	hypothetical protein	hypothetical protein pli0023	345/386 (89%)	0.0	*Listeria innocua* Clip11262
orfX	1559	2593	+	345	39.9	replication-associated protein, similar to OrfX	hypothetical protein pDB0R0p06	191/202 (94%)	8e-103	*L. lactis* subsp. *lactis* bv. *diacetylactis*
hsdRS	2607	3251	+	215	36.43	type I restriction-modification system restriction subunit, N-terminus; hsdS domain, C-terminus	restriction subunit	135/181 (74%)	7e-66	*L. lactis* subsp. *lactis* bv. *diacetylactis*
corA1	3440	4387	+	316	31.96	Mg2+ and Co2+ transport protein, CorA superfamily	CorA like magnesium and cobalt transport protein	316/316 (100%)	0.0	*L. lactis* subsp. *cremoris* MG1363
orf5	4772	5029	+	86	33.33	hypothetical protein; similar to repA	hypothetical protein pAH33_04	85/86 (98%)	2e-42	*L. lactis*
corA2	5126	6031	+	302	43.15	Mg2+ and Co2+ transport protein, CorA superfamily	hypothetical protein pNZ4000_44	300/302 (99%)	4e-174	*L. lactis* subsp. *cremoris*
orf7	7044	9155	+	704	40.29	ATP-dependent Clp protease, ATP-binding subunit ClpL	ATP-dependent Clp protease, ATP-binding subunit ClpL	697/704 (99%)	0.0	*Enterococcus faecium* TX1330
orf8	9346	9822	+	158	40.04	truncated transposase	truncated transposase	146/158 (92%)	8e-82	*Enterococcus faecium*
orf9	9917	10489	+	191	35.07	putative glycopeptide antibiotics resistance protein	hypothetical protein	189/191 (98%)	1e-101	*Streptococcus thermophilus*
pinR	10866	11417	+	184	34.78	Site-specific Serine Recombinase	DNA-invertase/resolvase	180/184 (97%)	1e-92	*L. lactis* subsp. *cremoris* MG1363
orf11	12130	11597	_	178	32.95	putative acetyltransferase, GNAT family	Acetyltransferase	87/177 (49%)	2e-40	*Streptococcus suis* 05ZYH33
tnp1-IS1216	12937	12296	_	214	38	transposase, IS1216 family	IS1216 transposase	212/214 (99%)	1e-120	*Enterococcus faecalis* V583
orf13	12971	13213	+	81	27.98	hypothetical protein	hypothetical protein pLK0018_p17	80/81 (99%)	9e-38	*Lactococcus garvieae* , pLK0018
orf14	14046	15983	+	646	33.38	tetracycline resistance protein	tetracycline resistance protein	592/592 (100%)	0.0	*Streptococcus thermophilus*
orf15	16074	16238	+	55	35.75	hypothetical protein	hypothetical protein pK214_p31	54/55 (98%)	2e-23	*L. lactis* subsp. *lactis*
tnp2-IS1216	17252	16569	_	228	37.71	transposase, IS1216 family	transposase	228/228 (100%)	5e-131	*Enterococcus faecium*
orf17	18401	18655	+	85	38.43	DNA recombinase, C-terminal fragment	DNA recombinase	82/85 (96%)	8e-42	*L. lactis* subsp. *lactis*
orf18	18787	19107	+	107	34.26	conserved hypothetical protein	conserved hypothetical protein	93/107 (86%)	9e-47	*L. lactis*
mobC1	19082	19573	+	164	34.34	MobC-like protein	conserved hypothetical protein	161/164 (98%)	3e-88	*Enterococcus faecalis* TX2141
mobA	19545	20784	+	410	40.4	MobA-like protein	Relaxase/Mobilisation nuclease domain	406/410 (99%)	0.0	*Enterococcus* sp. 7L76
mobB	20784	21404	+	207	37.35	MobB-like protein	putative mobilization protein	184/207 (88%)	3e-104	*L. lactis* subsp. *cremoris*
mobC2	21424	22023	+	200	32	MobC-like protein	putative mobilization protein	198/200 (99%)	5e-111	*L. lactis* subsp. *cremoris*

Table A2. List of ORFs identified in pVF21 (size 21,728 bp; GC content 33.59%)

Gene name	Start (bp)	End (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domain(s)	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
orf1	1488	2	–	495	28.03	Primosomal protein N' (replication factor Y), superfamily II helicase (COG1198)	primosomal protein N'	180/498 (37%)	1e-73	*Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293
tnp1A-IS981	2161	1664	–	166	35.14	transposase, IS981 family; C-terminus, frame shift	transposase	148/163 (90%)	3e-85	*L. lactis* subsp. *cremoris* SK11
tnp1B-IS981	2499	2143	–	119	40.33	transposase, IS981 family; N-terminus, frame shift	transposase	100/117 (85%)	2e-53	*L. lactis* subsp. *cremoris* SK11
tnp2-IS981	2756	2499	–	86	37.98	transposase, IS981 family	transposase	85/86 (99%)	8e-42	*L. lactis* subsp. *lactis* KF147
orf5	4964	3870	–	365	26.48	hypothetical protein containing the DGQHR domain (TIGR03187); several members annotated as putative bacteriophage proteins	DGQHR domain-containing protein	57/175 (32%)	2e-25	*Bacillus cereus* G9842
repB	6953	8101	+	383	33.5	replication protein RepB	replication protein	378/383 (98%)	0.0	*L. lactis* subsp. *cremoris*
orfX	8104	8730	+	209	33.33	replication-associated protein, similar to OrfX	hypothetical protein LACR_E7	126/222 (56%)	1e-50	*L. lactis* subsp. *cremoris* SK11
hsdR	8744	11818	+	1025	37.91	type I restriction-modification system, restriction subunit	hypothetical protein pAH82_p15	1017/1025 (99%)	0.0	*L. lactis* subsp. *lactis*
hsdM	11803	13413	+	537	39.78	type I restriction-modification system, modification subunit	putative type I site-specific deoxyribonuclease	534/537 (99%)	0.0	*L. lactis* subsp. *cremoris* MG1363
hsdS	13406	14647	+	414	36.47	type I restriction-modification system, specificity subunit	type I R/M system specificity subunit	159/430 (36%)	3e-58	*L. lactis* subsp. *lactis* bv. *diacetylactis*
gdh	15034	16377	+	448	44.27	NADP-dependent glutamate dehydrogenase	glutamate dehydrogenase	447/448 (99%)	0.0	*Lactococcus lactis*
tnp-IS1216	17187	16504	–	228	39.32	transposase for insertion sequence-like element IS1216, C-terminal fragment	hypothetical protein pGdh442_02	227/228 (99%)	7e-131	*Lactococcus lactis*
cadA	19461	17347	–	705	37.16	cadmium efflux ATPase CadA	cadmium efflux ATPase CadA	705/705 (100%)	0.0	*L. lactis* subsp. *lactis*
cadC	19461	19817	–	119	31.37	cadmium resistance regulator CadC	cadmium resistance regulator CadC	119/119 (100%)	1e-61	*L. lactis* subsp. *lactis*
orf15	20550	20230	–	107	20.87	putative cation efflux system protein	hypothetical protein LLKF_p0013	106/107 (99%)	8e-53	*L. lactis* subsp. *lactis* KF147, pKF147A
entA-im	21155	20823	–	111	33.33	Enterocin A Immunity domain (pfam08951)	ORF13	96/107 (90%)	9e-45	*Lactococcus lactis*, pNP40

Table A3. List of ORFs identified in pVF18 (size 18,977 bp; GC content 33.9%)

Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domain(s)	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
orf1	834	10	-	275	30.30	putative membrane protein	hypothetical protein llmg_0710	271/275 (99%)	1e-151	*L. lactis* subsp. *cremoris* MG1363
orf2	1175	834	-	114	31.87	transcriptional regulator, PadR-like family	hypothetical protein pMRC01_047	114/114 (100%)	2e-58	*Lactococcus lactis*
tnp1-1216	1340	2002	+	221	37.25	transposase, IS1216 family	IS1216, transposase	175/214 (82%)	2e-101	*Listeria grayi* DSM 20601
orf4	2230	2467	+	79	40.75	integral membrane protein; putative transglycosylase associated proteins, cl00978	hypothetical protein llmg_1257	78/79 (98%)	6e-26	*L. lactis* subsp. *cremoris* MG1363
orf5	2499	3056	+	186	32.61	putative membrane protein	hypothetical protein LLKF_2283	181/186 (98%)	6e-87	*L. lactis* subsp. *lactis* KF147
orf6	3069	3254	+	62	30.64	small integral membrane protein of unknown function, COG5547	hypothetical protein llmg_1259	62/62 (100%)	2e-07	*L. lactis* subsp. *cremoris* MG1363
orf7	3278	3826	+	183	35.15	general stress protein, Gls24 family	putative 20-kDa protein	183/183 (100%)	6e-80	*Lactococcus lactis*
orf8	4087	4545	+	153	28.32	putative acetyltransferase, GNAT family, cd04301	hypothetical protein pJB01p2	149/153 (97%)	2e-81	*Enterococcus faecium*
orf9	4884	5120	+	79	33.33	putative DNA polymerase III subunit epsilon, PRK06195	conserved hypothetical protein	73/74 (99%)	1e-34	*Enterococcus faecalis* E1Sol
tnp2-IS1216	7119	6442	-	226	36.57	transposase, IS1216 family	ORF-W2 protein	221/226 (97%)	2e-121	*Lactococcus lactis*
orf11	7288	8583	+	432	36.11	putative hydrolase; polysaccharide deacetylase domain, PFAM01522	conserved hypothetical protein	431/432 (99%)	0.0	*Enterococcus faecium* PC4.1
orf12	8743	8931	+	63	37.56	DNA integrase/recombinase, C-terminal fragment	DNA recombinase	61/63 (96%)	1e-28	*L. lactis* subsp. *lactis*
orf13	9099	9371	+	91	34.06	conserved hypothetical protein; putative cytochrome B-like	unknown	85/91 (94%)	4e-42	*L. lactis* subsp. *cremoris*
repB	10408	11562	+	385	33.93	replication protein RepB	replication protein	305/385 (79%)	2e-178	*L. lactis* subsp. *lactis*
orf15	11579	12103	+	175	27.04	hypothetical protein	hypothetical protein LACR_B7	165/175 (94%)	1e-84	*L. lactis* subsp. *cremoris* SK11
orfX	12103	12732	+	210	31.90	replication-associated protein, similar to OrfX	hypothetical protein pCI305_p2	186/210 (88%)	3e-99	*L. lactis* subsp. *lactis*
hsdS	12711	13976	+	422	34.91	type I restriction-modification system, specificity subunit	type I R/M system specificity subunit	177/436 (40%)	4e-63	*L. lactis* subsp. *lactis* bv. *diacetylactis*
orf18	14113	14646	+	182	30.95	transcriptional regulator, Xre family	putative transcriptional regulator	53/130 (41%)	1e-24	*Leuconostoc kimchii* IMSNU11154
orf19	14793	15017	+	75	40.44	hypothetical protein	hypothetical protein LKI_10701	72/79 (92%)	6e-32	*Leuconostoc kimchii* IMSNU11154
orf20	15017	15664	+	216	44.13	ABC_cobalt_CbiO domain; cl09099: P-loop NTPase	ABC transporter, ATP-binding protein	215/216 (99%)	4e-123	*Leuconostoc kimchii* IMSNU11154
orf21	15664	16416	+	251	46.08	ABC-type uncharacterized transport system, permease component, COG0390; putative YbbM family protein	ABC-type uncharacterized transport system, permease component	251/251 (99%)	9e-139	*Leuconostoc citreum* KM20
orf22	16597	16902	+	102	40.84	DNA integrase/recombinase, N-terminal fragment	integrase/recombinase plasmid associated, putative	101/102 (99%)	2e-51	*Leuconostoc kimchii* IMSNU11154
orf23	16940	17368	+	143	29.13	putative acetyltransferase, GNAT family, cd04301	GNAT family acetyltransferase	114/136 (84%)	6e-51	*Weissella paramesenteroides* ATCC33313
tnp-IS6770	17986	17597	-	130	35.64	transposase, IS30 family, COG2826; N-terminal fragment	transposase	130/130 (100%)	6e-71	*Enterococcus faecalis* TUSoD Ef11
orf25	18201	18620	+	140	35.95	protein containing cupin 2 domain, PFAM07883	hypothetical protein SUB0906	127/139 (92%)	2e-72	*Streptococcus uberis* 0140J

Table A4. List of ORFs identified in contig1 (size 23,150 bp) and predicted to be contained in pVF50

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domain(s)	Highest homology protein	aa identity(%)	E-value	Organism, plasmid
ctg1.gene1		13	330	+	110	32.12	Peptidase S24_S26_LexA-like superfamily (cd06462); truncated, C-terminus	phage repressor-like protein	44/93 (48%)	2e-11	*Streptococcus equi* subsp. *zooepidemicus*
ctg1.gene2	repA	838	2187	+	450	31.18	Initiator Replication protein (pfam01051)	replication protein	448/450 (99%)	0.0	*Lactococcus lactis*, pGdh442
ctg1.gene3	parA	2943	3698	+	252	28.83	ParA domain (cd02042); Soj domain, ATPases involved in chromosome partitioning (COG1192)	partition protein A	250/252 (99%)	1e-138	*Lactococcus lactis*, pGdh442
ctg1.gene4	parB	3706	4431	+	242	34.02	ParB-like nuclease domain (pfam02195)	partition protein B	241/242 (99%)	3e-133	*Lactococcus lactis*, pGdh442
ctg1.gene5	tnp-IS110	5182	6435	+	418	38.03	Transposase belonging to the IS110 family; Transposase_20 domain (pfam02371); Transposase_9 domain (pfam01548)	transposase, IS116/IS110/IS902 family protein	314/419 (75%)	0.0	*Streptococcus pneumoniae* SP19-BS75
ctg1.gene6		6604	6936	+	111	35.13	Transposase and inactivated derivatives, Tra5 (COG2801); truncated, C-terminus	transposase	106/110 (97%)	1e-58	*L. lactis* subsp. *cremoris* SK11, pSK11P
ctg1.gene7		6950	7300	+	117	36.75	Transposase and inactivated derivatives, Tra5 (COG2963); Helix-turn-helix domain of Hin and related proteins (cl01116); truncated, C-terminus	IS-LL6 transposase/hypothetical protein fusion	115/115 (100%)	8e-60	*L. lactis* subsp. *cremoris* SK11, pSK11P
ctg1.gene8	tnp-IS3	7261	8157	+	299	38.46	Transposase belonging to the IS3 family; putative transposase OrfB (PHA02517)	unnamed protein product	298/299 (99%)	5e-177	*Lactococcus lactis*
ctg1.gene9		8419	10278	+	620	40.91	Cation transport ATPase (COG2217); Haloacid Dehalogenase superfamily (TIGR01488); E1-E2 ATPase (pfam00122)	cation transport ATPase	617/620 (99%)	0.0	*L. lactis* subsp. *lactis* KF147, pKF147A
ctg1.gene10		10984	11208	+	75	37.77	Heavy-metal-associated domain (pfam00403); Copper chaperone [Inorganic ion transport and metabolism] (COG2608)	copper chaperone	75/75 (100%)	3e-34	*L. lactis* subsp. *lactis* KF147, pKF147A
ctg1.gene11	dpsB	11260	11802	+	181	35.91	DPS protein, ferritin-like diiron-binding domain (cd01043)	DNA binding protein	181/181 (100%)	9e-101	*L. lactis* subsp. *lactis* KF147, pKF147A
ctg1.gene12	flp	11891	12577	+	229	38.71	cAMP-binding proteins (COG0664); effector domain of the CAP family of transcription factors (cd00038); Winged helix-turn-helix (WHTH) DNA-binding domain of the GntR family of transcriptional regulators (cl00088)	FNR family transcriptional regulator	225/229 (99%),	5e-129	*L. lactis* subsp. *lactis* KF147, pKF147A
ctg1.gene13	oppD	13554	14567	+	338	37.77	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component, DppD (COG0444); ABC_NikE_OppD_transporters (cd03257)	oligopeptide ABC trasporter ATP binding protein	333/338 (99%)	0.0	*L. lactis* subsp. *lactis* Il1403
ctg1.gene14	oppF	14567	15523	+	319	35.63	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component, DppF (COG0444); ABC_NikE_OppD_transporters (cd03257)	oligopeptide transporter F	316/319 (99%)	0.0	*Lactococcus lactis*, pGdh442
ctg1.gene15	oppB	15507	16463	+	319	38.14	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components (COG0601); Transmembrane subunit found in Periplasmic Binding Protein (PBP)-dependent ATP-Binding Cassette (ABC) transporters (cd06261)	oligopeptide transporter B	316/319 (99%)	0.0	*Lactococcus lactis*, pGdh442
ctg1.gene16	oppC	16476	17357	+	294	36.39	ABC-type uncharacterized transport system, permease component (COG4239); Transmembrane subunit found in Periplasmic Binding Protein (PBP)-dependent ATP-Binding Cassette (ABC) transporters (cd06261)	oligopeptide transporter C	289/294 (99%)	1e-164	*Lactococcus lactis*, pGdh442
ctg1.gene17	oppA	17465	19262	+	599	37.37	PBP2_Lactococcal_OppA_like transport system, substrate binding domain (cd08510)	oligopeptide transporter A	576/599 (97%)	0.0	*Lactococcus lactis*, pGdh442
ctg1.gene18	pepO	19392	21270	+	626	35.17	Predicted metalloendopeptidase PepO (COG3590); Peptidase family M13 (pfam01431)	oligoendopeptidase O	623/626 (99%)	0.0	*L. lactis* subsp. *lactis* KF147, pKF147A
ctg1.gene19	tnp-IS946	22577	21942	-	212	39.93	rve, Integrase core domain (pfam00665); Transposase and inactivated derivatives (COG3316)	transposase	201/213 (95%)	3e-113	*Lactococcus lactis*, pTR2030

Table A5. List of ORFs identified in contig2 (size 18,225 bp) and predicted to be contained in pVF50

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domain(s)	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
ctg2_gene1	nah	327	1469	+	381	30.44	Sodium/hydrogen exchanger family (pfam00999); Kef-type K+ transport systems, membrane components (COG0475)	Na+/H+ antiporter	374/374 (100%)	0.0	*Lactococcus lactis*, pGdh442
ctg2_gene2		1485	2819	+	445	34.23	potassium uptake protein, TrkH family (TIGR00933); Trk-type K+ transport systems, membrane components, TrkG (COG0168)	putative K+ transporter	443/445 (99%)	0.0	*Lactococcus lactis*, pGdh442
ctg2_gene3		2842	3510	+	223	32.88	K+ transport systems, NAD-binding component, TrkA (COG0569); Anion permease ArsB/NhaD (cl09110); Rossmann-fold NAD(P)(+)-binding proteins (cl09931)	putative K+ transport system TrkA	213/214 (99%)	5e-118	*Lactococcus lactis*, pGdh442
ctg2_gene4		4348	3892	-	152	39.6	transposase IS1216E belonging to IS6 family; truncated, C-terminus	transposase	151/152 (99%)	8e-84	*L. lactis* subsp. *lactis* K214, pK214
ctg2_gene5		13315	13037	-	93	33.69	hypothetical protein	hypothetical protein pGdh442_p07	92/93 (99%)	2e-47	*Lactococcus lactis*, pGdh442
ctg2_gene6		5215	5625	+	137	33.81	Protein family of unknown function, DUF322 (PFAM03780); may be involved in stress response; truncated, C-terminus	General stress protein, Gls24 family	123/125 (99%)	3e-60	*L. lactis* subsp. *lactis* KF147, pKF147A
ctg2_gene7	cspA	6601	6798	+	66	33.33	putative cold shock protein	putative cold shock protein	65/66 (99%)	1e-29	*L. lactis* subsp. *cremoris* SK11, pSK11P
ctg2_gene8	cspC	6932	7129	+	66	32.82	Cold-shock' DNA-binding domain (pfam00313); Ribosomal protein S1-like RNA-binding domain (cl09927)	cold shock protein	64/66 (97%)	1e-29	*L. lactis* subsp. *lactis* KF147
ctg2_gene9	cspB	7337	7609	+	91	32.6	Cold-shock' DNA-binding domain (pfam00313); Ribosomal protein S1-like RNA-binding domain (cl09927)	cold shock protein	90/91 (99%)	9e-46	*L. lactis* subsp. *lactis* KF147
ctg2_gene10		8108	8698	+	197	31.47	Helix-turn-helix XRE-family like proteins (cd00093)	XRE family transcriptional regulator	169/179 (95%)	1e-85	*L. lactis* subsp. *lactis* KF147, pKF147A
ctg2_gene11	mobC	9891	10256	+	148	26.8	Bacterial mobilisation protein, MobC (pfam05713)	ORF57	104/122 (86%)	4e-52	Lactococcus lactis, pNP40
ctg2_gene12	mobD	10264	10773	+	170	32.15	Relaxase/Mobilisation nuclease domain (pfam03432)	MobD	157/168 (94%)	5e-89	*Lactococcus lactis*, pGdh442
ctg2_gene13	tnp-ISLpl1	7123	7542	+	140	36.19	Transposase and inactivated derivatives, IS30 family (COG2826)	putative transposase of ISLpl1	139/140 (99%)	1e-75	*Lactococcus lactis*, pGdh442
ctg2_gene14	dld	11363	13039	+	559	38.87	D-lactate dehydrogenase, membrane binding (pfam09330); FAD/FMN-containing dehydrogenases (COG0277)	D-lactate dehydrogenase	558/559 (99%)	0.0	*L. lactis* subsp. *cremoris* SK11, pSK11P
ctg2_gene15	tnp1-IS150	13146	13694	+	183	36.24	Transposase and inactivated derivatives, IS3 family (COG2963)	transposase	182/183 (99%)	6e-103	*L. lactis* subsp. *cremoris* SK11
ctg2_gene16	tnp2-IS150	13700	14518	+	273	34.67	Integrase core domain (pfam00665); Transposase and inactivated derivatives, IS3 family (COG2801)	transposase	269/273 (99%)	2e-158	Enterococcus faecalis T1
ctg2_gene17	tnpA1-IS1068	14532	14882	+	117	36.75	Transposase and inactivated derivatives, IS3 family (COG2963); Helix-turn-helix DNA-binding domain of Hin and related proteins (cl01116)	IS-LL6 transposase/hypothetical protein fusion	115/115 (100%)	8e-60	*L. lactis* subsp. *cremoris* SK11, pSK11P
ctg2_gene18	tnpB1-IS1068	14855	15739	+	295	38.75	Transposase and inactivated derivatives, IS3 family (COG2963); Helix-turn-helix DNA-binding domain of Hin and related proteins (cl01116)	transposase AB of IS1068	277/279 (99%)	8e-165	*Lactococcus lactis*, pGdh442
ctg2_gene19		16327	15884	-	148	36.93	Integrase core domain (pfam00665); Transposase and inactivated derivatives, IS3 family (COG2801); truncated	IS981 transposase B	92/136 (68%)	3e-42	*L. lactis* subsp. *lactis* KF147
ctg2_gene20	pepF	18126	16315	-	604	33.94	Oligoendopeptidase F (COG1164); Peptidase family M3B Oligopeptidase F (cd06459)	oligopeptidase F	597/602 (99%)	0.0	*L. lactis* subsp. *cremoris* SK11, pSK11P

Table A6. List of ORFs identified in contig3 (size 8,252 bp) and predicted to be contained in pVF50

Code	Gene name	start (bp)	end (bp)	Strand	Length (aa)	G+C (%)	Product / Predicted function / Conserved domain(s)	Highest homology protein	aa identity (%)	E-value	Organism, plasmid
ctg3.gene1	lacX	631	2	-	210	36.19	Aldose 1-epimerase, similar to Lactococcus lactis lacX (cd09024); Galactose mutarotase and related enzymes (COG2017); truncated, N-terminus	galactose mutarotase-like protein	208/210 (99%)	6e-120	*L. lactis* subsp. *cremoris* SK11, pSK11L
ctg3.gene2	lacG	2350	920	-	477	38.15	Glycosyl hydrolase family (pfam00232); 6-phospho-beta-galactosidase (TIGR01233)	6-phospho-beta-galactosidase	475/477 (99%)	0.0	*Lactobacillus sakei*
ctg3.gene3	lacE	4167	2511	-	552	40.97	phosphotransferase system, lactose specific, IIC component (TIGR00394); PTS_IIB_lactose (cd05565)	lactose-specific phosphotransferase system, enzymes IIBC	541/552 (99%)	0.0	*Enterococcus faecalis* DS5
ctg3.gene4	lacF	4484	4170	-	105	42.53	PTS_IIA, PTS system, lactose/cellobiose specific IIA subunit (cd00215)	PTS family lactose-N,N-diacetylchitobiose-beta-glucoside	105/105 (100%)	3e-54	*Enterococcus faecium* TX1330
ctg3.gene5	lacD	5492	4515	-	326	39.05	Tagatose 1,6-diphosphate aldolase (TIGR01232)	tagatose 1,6-diphosphate aldolase	322/326 (99%)	0.0	*Enterococcus faecium* TX1330
ctg3.gene6	lacC	6428	5499	-	310	36.66	1-phosphofructokinase (FruK), minor 6-phosphofructokinase (pfkB) and related sugar kinases (cd01164); tagatose-6-phosphate kinase, lacC (TIGR01231)	tagatose-6-phosphate kinase	307/310 (99%)	2e-176	*Enterococcus faecium* TX1330
ctg3.gene7	lacB	6954	6442	-	171	39.76	Galactose-6-phosphate isomerase subunit LacB (TIGR01119)	galactose-6-phosphate isomerase, subunit LacB	170/171 (99%)	2e-95	*Lactobacillus sakei*
ctg3.gene8	lacA	7396	6974	-	141	39.0	Galactose-6-phosphate isomerase subunit LacA (TIGR01118)	galactose-6-phosphate isomerase, subunit LacA	140/141 (99%)	1e-74	*Lactococcus lactis*, pLP712
ctg3.gene9	lacR	8252	7896	-	119	33.89	Winged helix-turn-helix DNA-binding domain of the GntR family of transcriptional regulators (cd07377); Bacterial regulatory proteins, deoR family (pfam00455); truncated, N-terminus	lactose phosphotransferase system repressor	119/119 (100%)	1e-60	*Enterococcus faecium* 1,230,933

Chapter V

The presence of pMRC01 promotes greater cell permeability and autolysis in lactococcal starter cultures

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ABSTRACT

Conjugative transfer of plasmid-associated properties is routinely used to generate food-grade derivatives of lactococcal starter strains with improved technological traits. However, the introduction of one or more plasmids in a single strain is likely to impose a burden on regular cell metabolism and may affect the growth characteristics of the transconjugant culture. The aim of this study was to evaluate the impact of the 60.2-kb plasmid pMRC01 (encoding for a bacteriophage resistance system and production of the antimicrobial, lacticin 3147) on starter performance. Five lactococcal strains (*L. lactis* HP, 255A, SK1, 712 and IL1403) and their pMRC01-containing derivatives were compared in terms of technological properties, including analysis of growth, acidification and autolysis rates. The transconjugants exhibited lower specific growth rates and higher generation times compared to the parental strains when grown at 30 °C in glucose-M17, but the presence of pMRC01 did not significantly affect the acidification capacity of strains in 11% reconstituted skimmed milk and synthetic media. Levels of lactate dehydrogenase were two-fold higher in supernatants of transconjugants than in those of parental strains, after 24 and 72 h of growth at 30 °C in glucose-M17, suggesting that the presence of pMRC01 somehow accelerates and promotes cellular autolysis. Analysis by flow cytometry following live/dead™ staining confirmed this result by showing larger populations of injured and dead cells in pMRC01-carrying cultures compared to the parental strains. The results of this study reveal that the plasmid pMRC01 places a burden on lactococcal host metabolism, which is associated with an increased cell permeability and autolysis, without significantly affecting the acidification capacity of the starter. While the magnitude of these effects appear to be strain dependent, production of the bacteriocin lacticin 3147 may not be involved.

INTRODUCTION

Lactococcus lactis is a gram-positive homofermentative microorganism exploited worldwide as one of the main components of starter cultures for the manufacture of dairy and other fermented products. It has been estimated that over 10^9 litres of starter bacteria are annually used in food fermentation, resulting in cheese production of 10^7 tons and human consumption close to 10^{18} lactococci: these figures adequately depict the biotechnological and economical value of lactococcal strains (Bolotin *et al*., 2001). Much of the genetic basis associated with the diversity and technological superiority of commercial *L. lactis* starters is recognized to reside in their plasmid complement. Plasmids are omnipresent among dairy *L. lactis* strains, and most isolates contain multiple plasmids ranging in size from 2 to 80 kb. Modern DNA sequencing technologies have decisively boosted the number of plasmid sequences available, unravelling the diversity and contribution of these genetic necessities to the performance of lactococcal starter. Many industrially important traits, including lactose metabolism, proteinase production, citrate metabolism, bacteriophage resistance, and bacteriocin and exopolysaccharide production are generally plasmid-encoded. The self-transmissible nature of some plasmids has either allowed the exploitation of these genotypes to improve the performance of industrial starters or provided the genetic markers for the construction of food-grade vectors for *L. lactis* (Lee and Moon, 2003, Mills *et al.*, 2006).

A disadvantage associated with plasmid-mediated strategies for improvement of starters is that the introduction of one or more plasmids in a single strain may impose an additional burden to the regular cell metabolism. Plasmid genes compete with the chromosomally-encoded genes for the limiting cellular resources available for DNA replication and expression. Reallocation of energy and precursor metabolites into plasmid replication and synthesis of plasmid proteins places a metabolic burden that usually results in alteration of the growth kinetics of the transconjugant strain (Diaz Ricci and Hernandez, 2000, Rozkov *et al*., 2004). Plasmid-host interaction has been the focus of considerable research in recombinant microorganisms, most notably in *Escherichia coli*. Diaz Ricci and Hernandez (2000), in reviewing these studies, concluded that size, copy number, and gene expression are the plasmid properties most likely to affect cell growth rate, and that the impact of gene over-expression is significantly higher than that of size or copy number. The few studies that do exist in

lactococci (el Alami *et al.*, 1992, Kobayashi *et al.*, 2002, Lee and Moon, 2003, Pillidge *et al.*, 2000) report of a negative impact of plasmid content on growth and acidification rates of the lactococcal host cell, and tend to associate the metabolic burden with the genetic information encoded on the plasmid (Kobayashi *et al.*, 2002) rather than with size or copy number (el Alami *et al.*, 1992).

Nevertheless, the analysis, mobilization and exploitation of the genetic diversity of plasmids naturally occurring in lactococci remains one of the most utilized and promising tools for the food-grade improvement of industrial starters. Many plasmids carrying industrially valuable traits have been recently described (Mills *et al.*, 2006). One of these is the 60.2-kb plasmid pMRC01 that was found in *Lactococcus lactis* subsp. *lactis* DPC3147, a strain isolated from an Irish kefir grain (Ryan *et al.*, 1996). Useful characteristics of the plasmid include determinants encoding for conjugative functions, bacteriophage resistance and lacticin 3147 production. Conjugal transfer of this plasmid has been successfully performed in over 30 starter cultures giving rise to strains with improved phage resistance and broad anti-microbial properties while retaining sufficient acidification capacity (Coakley *et al.*, 1997, Ryan *et al.*, 1996).

Initial observations that pMRC01-bearing strains generally grow more slowly and exhibit reduced rates of acid production compared to the parent strains prompted a more detailed investigation of the metabolic load associated with the presence of pMRC01 in lactococcal starters through a comparative analysis of growth, acidification and viability rates of wild-type and pMRC01-transconjugant strains. The results of this study reveal that the acquisition of pMRC01 places a metabolic burden on lactococcal host that results in lower growth rate in synthetic media and greater cell permeability and autolysis. Nevertheless, the plasmid does not significantly affect the milk acidification times of the strains, thus allowing their use as starter cultures in dairy industry. In this respect, it is arguable that the permeable physiological state of plasmid-carrying strains may also be significant in terms of contribution to cheese ripening and flavour.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains used in this study are listed in Table 1. Lactococcal strains were routinely propagated at 30 °C in M17 medium (Oxoid, Hampshire, England) supplemented with 0.5% (wt/vol) glucose (GM17). 11% reconstituted skim milk (RSM) was also used during the course of this study. Solid media contained 1.0% (wt/vol) bacteriological agar (Oxoid, Hampshire, England). Where required, the antibiotic rifampicin was added to agar at a concentration of 100 μg/ml. Bacterial strains were stocked in M17 containing 40% glycerol at -80 ℃. Working cultures were stored at 4 °C and transferred periodically. Selective plates containing lacticin 3147 and rifampicin were prepared as follows: an overnight culture of *L. lactis* DPC3147 was centrifuged at 8,000 x *g* for 30 min and the resulting supernatant was adjusted to pH 7.0 with concentrated NaOH, filter sterilized with Millipore (Middlesex, England) HVLP filters (0.45 μm pore size) and tempered to 45°C. The bacteriocin preparation was added to an equal volume of double-strength lactose indicator agar (LIA) (McKay *et al.*, 1972), containing rifampicin (100 μg / ml) and bromocresol purple (0.004%), to generate a final lacticin 3147 concentration of 400 arbitrary units / ml.

Detection of plasmid pMRC01 in lactococcal transconjugants

Plasmid DNA was extracted according to the method of (O'Sullivan and Klaenhammer, 1993). Plasmid profiles were analyzed by electrophoresis on horizontal 0.6% agarose gels (Sigma, St. Louis, USA) in TAE buffer (40 mM Tris–HCl, 20 mM Acetic acid, 2 mM EDTA, pH 8.0) for 4 h at 80 V. Plasmid DNA molecules were sized using HyperLadder I (0.2-10 kb) (Bioline, London, UK) and the strain *L. lactis* subsp. *lactis* biovar. diacetylactis DRC3 as a source of high molecular size markers (35, 51, 65 and 78 kb) (McKay and Baldwin, 1984). Where difficult to visualize on plasmid profile, presence of pMRC01 in transconjugants was confirmed by multiplex-PCR by using primers 5'-GGAGATGATTGACATGGA-3' and 5'-CTACTCTCTAACTCATCC-3' designed to amplify a ca. 500-bp fragment spanning both structural genes *ltnA1* and *ltnA2* (Dougherty *et al.*, 1998), and primers 5'-AGGTACGATCCCACTCACAGA-3' and 5'-

AATAGACTTTTTCAATCGCAACTC-3' targeting a ca. 260-bp fragment in the ORF61 of the replication region that is located just opposite the lacticin operon. Lacticin 3147 production by transconjugants was estimated by an agar well diffusion assay, using *L. lactis* subsp. *cremoris* HP as the indicator strain, and levels of bacteriocin were calculated and expressed in arbitrary units (AU)/ml as previously described (Ryan *et al.*, 1996).

Measurement of bacterial growth rate

Standardization of culture inoculum prior to each of the analyses described hereafter was considered crucial to establish a reliable correlation between the presence of plasmid pMRC01 and any putative effect. Following overnight growth, the bacterial density of each culture was thus measured by flow cytometry (FCM) using the BD Cell Viability Kit and BD Liquid Counting Beads (BD Biosciences, San Jose, USA) as described in the next subsection. Within each pair of wild-type and pMRC01-carrying strains, inoculums were standardized to the culture with the lowest count. The growth rate of *L. lactis* strains was measured in GM17 at 30 °C. After overnight propagation, the cultures were inoculated at 2% into GM17 and grown over a 6-h incubation time. Samples were withdrawn at 1-h intervals to measure turbidity with a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan) at 600 nm and to determine growth rate parameters by plate counts. The max growth rate (μ_{max}) of each culture was calculated in the middle of the log phase according to the equation:

$$(\log N_t - \log N_{t+1}) / (0.301 \times \Delta t)$$

where N_t and N_{t+1} are the number of cells (cfu/ml) respectively after t and t+1 time, 0.301 corresponds to $\log_{10} 2$, and Δt is the interval between times t+1 and t. The generation time (G) was calculated using the equation: $1 / \mu_{max}$.

Starter Activity Test

Acidification properties of wild-type and transconjugant strains were compared by using the Heap-Lawrence starter culture activity test (Heap and Lawrence, 1981) where strains are grown in RSM over a 5-h incubation time through a simulated Cheddar cheese making temperature profile. Briefly, cultures were propagated over

16 h at 22 °C in 11% RSM containing 1.9% (w/vol) β-glycerophosphate and, where required, 0.5% (wt/vol) glucose and 0.25% (wt/vol) yeast extract. For each pair of wild-type and pMRC01-carrying strains, starter inoculums for the activity test were standardized to the culture with the lowest turbidity at 410 nm by using the EDTA turbidimetric method as modified by Heap and Lawrence (1981). Strains were then inoculated into 11% RSM and incubated at 32 °C for 70 min. The temperature was then increased to 40°C over 30 min, held for 160 min, and finally decreased to 32 °C over 40 min. Starter activity was evaluated based on final pH. Acidification properties of wild-type and transconjugant strains were also compared by measuring the final pH after growth for 5 h in GM17 at 30 °C.

Viability of lactococcal strains

The degree of autolysis of *Lactococcus* strains was evaluated by monitoring the levels of lactate dehydrogenase (LDH) released after 24 and 72 h of growth in GM17 at 30°C. LDH assay was performed according to a modified version of the method outlined by (Wittenberger and Angelo, 1970), by measuring the decrease in absorbance at 340 nm resulting from the pyruvate-dependent oxidation of NADH (Sigma, St. Louis, USA) in the presence of fructose-1,6-diphosphate (Fluka, Buchs, Germany). Activity is expressed as units per millilitre, where 1 U is the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH per min per ml.

Qualitative assessment of the autolytic phenotype of *Lactococcus* strains after 24 and 72 h of growth in GM17 at 30 °C was conducted by FCM. FCM analyses were performed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, USA) equipped with two air-cooled lasers, a 20-mW solid state (emission, 488 nm) and a 17-mW HeNe (emission, 633 nm), and five sensors for the detection of forward (FSC) and sideward (SSC) light scatter, green (FL1, 525 nm), yellow (FL2, 575 nm), and far red (FL3, 695 nm) fluorescence. Cell viability was estimated by using the BD Cell Viability Kit (BD Biosciences, San Jose, USA) as follows. Cultures were diluted in 0.22 μm-filtered 50 mM sodium phosphate buffer (pH 7), containing 0.01% Tween-20 and 1 mM EDTA, to an approximate concentration of 10^6 cells per ml. 500 μl aliquots of each culture were incubated with 80 nM thiazole orange (TO) and 8 μM propidium iodide (PI) for 5-10 min at room temperature. Whenever cell enumeration

was required, 10 µl of BD Liquid Counting Beads were added to each sample prior to FCM analysis, and absolute counts were determined according to manufacturer's instructions (BD Biosciences, San Jose, USA). Validity of cell counts by FCM was confirmed by running plate counting in parallel on some samples. Cell samples were delivered at the low flow rate, corresponding to 300 to 500 cells per sec, until 10,000 cells were measured. Fluorescence signals were recorded by using the following detector settings: FSC, 600; SSC, 400; FL1, 550; FL2, 500; and FL3, 700 with logarithmic amplifications. A threshold was set at an SSC signal of 400 to reduce background noise deriving from cellular debris. A total number of 10,000 events were acquired by using FACSDiva software v.5.0.2 (BD Biosciences, San Jose, USA) and analyzed using dot plots (i.e., bivariate displays in which each dot represents one measured event) and population gates.

Statistical analysis

Analyses of growth, acidification and viability rates in *L. lactis* strains were performed in triplicate, and the putative impact of the plasmid pMRC01 on these cellular parameters was statistically evaluated by using unpaired Student's *t* test at a significance level of $P < 0.05$.

RESULTS

Transconjugants harbouring pMRC01 generally exhibit lower growth rates than parent strains

The physiological consequences of carrying the plasmid pMRC01 were evaluated by using a panel of five well-known lactococcal starters (*L. lactis* HP, 255A, SK1, 712 and IL1403) that were compared to their pMRC01-bearing transconjugants in terms of growth, acidification and viability rates. Preliminary tests were performed in order to detect the presence of the plasmid pMRC01 in transconjugants. All cultures were first tested for the lacticin 3147-producing phenotype by agar well diffusion assay using *L. lactis* HP as the indicator strain (Figure 1b). All transconjugants were found to produce lacticin 3147 at similar levels (data not shown); in contrast, none of the parent strains produced the bacteriocin. Agarose gel electrophoresis of plasmid profiles was performed to verify the acquisition of the 60.2-kb pMRC01 by the transconjugants, using DPC3147 as marker of pMRC01 position (Figure 1a). An additional 60-kb band, corresponding to pMRC01, was evident in the profiles of most transconjugants. However, in some of the transconjugants, the presence of pMRC01 was masked by similarly-sized resident plasmids. In these cases, the presence of pMRC01 was confirmed by multiplex PCR using two different sets of pMRC01-specific primers (Figure 1c).

It is known that the metabolic burden imposed by certain plasmids primarily results in an alteration of the growth kinetics of the host cell (Kobayashi *et al.*, 2002, Lee and Moon, 2003, Pillidge *et al.*, 2000). The growth characteristics of wild-type and pMRC01-bearing *L. lactis* strains in GM17 at 30 °C were therefore analyzed and the results are compared in Table 2. In general, the transconjugants exhibited lower μ_{max} and higher generation times compared to the parental strains, with differences being significant ($P < 0.05$) in three (*L. lactis* 255A, SK1 and IL1403) of the five strains tested. The extent of the impact of pMRC01 on the host growth rate in terms of percentage difference between transconjugant and parent varied from 4 to 33%. *L. lactis* 255A showed the largest percent reduction (33%) in growth rate, followed by strains SK1 (14%) and IL1403 (11%), while differences for strains HP (8%) and 712 (4%) were statistically not significant ($P > 0.05$).

Introduction of pMRC01 does not significantly affect acid production

Construction of improved derivatives of dairy starters through genetic manipulation can pose the risk of negatively impacting on the strains' technological properties, e.g. by affecting the rate of acid production in milk. The acidification capacities of the wild-type and pMRC01-bearing strains were first compared by using the Heap-Lawrence starter activity test (Heap and Lawrence, 1981). Results of the assay (Table 3) revealed that, in general, the transconjugants were slightly, but not significantly ($P > 0.05$), less efficient acidifiers of milk compared to the parent strains. Testing the acidification capacities of the strains in GM17 did result in similar non-significant trends (Table 3).

Acquisition of pMRC01 is associated with greater cell permeability and autolysis

Bacterial cell viability is a very complex phenomenon, and cannot be merely defined in terms of cells with an intact or a lysed membrane. A variety of physiological states have been demonstrated to exist between live and dead, ranging from dormant and injured cells (i.e. intact and metabolically active but noncultivable) to dead cells with a permeabilized membrane (a stage preceding cell lysis) (Bunthof and Abee, 2002, Bunthof *et al.*, 1999, Bunthof *et al.*, 2001, Niven and Mulholland, 1998). This is particularly the case after exposure to stress. The impact of carrying pMRC01 on viability of the five lactococcal starters was evaluated in terms of cell lysis as measured by released LDH and cell permeability as measured by the ability to uptake PI.

In this respect, LDH is a key enzyme of the metabolism of most lactic acid bacteria and given its cytoplasmic location, is commonly regarded as a good marker of cell lysis (Bunthof *et al.*, 2001, Wittenberger and Angelo, 1970). To examine the impact of pMRC01 on cell integrity, LDH activities were measured in supernatants of wild-type and pMRC01-bearing *L. lactis* strains during incubation at 30 °C in GM17 (Table 4). Following 24 h of growth, the mean levels of LDH were ca. two-fold higher in supernatants of transconjugants than in those of parental strains, with significant ($P < 0.05$) differences found in four (*L. lactis* 255A, 712, SK1 and IL1403) out five strains. Cell autolysis increased with incubation time and, after 72 h of growth, all the transconjugants tested exhibited significantly higher LDH activities.

Qualitative discrimination between intact, injured/permeabilized, and dead populations in wild-type and pMRC01-bearing strains of *L. lactis* was then accomplished using the fluorescent nucleotide-binding dyes TO and PI. TO is commonly used as a live stain, whereas PI is excluded by the intact cell membrane, thus staining a cell only when the integrity of the membrane is compromised, i.e. dead or injured. When used in combination, live cells are labelled green by TO while injured or dead cells are labelled red by PI. Figure 2 shows the biplots of green (FL1) and red (FL3) fluorescence of *L. lactis* strains at different stages of growth in GM17 at 30 °C after live/dead staining with TO and PI dyes. Total cell populations were gated on dot plots of SSC vs. FL2, while TO- and PI-labelled cells were identified and gated on dot plots of SSC vs. FL1 and SSC vs. FL3, respectively. Differentially-labelled cells were best discriminated on dot plots of FL1 vs. FL3. FCM biplots of cultures during log phase appeared to consist of a major group of cells that were alive and replicating, and by a small subpopulation of cells whose membranes were damaged or injured, as indicated by an increased permeability to PI; levels of these injured cells were found to be significantly higher in four out of five transconjugants compared to wild-type strains (Table 5). Within this population, cells with higher TO fluorescence intensities were still sufficiently intact to retain their macromolecular contents and they were thus metabolically active, while cells with lower TO fluorescence intensities were most likely non-viable. Cell autolysis increased notably with incubation time in both wild-type and transconjugant strains. After 24 h of growth, cell injury was still significantly higher in four out of five strains carrying pMRC01, with the transconjugant of *L. lactis* SK1 showing the highest percent of permeabilization (24.9% of the total population), followed by transconjugants of strains 255A (7.3%), IL1403 and 712 (3%). At this stage, cell lysis did not show any particular trend being larger in two transconjugants (255A and IL1403) as well as in two wild-type (SK1 and HP) strains. The increase in cell lysis, after 72 h of growth, was generally associated with the corresponding reduction in injured and live cells. Nevertheless, large groups of injured cells were still present in transconjugants of strains 712 and SK1, respectively accounting for 14.6% and 12.2% of the total population. As a general trend, transconjugants exhibited larger cell permeabilization (SK1, 712 and IL1403) and lysis (SK1, 255A and IL1403) than wild-type strains even after 72 h of growth.

Lacticin 3147 production is not responsible for the observed plasmid-associated metabolic burden

The introduction of pMRC01 seems to place a metabolic load on the lactococcal starters tested by affecting growth rate in synthetic media and the strains ability to lyse over time. Therefore, we investigated the role of lacticin 3147 gene expression as potential factor for the observed effect by using a strain derivative (DPC5559) of the Cheddar cheese starter DPC4268 that carries the cointegrate plasmid pMRC02 (Trotter *et al*., 2004). This construct originated from the spontaneous integration of a 17-kb region from pMRC01, encompassing the lacticin 3147 operon, into pMT60, a plasmid resident in DPC4268. The DPC5559 strain containing the cointegrate displayed bacteriocin production and immunity in addition to the native phenotypes of pMT60 as exhibited in DPC4268 (Trotter *et al.*, 2004).

The DPC5559 strain was first tested for the level of lacticin 3147 production that was found to be similar to those of the other transconjugants used in this study (data not shown). Growth, acidification and viability rates of DPC4268 (ltn^-) and DPC5559 (ltn^+) strains were then compared and the results are reported in Table 6. Although the lacticin 3147-producing strain exhibited slightly lower rates of growth and acidification, and higher levels of LDH activity compared with the wild-type, these differences were found to be not significant ($P > 0.05$). FCM analysis (Figure 3) confirmed these results by showing not significantly different ($P > 0.05$) populations of injured and dead cells in the fluorescence dot plots of DPC4268 and DPC5559 strains over a 72-h incubation time.

DISCUSSION

The plasmid pMRC01 has proved to be an extremely versatile food-grade tool for the genetic improvement of dairy starters, as it provides cultures with significant phage resistance and also broad anti-microbial properties without significantly affecting their technological characteristics (Coakley *et al.*, 1997, Ryan *et al.*, 1996). However, observations that pMRC01-containing strains grow more slowly and exhibit reduced rates of acid production compared to the parent strains prompted a more detailed investigation of the metabolic burden of the presence of pMRC01 in lactococcal starters.

Comparative analyses of growth, acidification and viability rates in wild-type and pMRC01-containing strains clearly revealed a plasmid impact on cell metabolism. The first and most visible effect of the plasmid load was an alteration of the growth kinetics of the host cell. All the transconjugants exhibited lower growth rates and higher generation times compared to the parental strains, with the extent of pMRC01 impact being strain dependent and fluctuating between 4 and 33%. Similar results have already been reported in lactococci. Pillidge and coworkers (2000) noted a decrease in the growth rate of four commercial *L. lactis* subsp. *cremoris* starter strains after conjugal transfer of phage resistance plasmids. In another study, Kobayashi and coworkers (2002) found that a small cryptic plasmid was able to significantly affect the growth rate of *L. lactis* subsp. *lactis* biovar. *diacetylactis* DRC1. Considerable research has been performed on the plasmid-host interaction in other microorganisms, mostly in *Escherichia coli* (Diaz Ricci and Hernandez, 2000, Rozkov *et al.*, 2004). From these studies, it is well recognized that the presence of certain plasmids places a metabolic burden on the biochemical capacities of the host cell. The accepted explanation for this phenomenon is that plasmid DNA replication and expression rely on the same cellular pool of metabolic components generally exploited only by chromosomal genes. As soon as the extra demand of energy and metabolites exhausts the limiting cellular reserves, the cell enters a phase of metabolic starvation that in turn reduces the growth rate of the culture (Diaz Ricci and Hernandez, 2000). Therefore, it is not surprising to find that plasmid-free cells grow more quickly than plasmid-bearing cells.

The observation that the introduction of pMRC01 did not significantly affect the technological properties of the lactococcal starters tested was unexpected: the

transconjugants retained milk acidification capacities similar to those of the parent strains and sufficient for commercial cheese manufacture. This significant finding is of major importance as it means that pMRC01-mediated improvement of strains does not preclude their use as starter cultures for dairy industry. However, this finding was in apparent contradiction to the aforementioned decrease in growth rate of the pMRC01-bearing strains and also to the results of other studies reporting a plasmid-mediated impact on both growth and acidification rates of the host cell (Lee and Moon, 2003, Pillidge *et al.*, 2000). FCM analysis of cell viability in log phase offered a plausible explanation for this result. Fluorescence dot plots of pMRC01-carrying transconjugants differed from those of wild-type strains by the presence of a larger percent of injured and permeabilized cells in the transconjugant population. The FCM results suggested that these cells were not lysed but that their membrane integrity was compromised. It is well known that after exposure to various forms of sub-lethal stress, cultures may contain injured bacteria that fail to grow on common growth media but nevertheless are still metabolically active, as demonstrated by the observation that they may recover their ability to grow when given a suitable environment (Bunthof and Abee, 2002). These uncultivable but metabolically-active cells would not be detected by viable plate counting techniques used for growth rate calculation, but they may well contribute to fermentation processes. Hypothetically, with their metabolic routes still functioning, injured cells are likely to contribute to the acidification process. Similar contribution might derive from the cells exhibiting low TO and high PI fluorescence. These cells are dead as they are intensely stained by PI, but the fact that they are also capable of taking up the TO stain suggests the presence of an essentially intact but permeabilized membrane. This permeabilized state has been demonstrated to represent an intermediate stage following cell death and preceding cell lysis (Niven and Mulholland, 1998). Permeable cells have previously been detected by FCM analysis in *L. lactis* cultures (Niven and Mulholland, 1998) and in dairy starters and probiotic products (Bunthof and Abee, 2002). Again, such cells would not contribute to the growth kinetics of the cultures but might participate in the fermentation process. Membrane permeabilization may in fact lead to a higher acid production rate by facilitating the access of the substrates to intracellular enzymes. It is thus plausible that the higher population of injured and permeabilized cells present in pMRC01-bearing strains might account for the

different growth rates and the similar acidification capacity found between transconjugants and wild-type starters.

Overall, LDH and FCM assays showed that the presence of pMRC01 somehow promotes cell permeabilization and accelerates autolysis in lactococcal strains. It is reasonable to speculate that these physiological states of pMRC01-carrying strains may be relevant in terms of contribution to cheese ripening and flavour development, when used as cheese starters. Finally, we note that strains belonging to the *lactis* subspecies (*L. lactis* SK1, IL1403 and 712) were found to be the most permeabilized and, overall, the most affected by the presence of pMRC01 compared to members of the *cremoris* subspecies. This finding is in agreement with the general view that *cremoris* strains are more robust and suitable as starters for cheese manufacture than strains belonging to the *lactis* subspecies (Feirtag and McKay, 1987, Pillidge *et al.*, 2000).

The finding that the presence of pMRC01 places a metabolic burden on the lactococcal host prompted us to investigate the putative mechanism behind this phenomenon. Many of the *L. lactis* starters used in this study contain numerous endogenous plasmids of various sizes that bear genes involved in important technological properties (lactose metabolism, proteolysis, bacteriocin production, phage resistance). These plasmids often have similar theta replicons with tight copy number control and it is likely that the introduction of pMRC01 in these strains could affect stability and copy number of the resident plasmids; incompatibility between pMRC01 and the endogenous plasmids might be one of the reasons for the modified properties of transconjugants. However, we noted that *L. lactis* IL1403, a plasmid-free strain, was among the transconjugants to be most affected by acquisition of pMRC01; in this case, it is clear that the metabolic load may not be explained with any compatibility issues and must be associated with some intrinsic characteristics of pMRC01. Size, copy number, and gene expression are plasmid characteristics commonly acknowledged to impact on cellular growth rate, with the impact of gene over-expression being strikingly higher than the effect of size or copy number (Diaz Ricci and Hernandez, 2000). Similarly, the few studies carried out on this topic in lactococci point to the genetic information encoded on the plasmid as the main cause of the decline of the growth rate in the host cell (Kobayashi et al., 2002). The most significant genetic determinants located on the plasmid pMRC01 are those encoding

for an abortive infection phage resistance mechanism, and those responsible for the production of and immunity to lacticin 3147 (Coakley *et al.*, 1997). Considering that the expression of lacticin 3147 structural proteins has been shown to be constitutive (McAuliffe *et al.*, 2001), we targeted the lacticin 3147 genetic information as a potential factor in the observed pMRC01-asscociated metabolic burden. However, no significant differences were found in terms of growth, acidification and viability rates between DPC4268 and its derivative carrying the 17-kb lacticin gene cluster. Considering that all transconjugants were also found to produce lacticin 3147 at similar levels, these results overall suggest that the expression of lacticin 3147 genes might not be responsible for the metabolic load of lactococcal starters reported in this study.

Speculation that the phage resistance mechanism encoded by pMRC01 might play a role in determining the plasmid burden is an alternative idea supported by some reports. Kobayashi and coworkers (2002) demonstrated that, out of seven resident plasmids, only a small cryptic plasmid significantly reduced the growth rate of *L. lactis* subsp. *lactis* biovar. *diacetylactis* DRC1. The 7.4-kb plasmid contained just six open reading frames and encoded only for a relevant *hsdS* subunit of a type I restriction-modification system. Additionally, the four lactococcal plasmids, reported by Pillidge and coworkers (2000) to exert a negative impact on growth and acidification rates of four cheese starters, were all encoding a phage resistance mechanism, and particularly an Abi system in the case of pNP40 and pMU1311. The Abi mechanism of pMRC01 has been shown to target the phage-lytic cycle at a point after phage DNA replication (Coakley *et al.*, 1997). However, although the complete sequence of pMRC01 has been elucidated, the identification of the Abi system has proven difficult due to low levels of sequence homology between known Abi genes (Dougherty *et al.*, 1998). This renders difficult the creation of an accurate knock-out of the phage resistance mechanism in order to investigate its contribution to the plasmid-mediated effect.

To conclude, strains carrying pMRC01 have thus been shown to be sufficiently good acid producers, phage-resistant, and with the ability to produce bacteriocin to control spoilage and non-starter microorganisms (Coakley *et al.*, 1997, Ryan *et al.*, 1996); this study). After considering all these characteristics and the disadvantages reported for other plasmid- or nisin-mediated strategies (Lee and Moon, 2003,

Lipinska, 1973, Pillidge *et al*., 2000), the plasmid pMRC01 may be fairly regarded as one of the most efficient and versatile tools for the improvement of cheese starter cultures available to date.

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TABLES

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source[a] or reference
Strains		
L. lactis subsp. *lactis*		
SK1	Cheese starter	TFRC culture collection
712	Cheese starter; contains plasmids of 2.7; 3.8; 7.9; 13.7 and 50.2 kb	TFRC culture collection
IL1403	Plasmid-free derivative of IL594	Chopin et al. (1984)
DPC3147	Lacticin 3147 producer	Ryan et al. (1996)
L. lactis subsp. *lactis* biovar. *diacetylactis*		
DRC3	DNA size marker; contains plasmid of 2; 3.4; 4.8; 5.2; 35.8; 51; 65 and 78 kb	McKay and Baldwin (1984)
L. lactis subsp. *cremoris*		
HP	Cheese starter	TFRC culture collection
255A	Cheese starter	TFRC culture collection
DPC4268	Cheese starter; contains plasmids of 40; 44; 55 and 60 kb	TFRC culture collection
DPC5559	DPC4268 transconjugant containing pMRC02; contains plasmids of 40; 44; 55 and 80 kb	Coakley et al. (1997)
Plasmids		
pMRC01	Conjugative 60-kb plasmid from L. lactis DPC3147; encodes abortive infection mechanism and lacticin 3147 production and immunity	Ryan et al. (1996); Coakley et al. (1997)
pMRC02	Cointegrate non-conjugative 80-kb plasmid from L. lactis DPC5559; encodes proteinase activity and lacticin 3147 production and immunity	Trotter et al. (2004)

[a]TFRC, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

Table 2. Comparison of maximum growth rate (μ_{max}) and generation time in wild-type (Wt) and pMRC01-containing transconjugant (Tc) strains of *L. lactis* grown at 30 °C in GM17

L. lactis strain	μ_{max} (h^{-1})		Generation time (h^{-1})	
	Wt	pMRC01-Tc	Wt	pMRC01-Tc
HP	0.86 (± 0.12)	0.79 ± (0.06)	1.18 (± 0.17)	1.27 (± 0.10)
SK1	1.18 (± 0.01) [a]	1.01 (± 0.03) [b]	0.85 (± 0.01) [b]	0.99 (± 0.03) [a]
712	0.74 (± 0.07)	0.71 (± 0.05)	1.36 (± 0.14)	1.41 (± 0.10)
255A	1.42 (± 0.02) [a]	0.96 (± 0.07) [b]	0.70 (± 0.01) [b]	1.04 (± 0.07) [a]
IL1403	1.05 (± 0.06) [a]	0.94 (± 0.03) [b]	0.96 (± 0.05) [b]	1.07 (± 0.03) [a]

[a,b]Means with different superscripts were significantly different ($P < 0.05$); Values are means of three replicates (± SD).

Table 3. Acidification capacity of wild-type (Wt) and pMRC01-containing transconjugant (Tc) strains of *L. lactis* grown in different media and conditions

L. lactis strain	11% RSM		GM17	
	Wt	pMRC01-Tc	Wt	pMRC01-Tc
HP	5.37 (± 0.18)	5.68 (± 0.35)	6.06 (± 0.04)	6.08 (± 0.04)
SK1	4.91 (± 0.24)	4.94 (± 0.21)	5.67 (± 0.03)	5.70 (± 0.02)
712	5.15 (± 0.01)	5.19 (± 0.03)	5.75 (± 0.05)	5.77 (± 0.03)
255A	4.83 (± 0.17)	4.95 (± 0.13)	5.51 (± 0.02)	5.55 (± 0.02)
IL1403	6.34 (± 0.05)	6.36 (± 0.05)	6.31 (± 0.03)	6.34 (± 0.02)

Values are means of three replicates (± SD).

Table 4. Levels of lactate dehydrogenase (LDH) in supernatants of wild-type (Wt) and pMRC01-containing transconjugant (Tc) strains of *L. lactis* grown at 30 °C in GM17

L. lactis strain	LDH (24 h)		LDH (72 h)	
	Wt	pMRC01-Tc	Wt	pMRC01-Tc
HP	0.76 (± 0.30)	1.90 (± 1.12)	9.19 (± 1.52) [b]	23.40 (± 5.10) [a]
SK1	3.44 (± 1.17) [b]	6.46 (± 0.87) [a]	13.49 (± 1.25) [b]	19.57 (± 2.17) [a]
712	2.97 (± 0.33) [b]	6.27 (± 0.58) [a]	15.82 (± 0.65) [b]	47.33 (± 1.87) [a]
255A	27.20 (± 1.94) [b]	46.37 (± 0.40) [a]	67.97 (± 0.51) [b]	81.53 (± 3.30) [a]
IL1403	29.47 (± 1.91) [b]	56.32 (± 3.29) [a]	41.39 (± 3.03) [b]	62.34 (± 4.89) [a]

[a,b]Means with different superscripts were significantly different ($P < 0.05$); Values are means of three replicates (± SD).

Table 5. Numbers of events in populations of injured and dead cells, as obtained after gating in FCM biplots, in wild-type (Wt) and pMRC01-containing transconjugant (Tc) strains of *L. lactis* grown at 30 °C in GM17

		HP		SK1		712		255A		IL1403	
		Wt	pMRC01-Tc	Wt	pMRC01-Tc	Wt	pMRC01-Tc	Wt	pMRC01-Tc	Wt	pMRC01-Tc
log phase	Injured	80 (± 15) [b]	241 (± 24) [a]	34 (± 10)	44 (± 9)	45 (± 10) [b]	166 (± 20) [a]	34 (± 5) [b]	63 (± 8) [a]	73 (± 8) [b]	331 (± 40) [a]
	dead	6 (± 3)	9 (± 3)	5 (± 4)	6 (± 3)	7 (± 2)	8 (± 3)	9 (± 4)	12 (± 3)	6 (± 2)	11 (± 3)
24 h	Injured	456 (± 39)	427 (± 20)	152 (± 14) [b]	2493 (± 114) [a]	131 (± 14) [b]	283 (± 8) [a]	38 (± 6) [b]	735 (± 24) [a]	137 (± 9) [b]	319 (± 16) [a]
	dead	700 (± 26) [a]	475 (± 35) [b]	1053 (± 82) [a]	884 (± 35) [b]	82 (± 17)	114 (± 20)	31 (± 7) [b]	379 (± 35) [a]	495 (± 24) [b]	1357 (± 31) [a]
72 h	Injured	168 (± 12)	161 (± 17)	330 (± 14) [b]	1224 (± 57) [a]	289 (± 28) [b]	1461 (± 34) [a]	282 (± 24)	273 (± 47)	183 (± 27) [b]	243 (± 26) [a]
	dead	1068 (± 76)	981 (± 59)	1233 (± 199) [b]	2309 (± 311) [a]	884 (± 38) [a]	791 (± 36) [b]	1023 (± 85) [b]	1250 (± 96) [a]	722 (± 23) [b]	1259 (± 236) [a]

[a,b]Means with different superscripts were significantly different ($P < 0.05$); Values are means of three replicates (± SD).

Table 6. Comparative analysis of maximum growth rate (μ_{max}), generation time, acidification capacity and levels of released lactate dehydrogenase (LDH) of lacticin 3147-negative DPC4268 and lacticin 3147-positive DPC5559 strains of *L. lactis* grown at 30 °C in GM17

Parameters	*L. lactis*	
	DPC4268	DPC5559
μ_{max} (hr^{-1})	1.10 (± 0.13)	0.98 (± 0.22)
Generation time (hr^{-1})	0.92 (± 0.11)	1.06 (± 0.27)
pH (in 11% RSM)	4.78 (± 0.13)	4.88 (± 0.11)
pH (in GM17)	5.41 (± 0.01)	5.45 (± 0.02)
LDH (24 h)	13.21 (± 1.08)	14.21 (± 1.80)
LDH (72 h)	21.58 (± 3.03)	27.27 (± 3.31)

Values are means of three replicates (± SD).

FIGURES

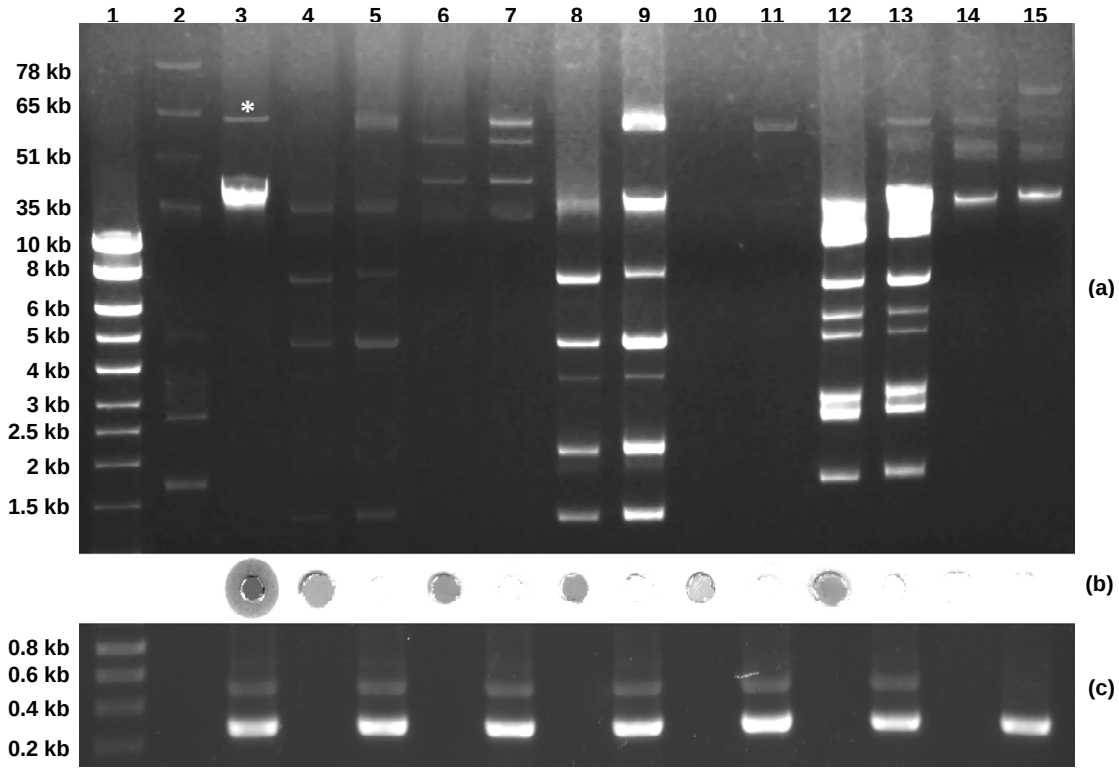


Figure 1. (a) Plasmid DNA profiles of lactococcal starters and their pMRC01 derivatives: lane 4, SK1; 5, SK1.pMRC01; 6, 255A; 7, 255A.pMRC01; 8, 712; 9, 712.pMRC01; 10, IL1403; 11, IL1403.pMRC01; 12, HP; 13, HP.pMRC01; 14, DPC4268; 15, DPC5559. Lane 1 contains a 0.2-10 kb molecular weight marker, and lane 2 the strain *L. lactis* DRC3 as a source of high molecular size markers (35, 51, 65 and 78 kb). The plasmid profile of DPC3147 (lane 3) was used as reference for pMRC01 position (indicated by the asterisk). (b) Agar well diffusion assay using *L. lactis* HP as indicator strain to test lacticin 3147 production in pMRC01 derivatives of lactococcal starters. Samples and lanes are as indicated in panel a. (c) Multiplex-PCR by using primers targeting the replication region (~ 260 bp) and lacticin operon (~ 500 bp) of pMRC01 in order to confirm the acquisition of the entire plasmid in transconjugants.

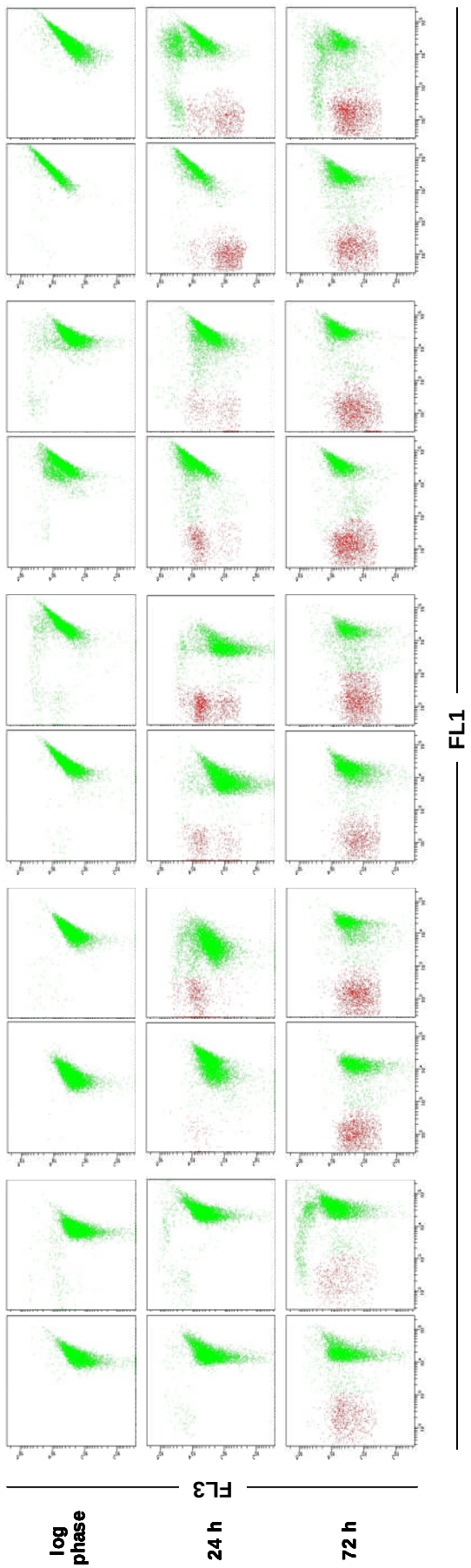


Figure 2. FCM analysis of cell viability after TO and PI labelling in wild-type (x) and pMRC01-containing derivative (x') of *L. lactis* starters 712 (a, a'), 255A (b, b'), IL1403 (c, c'), HP (d, d') and SK1 (e, e') during growth at 30 °C in GM17.

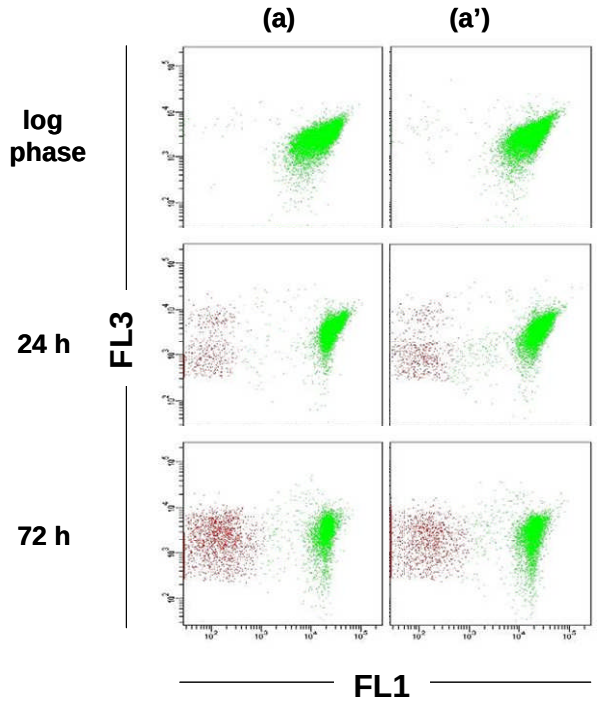


Figure 3. FCM analysis of cell viability after TO and PI labelling in lacticin 3147-negative DPC4268 (a) and lacticin 3147-positive DPC5559 (a') during growth at 30 °C in GM17.

Chapter VI

Microarray-based targeting of the abortive infection mechanism of pMRC01 during bacteriophage infection of the lactococcal host

ABSTRACT

The lactococcal plasmid pMRC01 harbours a potent abortive infection (Abi) mechanism whose genetic determinant(s) are yet to be identified. Changes in pMRC01 gene expression at an early (15 min) and late (30 min) stage of infection with the lytic phage c2 were analyzed using pMRC01-based microarrays in order to detect the putative activation of the Abi system, or elements of the system. Three genes (*orf51*, *orf58* and *orf62*) were differentially regulated at both time-points, whereas six additional genes (*orf5*, *orf9*, *orf18*, *orf45*, *orf46* and *orf56*) were differentially expressed only after 30 min. Of the genes differentially regulated at both time points, *orf58* and *orf62* were negatively regulated throughout phage infection, whereas a switch from repression to activation was observed for *orf51*. *Orf62* encodes an initiator of plasmid replication whose down-regulation could be associated to the phase where the phage switches host metabolism off to use the cell replication machinery for its own replication. *Orf51* and *orf58* both encode transcriptional regulators and their differential regulation throughout phage infection implies that the functioning of the Abi system is most likely subject to complex regulatory control. The Rgg-like protein ORF51 is a bifunctional regulator similar to many phage repressors. The initial down-regulation of *orf51* might serve to activate the Abi system of pMRC01 by removing the ORF51-mediated transcriptional inhibition, whereas the successive gene up-regulation would restore a negative control to avoid accumulation of the Abi effector/s above concentrations that are toxic for the cell. ORF58 is a CopG-like regulator, whose homologues are also known to affect phage development. ORF58 might interact with ORF51 to create a complex regulatory system modulating the activity of the putative Abi effectors, which we suggest to be *orf50* and *orf49*. These constitutively expressed genes encode proteins exhibiting topology and functional similarities to the two-component Rex system that aborts lytic growth in phage λ by causing membrane depolarization. Similarly to RexA, the membrane-anchored ORF50 might use its protein kinase-like activity to cause a conformational change in the putative channel protein RexB-like ORF49, thereby triggering channel opening and consequent membrane depolarization. The ORF58 regulator might be involved, directly or in network with ORF51, in controlling ORF50 activity.

INTRODUCTION

Lactococcus lactis strains are extensively used by the dairy industry for their fermentation abilities, but they are susceptible to the action of virulent bacteriophages, which are ubiquitous and frequently outnumber these bacteria in the dairy environment. Lactococcal strains have thus developed several molecular mechanisms to defend themselves against invading phages. These anti-phage systems can affect viral multiplication by preventing phage adsorption to the cell, blocking DNA injection, cutting the foreign DNA that has entered the cell or halting phage development at any stage between DNA injection and cell lysis. This latter mechanism, denoted as abortive infection (Abi), is characterised by a premature death of the infected cell and release of few or no progeny particles. As a consequence, further propagation of phage is prevented and the bacterial population survives. Whether cell death results from the irreversible damage caused by phage infection or from the Abi mechanism itself is still unclear but may vary given the diversity of Abi systems identified to date (Chopin *et al.*, 2005, Emond and Moineau, 2007, Forde and Fitzgerald, 1999).

The majority of these mechanisms are located on plasmids, except for the chromosomally-encoded AbiH and AbiN. Another common feature of Abi genes is a surprisingly low GC content (24-29%) when compared to that of the lactococcal host (35.4%). Additionally, protein homology is rarely observed between lactococcal Abis: AbiC and AbiP are only 22% identical, AbiA and AbiK share 23% homology, whereas AbiD, AbiD1 and AbiF identities range from 28 to 46% (Chopin *et al.*, 2005). Very little is still known about the mode of action of most Abi systems, but those characterized have revealed ability to interfere with various stages of the phage lytic cycle. AbiA (Dinsmore and Klaenhammer, 1994), AbiF (Garvey *et al.*, 1995), AbiK (Boucher *et al.*, 2000), and AbiR (Twomey *et al.*, 2000) rapidly halt phage DNA replication by an unknown mechanism. AbiD1 blocks phage DNA replication and packaging through a decrease of a RuvC-like endonucleolytic activity, which generally facilitates the removal of branched DNA structures (Bidnenko *et al.*, 1995). AbiP prevents both phage DNA replication and temporal transcription switch (Domingues *et al.*, 2004). AbiG inhibits mRNA synthesis (O'Connor *et al.*, 1999), whereas AbiB triggers the breakdown of phage transcripts through the induction of an RNase 10 to 15 min after infection (Parreira *et al.*, 1996). AbiT has been suggested to

be activated by one of the late viral mRNAs or proteins and to rapidly cause premature cell death (Bouchard *et al.*, 2002).

An Abi mechanism has been previously associated with the fully sequenced 60-kb lactococcal plasmid pMRC01, which also includes the genetic determinants for production of and immunity to the bacteriocin lacticin 3147 and for conjugative transfer of the plasmid (Coakley *et al.*, 1997, Dougherty *et al.*, 1998, Ryan *et al.*, 1996). Using bacteriocin immunity as a selectable marker, pMRC01 has been successfully transferred to a variety of lactococcal starters, resulting in the generation of strains with improved phage resistance. For example, the presence of pMRC01 in *L. lactis* MG1363 was shown to reduce by 6-fold the size of plaques formed by the prolate-headed phage c2 compared to those produced in the plasmid-free host. The anti-phage system was assessed to be of the Abi type and to target the phage-lytic cycle at a point after phage DNA replication (Coakley *et al.*, 1997). Despite many efforts, the exact location of this Abi mechanism still remains unknown.

Induction of Abi mechanisms following phage infection is known to occur both in *E. coli* and in *L. lactis* systems (Bidnenko *et al.*, 2002, Molineux, 1991). In this study, we have used oligonucleotide microarrays to investigate changes in expression levels of pMRC01 genes during an early and late stage of infection with the lytic phage c2 in an attempt to identify the Abi components. Our transcriptional analyses showed two transcriptional regulators (ORF51 and ORF58) to be differentially regulated throughout phage infection. This finding could imply that both proteins interact to create a complex regulatory system modulating the activity of the pMRC01 Abi effectors, which we suggest may be represented by ORF50 and ORF49.

MATERIALS AND METHODS

Bacterial strains

A pMRC01-containing derivative of *L. lactis* subsp. *lactis* IL1403 was used to investigate the genes responsible for the Abi phenotype of pMRC01 during phage attack. *L. lactis* subsp. *cremoris* MG1363 served as the sensitive host for propagation of the lytic phage c2 at high titers. Lactococcal strains were routinely propagated at 30 °C in M17 medium (Oxoid, Hampshire, England) supplemented with 0.5% (wt/vol) glucose (GM17). Solid media contained 1.0% (wt/vol) bacteriological agar (Oxoid, Hampshire, England). Bacterial strains were stocked in M17 containing 40% glycerol at -80 °C. Working cultures were stored at 4 °C and transferred periodically. Propagation of phage c2 to high titres, 10^{13} plaque forming unit (pfu)/ml, was obtained by polyethylene glycol precipitation followed by caesium chloride gradient centrifugation as previously described (Chapter I, this thesis).

Microarray design

The whole genome sequences of *L. lactis* IL1403 (2,310 genes), pMRC01 (64 genes) and phage c2 (39 genes) were used to design 50-mer oligonucleotide probes for each gene which were subsequently spotted onto glass slides in duplicate. Control elements on each array included negative-control probes from unrelated *Arabidopsis thaliana* genes. Oligonucleotide probe design and microarray production were carried out by Ocimum Biosolutions Ltd. (Hyderabad, India).

Transcriptional analyses of the global gene response of pMRC01 during phage infection

An overnight culture of *L. lactis* IL1403.pMRC01 was used to prepare a fresh 0.5% inoculum in 40 ml of GM17 broth, which was propagated at 30 °C to mid-log phase (OD_{600} = 0.4). The culture was split in two 20-ml aliquots (experimental and reference), to which were added phage c2 (MOI = 800) (experimental) or TMN buffer (reference), and 10 mM $CaCl_2$. Experimental and reference cultures were further incubated at 30 °C to allow phage infection. Samples were withdrawn after 15

and 30 min of phage infection and immersed in a -80 °C ethanol bath for 5 min to prevent any changes in gene expression profiles.

Total RNA extraction, mRNA enrichment, cDNA preparation and labelling, microarray hybridization, scanning, and data analysis were performed as previously described (Chapter I, this thesis). Validation of microarray data was obtained by quantitative Real-Time PCR (qRT-PCR) analysis as previously described (Chapter I, this thesis).

Computational prediction of subcellular localization and function of pMRC01 proteins.

A number of computational tools were used to predict sub-cellular localization and function of pMRC01 proteins. Prediction of protein function was attempted by using the PFAM database (Finn *et al.*, 2010), the Conserved Domain Database (CDD) (Marchler-Bauer *et al.*, 2009), and the InterProScan service (www.ebi.ac.uk/Tools/InterProScan). This latter tool predicts the occurrence of functional domains and motifs/signatures in a protein by combining 12 different databases and their relative protein signature recognition methods (Zdobnov and Apweiler, 2001). Prediction of topology in putative alpha-helical membrane proteins, including a specification of the membrane spanning segments and their in/out orientation relative to the membrane, was obtained by using the TOPCONS server (http://topcons.cbr.su.se/index.php). This server uses the prediction from five different topology prediction algorithms (SCAMPI-seq, single sequence mode; SCAMPI-msa, multiple sequence mode; PRODIV-TMHMM; PRO-TMHMM; and OCTOPUS) as input to the TOPCONS hidden Markov model (HMM), which gives a consensus prediction for the protein, together with a reliability score based on the agreement of the included methods across the sequence (Bernsel *et al.*, 2009). Finally, presence of a putative signal peptide sequence was predicted with the SignalP 3.0 Server (www.cbs.dtu.dk/services/SignalP) by using neural networks (NN) and hidden Markov models (HMM) trained on Gram-positive bacteria (Emanuelsson *et al.*, 2007).

RESULTS

Global transcriptional analysis of pMRC01 response during phage infection.

Activation of Abi mechanisms in response to phage infection is a well documented phenomenon, which has been demonstrated in all *Escherichia coli* systems that have been studied in detail (Molineux, 1991). In lactococci, most *abi* genes appear to be expressed in a constitutive manner, often at low levels, and are not subjected to increased expression following phage infection (Chopin *et al.*, 2005). However, an exception to this is represented by AbiD1, where the expression of an early-acting phage gene has been shown to stimulate the translation and drastically increase the efficiency of the Abi system (Bidnenko *et al.*, 2002). In an attempt to identify the genes responsible for the Abi phenotype of pMRC01, whole-genome DNA microarrays were used to analyse changes in plasmid gene expression occurring during host infection with the lytic phage c2. According to Garvey et al. (1995), Abi systems may be segregated depending on whether they act prior to or at the level of DNA replication (early) or after replication has occurred (late). The Abi system of pMRC01 has been shown to delay phage DNA replication by 5 min, which is suggestive of an early-acting mechanism (Coakley *et al.*, 1997). However, the exact step of the infective process being critically affected by this Abi system, the components involved, and its mode of action still remain unsolved. Therefore, global expression of pMRC01 genes was analysed after 15 and 30 min of phage infection in order to identify the genetic components responding to phage challenge.

Analysis of the DNA microarray data revealed that the presence of phage c2 within the host triggered the differential expression of 3 and 9 genes in pMRC01 after 15 and 30 min, respectively (Figure 1). All genes (*orf51, orf58* and *orf62*) repressed after 15 min of phage infection were also found to be differentially regulated after 30 min. *Orf58* and *orf62* were negatively regulated throughout the infection process, whereas an interesting switch from repression to activation was noted for *orf51*. Computational analysis of amino acid (aa) sequence revealed that the products of *orf51, orf58* and *orf62* are likely to be involved in DNA replication or control of the replication/transcription processes. The gene product of *orf51* is a 282 aa-long protein containing a conserved N-terminal helix-turn-helix (HTH) DNA-binding domain (PFAM01381), typically found in bacterial plasmid copy control proteins, bacterial methylases, and various bacteriophage transcription control proteins (Wintjens and

Rooman, 1996). Additionally, its C-terminal domain (TIGR01716) exhibits homology to the Rgg/GadR/MutR family of transcriptional regulators, which are involved in modulating a variety of physiological processes (Dmitriev *et al.*, 2006, Redon *et al.*, 2005, Skaugen *et al.*, 2002). The small product (88 aa) of *orf58* contains an N-terminal Ribbon-helix-helix (RHH) domain (IPR002145) similar to that found in transcriptional repressors of the CopG family (PF01402), which are involved in the control of copy number in rolling circle-replicating plasmids. Interestingly, despite the structural resemblance of the RHH domain to the HTH region of DNA-binding proteins, this bi-helical region in CopG proteins has been shown to play a role not in DNA recognition but in oligomerization and maintenance of the functional structure (Gomis-Ruth *et al.*, 1998). *Orf62* encodes a protein of 384 aa containing domains typical of RepB proteins (PFAM06430; PFAM01051), which are widespread in theta-replicating lactococcal plasmids (Seegers *et al.*, 1994) and use their nicking/closing topoisomerase I-like activity to initiate plasmid replication.

Following 30 min of infection, six additional genes (*orf5*, *orf9*, *orf18*, *orf45*, *orf46*, and *orf56*) were differentially regulated in pMRC01. *Orf5* and *orf9* belong to the large conjugal region of pMRC01 that has been shown to promote efficient transfer of the plasmid to a variety of lactococcal strains, including derivatives of commercial starters (Coakley *et al.*, 1997, Dougherty *et al.*, 1998, Ryan *et al.*, 1996). This region is composed of 16 genes organized in an operon-like structure, many of which encode proteins believed to be involved in conjugation but whose functions are mostly unclear (Dougherty *et al.*, 1998, Grohmann *et al.*, 2003). The gene product of *orf5* (TraA) is a well conserved protein with relaxase (TIGR02768) and nickase (PFAM03389) activity, which is known to introduce a nick at the origin of transfer *oriT* to initiate single-stranded plasmid DNA transmission from the donor to a recipient cell (Grohmann *et al.*, 2003). *Orf9* encodes a 115-aa long protein (TraC) whose N-terminal region shows weak homology to a family of conserved but functionally uncharacterised proteins (DUF3487). Additionally, the C-terminus of TraC shows a low similarity match to proteins of unknown function that are predicted to be integral membrane proteins (PF01988). This was confirmed by protein topology analysis, which predicted TraC as being anchored to the membrane by both terminal segments, with the remainder of the protein projecting towards the cytoplasm to form two trans-membrane helices with an in/out (positions 27-47) and out/in (positions 52-

72) orientation relative to the membrane (Figure 2). Similarly, *orf18* was found to encode a protein (131 aa) exhibiting weak similarities to proteins of unknown function (PF10883) and with predicted localization on the cytoplasmic membrane (PF03748). The topology model predicted an N-terminal trans-membrane helix in ORF18, but this was later found to be a signal peptide by the SignalP algorithms, which also allocated the most likely cleavage site between positions 21 and 22 (VG-FK) or 28 and 29 (KS-TQ) (Figure 3). Thus, ORF18 is likely to be a secreted protein.

The genes *orf45* and *orf46* are located on the complementary strand of pMRC01 and overlap by 8 bp, which is indicative of translational coupling. Although, a putative ribosome binding site (RBS) AAGGAG was identified upstream of *orf46*, strongly resembling the Shine-Dalgarno (SD) sequence pattern (TAAGGAGG) (Shine and Dalgarno, 1974), no terminator sequence was found downstream of *orf45*. The 91-aa long protein encoded by *orf46* contains the DUF2089 domain (PF09862), which is found in various hypothetical prokaryotic proteins of unknown function. ORF46 is likely to be a cytosolic protein as no trans-membrane segments were detected by topology analysis. The protein encoded by *orf45* is 100 aa long and shows weak similarity to the DUF3139 family (PF11337) of proteins with unknown function. The topology model predicts ORF45 as being a membrane protein containing three trans-membrane helices alternating out/in (positions 4-24, 53-73) and in/out (positions 27-47) orientation relative to the membrane (Figure 4). Finally, the gene product (94 aa) of *orf56* is likely to be involved in site-specific DNA recombination as it exhibits a typical N-terminal resolvase (PF00239) domain, containing the active site and the dimerization interface, and a C-terminal HTH domain (PF02796) involved in DNA-binding.

DISCUSSION

The conjugative 60-kb lactococcal plasmid pMRC01 has been shown to confer phage resistance through an Abi system conferring complete resistance to the small isometric-headed phage 712 and resulting in a 6-fold reduction of plaques size produced by the prolate-headed phage c2. The phage resistance mechanism seems to target the phage-lytic cycle at a point after phage DNA replication (Coakley *et al.*, 1997). Induction of Abi mechanisms following phage infection is known to occur both in *E. coli* and in *L. lactis* systems (Bidnenko *et al.*, 2002, Molineux, 1991). The probability that phage infection may also trigger the activation of the Abi system of pMRC01 was therefore investigated by using DNA microarrays and following changes in plasmid gene expression occurring at an early (15 min) and late (30 min) stage of the viral infection. A total of 9 genes were found to be differentially regulated in pMRC01, with only three of them (*orf51, orf58,* and *orf62*) being regulated at both time-points. Repression of 7 (*orf5, orf18, orf45, orf46, orf56, orf58* and *orf62*) out of 9 genes after 30 min of infection might be related to the initiation of the process leading to cell death. Most of the regulated genes encode hypothetical proteins whose functions have been presumptively deduced by using algorithms predicting structural topology and presence of conserved domains.

Orf51, orf58, and *orf62* were the only genes regulated after 15 min of phage infection and at both time points. Analyses of conserved domains suggest that their gene products are likely to play a role in DNA replication or control of the replication/transcription processes. ORF62 acts as initiator protein of plasmid replication and the expression of its cognate gene is obviously down-regulated during phases of the infection where the phage is switching host metabolism off to use the cell machinery for its own replication. More interesting is the putative function of the proteins encoded by *orf51* and *orf58*. The protein encoded by *orf51* exhibits two different conserved domains that are both indicative of a regulatory activity for this protein. ORF51 possesses an N-terminal HTH DNA-binding domain found in many phage and bacterial regulators, including the repressor *c*I of the coliphage 434 (Wintjens and Rooman, 1996). This regulator protein has been shown to function both as transcription activator and repressor, thereby regulating the transcription at two adjacent, but divergent, promoters in order to develop or maintain the lysogenic state over the lytic one (Xu and Koudelka, 2001). Additionally, the C-terminal

domain of ORF51 exhibits homology to the conserved family of Rgg-like regulators that are widely distributed in low-GC Gram-positive bacteria, including both pathogenic and commensal species (Dmitriev *et al.*, 2006). Members of the Rgg family are involved in modulating a variety of physiological processes. Rgg of *Streptococcus gordonii* is an activator of the expression of the glucosyltransferase G gene involved in extracellular glucan formation (Sulavik and Clewell, 1996). In *S. mutans*, the Rgg-like protein MutR induces the transcription of the *mutAMTFEG* operon, which encodes the synthesis of the lantibiotic mutacin (Qi *et al.*, 1999), whereas in *Lactobacillus sakei*, the plasmid-encoded LasX protein regulates the synthesis of and immunity to the lantibiotic lactocin S (Rawlinson *et al.*, 2002, Skaugen *et al.*, 2002). In *L. lactis*, the Rgg-like regulator GadR activates transcription of the *gadBC* operon, which is associated with glutamate-dependent acid tolerance (Redon *et al.*, 2005). Some Rgg-like regulators, such as LasX, have been shown to possess a bivalent regulatory capacity enabling them to act both as an activator and a repressor (Rawlinson *et al.*, 2002). A temporally controlled expression program seems to regulate the transcription of *orf51* in pMRC01 during phage infection, with the gene being down-regulated (-2.16) after 15 min and then up-regulated (+1.22) after 30 min. This might correlate with the host growth phase, as already observed for other Rgg members. In the pathogen *S. pyogenes*, RopB regulates the transcription of the gene encoding for a secreted cysteine protease, the expression of which involves control of transcription in response to the growth phase. Interestingly, the transcription of the *ropB* gene itself was also found to be subjected to growth phase control (Neely *et al.*, 2003). Alternatively, down-regulation of *orf51* after 15 min of phage infection may suggest repression of a regulatory capacity in order to activate the Abi system of pMRC01, whereas its later up-regulation may imply restoration of the negative control over the same.

This putative association of a regulatory protein with the Abi gene/s of pMRC01 would resemble the structure and functioning of some known Abi systems. In *L. lactis*, the anti-phage efficiencies of AbiU and AbiK have been shown to be under control of the putative repressor genes *abiU2* and *orf4*, respectively (Dai *et al.*, 2001, Fortier *et al.*, 2005). In both cases, the repressor binds to the *abi* gene promoter, thus resulting in decreased expression of the Abi protein and a weaker phage resistance phenotype. A similar regulation scheme affects the polycistronic operon *pifCAB*

aborting T7 infection in *E. coli*, where PifC negatively controls the expression of the Abi protein PifA (Miller and Malamy, 1983). Interestingly, inactivation of the *abiK* repressor *orf4* has been shown to result in a stronger phage resistance phenotype, but not in increased amount of AbiK. This protein is present in the cell before infection and its expression is not affected by phage infection, a finding which was correlated with the efficacy of AbiK depending on the intracellular concentration of the protein (Fortier *et al.*, 2005). Over-expression of many Abi proteins has been shown to be lethal for the host, both in *L. lactis* and *E. coli*, which is probably the reason for the constitutive transcription of almost all *abi* genes (except *abiD1*) known to date (Chopin *et al.*, 2005). Based on these findings, it may be hypothesized that the initial down-regulation of *orf51* serves to activate the Abi system of pMRC01 by removing the transcriptional inhibition, whereas the successive up-regulation may restore a negative control to avoid accumulation of the Abi protein above concentrations that are toxic for the cell.

Assuming that *orf51* is involved in regulating the Abi phenotype of pMRC01, the gene(s) responsible for the antiphage mechanism itself may be located close to *orf51*. Analysis of the nucleotide region surrounding *orf51* on pMRC01 reveals the presence of four genes (*orf52-51-50-49*) transcribed in the same direction. They appear to be organized in a putative polycistronic operon, as it is possible to identify sequences resembling an RBS and a -10 promoter signal upstream of *orf52* and a *rho*-independent terminator downstream of *orf49* (Figure 5). The product of *orf52* (296 aa) is likely to be a transcription regulator as it contains an N-terminal HTH DNA binding domain (PF00165) and a C-terminal effector binding domain involved in protein dimerization (PF06445), which are typical of the AraC/XylS family of bacterial transcription activators (Gallegos *et al.*, 1993). Interestingly, the location of these domains within ORF52 appears to be inverted compared to what is generally observed in AraC/XylS regulators. *Orf50* possesses a much lower GC content (25.8%) than the average in pMRC01, which is a typical characteristic of *abi* genes (Chopin *et al.*, 2005), and encodes a protein (605 aa) containining a conserved protein kinase catalytic domain (PF00069), with predicted active sites in residues 300 and 318. Protein kinases use their phosphorylation activity to generate a conformational change of the target protein, thereby affecting protein functionality. This allows them to play a role in a multitude of cellular processes, including division, proliferation,

apoptosis, and differentiation (Hanks and Quinn, 1991). The C-terminus of ORF50 shares weak homology to a domain found in Lanthionine synthetase C-like proteins (PF05147), a family of highly divergent proteins thought to be lantibiotic modifying enzymes and that includes the nisin biosynthesis protein NisC from *L. lactis* (Lubelski *et al.*, 2008). The C-terminus of ORF50 is also predicted to contain a trans-membrane region (residues 543-563), whereas the remainder of the protein is located on the cytosolic side (Figure 6). ORF49 (407 aa) is likely to belong to the Major Facilitator Superfamily (MFS) of transporters (PF07690), which are single-polypeptide secondary carriers capable only of transporting small solutes in response to chemiosmotic ion gradients (Pao *et al.*, 1998). Topology analysis confirmed this by predicting ORF49 as an integral membrane protein with 12 trans-membrane domains spanning the entire length of the protein (Figure 7). *Orf50* and *orf49* genes overlap by 4 bp, which is indicative of transcriptional coupling (Figure 5). Their localization immediately downstream of the regulator gene *orf51* suggests that both genes may be under the direct control of *orf51*, as generally observed for Rgg regulated genes (Qi *et al.*, 1999, Rawlinson *et al.*, 2002, Skaugen *et al.*, 2002, Sulavik and Clewell, 1996).

The organization and functionality of the polycistronic operon *orf52-49* strikingly resembles that of the phage-encoded Rex system of phage λ (Parma *et al.*, 1992, Shinedling *et al.*, 1987). In this case, phage lytic growth is aborted by a two-component system encoded by *rexA* and *rexB* genes, which are under the control of the upstream *c*I repressor gene. Shortly after λ phages infect a sensitive host, these three genes are transcribed as a polycistronic RNA from the promoter for repressor establishment. Abortion of lytic growth in phage λ is characterized by termination of macromolecular synthesis, loss of active transport, hydrolysis of ATP, and cell death due to depolarization of the cytoplasmic membrane. RexB is an inner membrane protein with four trans-membrane domains forming an ion channel, which is predicted to remain in the closed conformation in absence of RexA. In response to a signal generated by lytic growth, two or more RexA molecules bind to RexB causing opening of the channel and depolarization of the membrane (Parma *et al.*, 1992). RexA is thought to reside in the membrane but little is known about its mode of action (Shinedling *et al.*, 1987). Structural and functional similarities suggest that ORF49 and ORF50 may be the pMRC01 counterparts of Rex proteins of phage λ. Twelve membrane-spanning domains and sequence similarity to MFS transporters

strongly suggest that ORF49 may play a RexB-like role as channel protein, whereas, similarly to RexA, ORF50 is membrane-anchored and may use its protein kinase-like phosphorylation activity to cause a conformational change in ORF49, thereby triggering channel opening and consequent membrane depolarization. Finally, the bifunctional regulator ORF51 might control the amount of ORF49 and ORF50 in the cell, in a similar fashion to the *c*I repressor-mediated regulation of the RexA/RexB (Parma *et al.*, 1992). In a previous study (Mills, 2005), the role exerted by each gene of the *orf52-49* operon in the Abi mechanism of pMRC01 was evaluated by using a sequential gene knockout approach to inactivate individual genes. Disruption of any of these genes led to an increase in plaque size from pinpoint (in the presence of pMRC01) to plaques of 1 to 2 mm, which is indicative of an involvement of the operon in the Abi phenotype as also suggested by our transcriptional and computational analyses. All these observations concur to support a role for *orf50* and *orf49* in the Abi system of pMRC01. Both genes were not differentially regulated during phage infection, which would imply that ORF50 and ORF49 are expressed in a constitutive manner. This is consistent with the constitutive levels observed for most lactococcal Abi proteins, even during phage infection, a finding that has been associated with a putative toxicity of the Abi proteins for the cell (Chopin *et al.*, 2005). However, differential expression of the ORF51 regulator throughout phage infection would be expected to be accompanied by regulation of the downstream genes *orf50* and *orf49*, which are putatively under its control. This apparent contradiction finds its support in the mechanism of action of AbiK. Although the anti-phage efficiency of AbiK is regulated by ORF4, inactivation of this repressor surprisingly results in a stronger phage resistance phenotype but not in increased amount of AbiK (Fortier *et al.*, 2005). Additionally, in *E. coli* strains lysogenic for λ, it has been demonstrated that the *rex* genes are sufficient to control the growth of phage T4 mutants and that the *c*I repressor is not required. This might suggest a partial independence of the expression of Rex-like genes *orf50* and *orf49* from the repressor *orf51*, which is supported by the finding of putative RBS and -10 promoter signals upstream of *orf50* (Figure 5).

The involvement of membrane proteins has been established for some Abi systems in lactococci. Two putative integral membrane-spanning helices have been identified near the N-terminal end of AbiC (Durmaz *et al.*, 1992). The AbiP protein

was shown to possess a topology strictly resembling that of ORF50, with an N-terminal membrane-spanning domain and a nucleic acid binding domain freely accessible in the cytoplasm. AbiP halts phage DNA replication 10 min after infection, which is consistent with the mechanism observed in pMRC01, and inhibits the switch-off of phage early transcripts normally observed 15 min after infection. AbiP effect on phage development has been suggested to depend on the nucleic acid binding activity displayed by the membrane-bound protein (Domingues *et al.*, 2008), which is consistent with the notion that replication of viral DNA occurs at the inner membrane at the site of injection (Edgar *et al.*, 2008). In pMRC01, it is tempting to speculate that the phosphorylation activity of ORF50 might also be involved in altering the functionality of one or more host enzymes important for phage DNA replication.

Another differentially regulated gene was *orf58*, whose putative protein product exhibits homology to the Cop family of transcriptional repressors controlling plasmid replication. Structure and mode of action of these repressors have been studied in the family prototype CopG, which is a small protein (45 aa) encoded by the promiscuous streptococcal plasmid pMV158. CopG controls plasmid replication by regulating the expression of the initiator *repB* gene, which is co-transcribed with gene *copG* from the single promoter P_{cr}. Upon binding to the P_{cr} promoter, CopG represses the synthesis of the bicistronic *cop-rep* mRNA and thus regulates both the expression of its own gene and that of the initiator *repB* gene (del Solar *et al.*, 2002). Transcriptional repression by CopG may be achieved by hindering RNA polymerase binding to the P_{cr} promoter or, alternatively, by displacing RNA polymerase once the enzyme has formed a stable complex with P_{cr} (Hernandez-Arriaga *et al.*, 2009). In pMRC01, a stretch of 2.5 kb of sequence, containing three genes encoding hypothetical proteins, separate *orf58* from the *repB* gene (*orf62*). Despite domain homology to CopG-like proteins, it seems therefore unlikely that ORF58 controls plasmid replication in pMRC01. Alternatively, a putative role for ORF58 in interacting with the incoming phage may be suggested by the function of another protein with similar structural topology to CopG. Within the RHH class of DNA-binding proteins, CopG is the smallest protein and represents the minimal DNA-binding structure as it contains no more than the RHH motif. As a member of this class, which exhibits cooperativity based on protein–protein interactions, CopG

generates two protein dimers upon binding to the target DNA (Gomis-Ruth *et al.*, 1998). Interestingly, structural studies found the CopG dimer to be almost identical to that of the Mnt repressor of the Arc superfamily from *Salmonella typhimurium* phage P22, although this latter protein is slightly bigger (53 aa) and contains an additional N-terminal region which also interacts with the target DNA (del Solar *et al.*, 2002, Raumann *et al.*, 1994). The Mnt repressor has been shown to be required for super-infection exclusion and maintenance of lysogeny in phage P22 (Gough, 1968), in addition to regulating the temperature-dependent induction of the prophage (Gough, 1970). During lysogeny or early lytic growth, the Mnt repressor maintains or induces the lysogenic state by preventing the expression of the *ant* gene, whose product is an anti-repressor that has the capacity to inactivate a second repressor (c2) involved in maintenance of lysogeny (Susskind and Botstein, 1978). In phage P22 channelled towards lysogeny, levels of c2 repressor are thus high and DNA synthesis shows to be delayed to a greater extent than protein synthesis (Steinberg and Gough, 1975). Similarly to Mnt, ORF58 might play a role in aborting the phage lytic cycle in pMRC01-containing hosts by interacting with a second regulatory protein (ORF51) or possibly with one of the putative Abi effectors (ORF50 and ORF49). Interestingly, the most potent and selective inhibitor of the cGMP-dependent protein kinase, namely DT-2, was found to use its bi-dentate structure to bind the catalytic domains of a dimeric PKG (Pinkse *et al.*, 2009). ORF58 possesses an RHH domain that has been shown to play a role in oligomerization of CopG-like regulators, which in turn is crucial to the protein functionality (Gomis-Ruth *et al.*, 1998). It is therefore plausible to speculate that dimers of ORF58 might interact with the protein kinase domain of ORF50, thereby blocking the phosphorylation activity of this latter protein and ultimately resulting in a negative regulatory control over the Abi system of pMRC01 in absence of phage. Thus, during phage infection, repression of *orf58* would remove the inhibition over ORF50 activity and lead to expression of the Abi phenotype. *Orf58* also exhibits a GC content of 26.2%, which is definitively lower than pMRC01 average (30.1%). This finding further supports a role for *orf58* in the Abi phenotype of pMRC01, as it is well known that Abi genes are characterized by a lower GC content compared to that of the DNA element containing them (Chopin *et al.*, 2005).

While results of our transcriptional analyses do not allow the clear identification of candidate genes for the Abi phenotype of pMRC01, they do provide some

evidence to support a proposed mode of action of this anti-phage mechanism. Two transcriptional regulators (ORF51 and ORF58) are shown to be differentially regulated throughout phage infection, a finding which may imply that the functioning of the Abi system relies on complex interactions among different regulators. One of these regulators is an Rgg-like protein (ORF51) that, according to ours and previous results (Mills, 2005), might be involved in the Abi phenotype together with other genes of the *orf52-49* operon. Some Rgg-like proteins, such as RopB of *S. pyogenes*, have been found to interact with additional regulators in order to alter gene expression (Loughman and Caparon, 2007). In pMRC01, the additional regulator might be represented by the CopG-like ORF58 protein, whose homologues are also known to affect phage development. Both proteins might interact to create a complex regulatory system modulating the activity of the Abi effector/s, which we suggest may be represented by ORF50 and ORF49. These proteins are constitutively expressed and share structural and functional similarities with the Rex two-component system of phage λ (Parma *et al.*, 1992, Shinedling *et al.*, 1987). Some structural characteristics of ORF58 also suggest that this regulator might be directly involved in controlling the switching of the Abi system. A potential cross-talk between the ORF51 and ORF58 regulators or a separate regulatory activity of ORF58 itself might also explain the failure of the individual gene inactivation approach in restoring full phage sensitivity in the host as observed in absence of pMRC01 (Mills, 2005). Further studies are needed to verify our hypotheses and to definitively identify the genetic determinants governing the Abi system of pMRC01.

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FIGURES

(a)

Organism / plasmid	Gene	Predicted function	Length (aa)	GC (%)	Microarrays		qRT-PCR	
					15 min	30 min	15 min	30 min
pMRC01	*orf 5*	Nicking enzyme (traA)	680	30.24		-1.16		
	orf 9	TrsC protein (traC)	115	26.14		1.25		
	orf 18	Hypothetical protein	131	27.27		-1.22		
	orf 45	Hypothetical protein	100	28.38		-2.01		
	orf 46	Hypothetical protein	91	29.71		-1.64		
	orf 51	Putative rgg protein	282	30.27	-2.16	1.22	-4.58	3.63
	orf 56	Conserved hypothetical protein	94	29.12		-1.28		
	orf 58	Hypothetical protein	88	26.21	-2.34	-1.01	-3.96	-2.64
	orf 62	RepB protein	384	33.59	-1.76	-1.67	-2.23	-1.61
L. lactis IL1403		Down-regulated genes			123	160		
		Up-regulated genes			84	106		

(b)

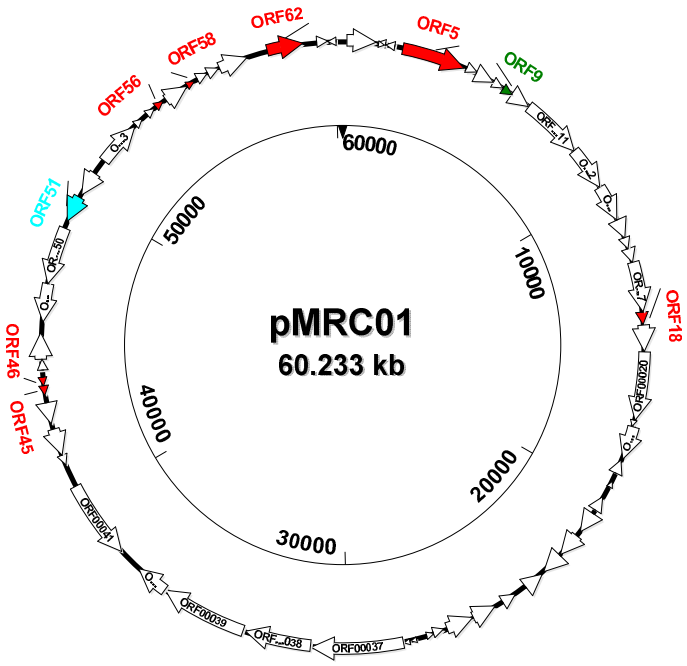


Figure 1. (a) Transcriptional response of pMRC01 genes after 15 and 30 min of phage infection. The numbers of up- and down-regulated genes of the host strain *L. lactis* IL1403 is also shown (b) Location of regulated genes on pMRC01 physical map. Genes that are either negatively or positively regulated at both time-points are shown in red or in green, respectively. Genes showing different regulation between time-points are shown in azure.

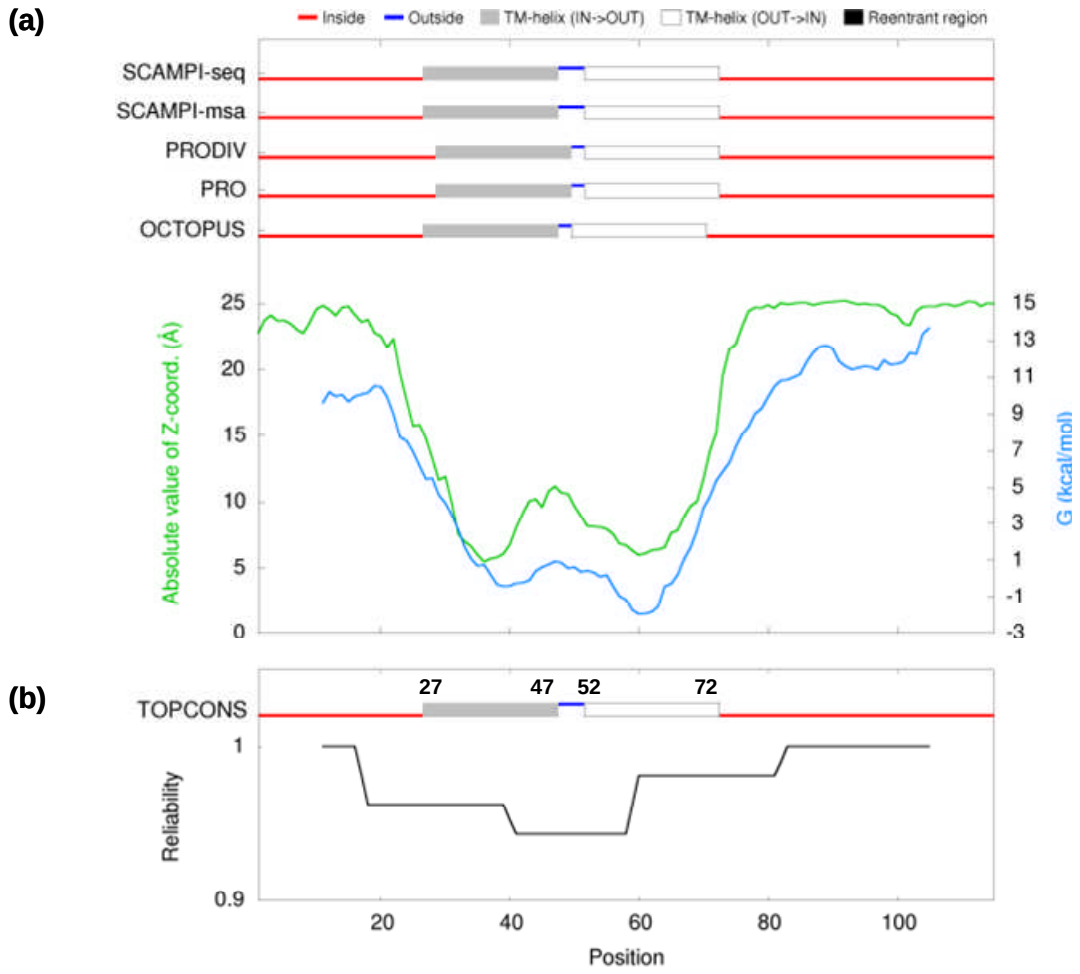


Figure 2. Topology prediction in ORF9 (TraC) according to the algorithms SCAMPI-seq, SCAMPI-msa, PRODIV, PRO, and OCTOPUS (a), and determination of the consensus prediction for ORF9 by the TOPCONS hidden Markov model (b).

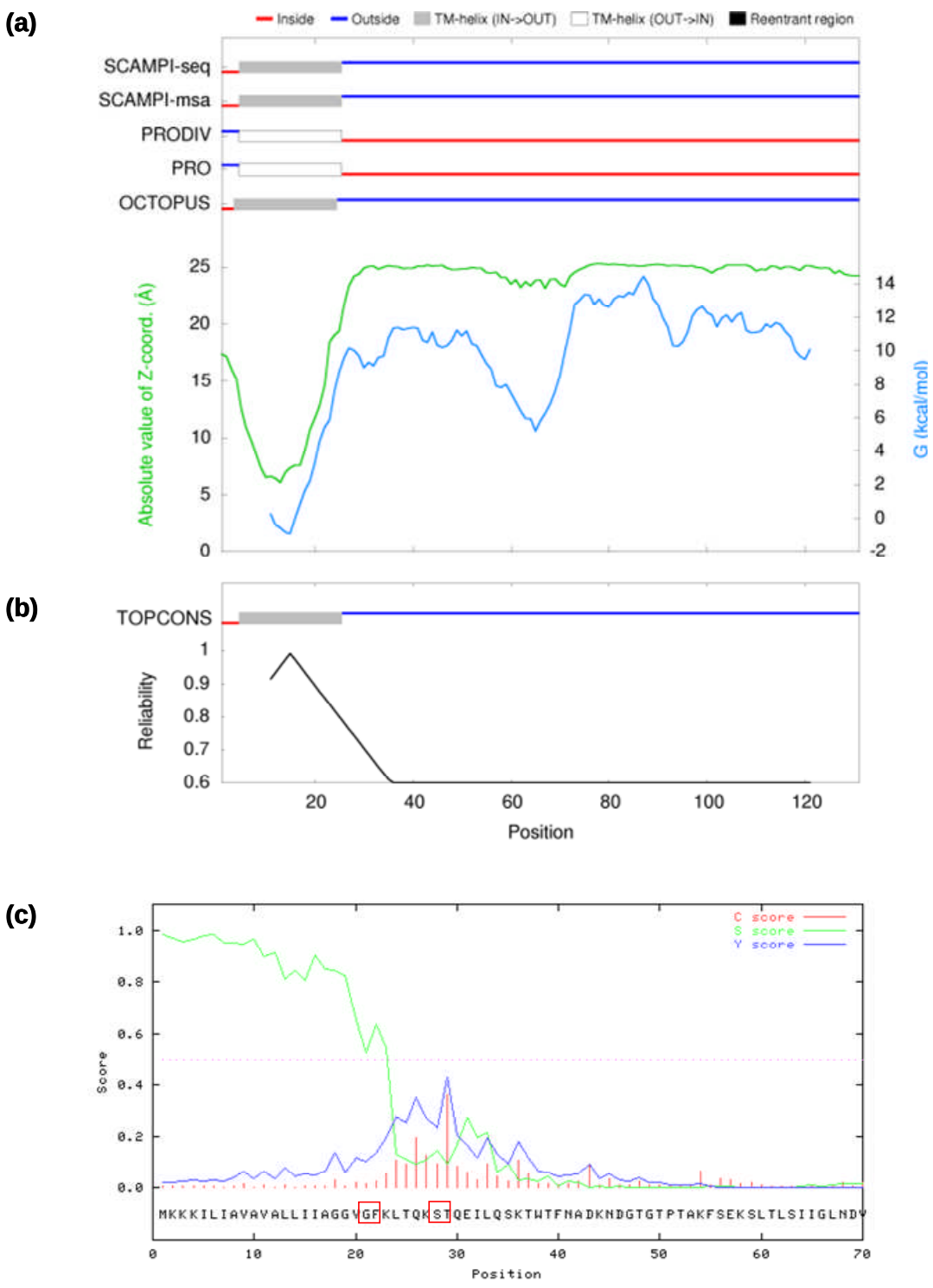


Figure 3. Topology prediction in ORF18 according to the algorithms SCAMPI-seq, SCAMPI-msa, PRODIV, PRO, and OCTOPUS (a), and determination of the consensus prediction for the protein by the TOPCONS hidden Markov model (b). Prediction of signal peptide sequence in ORF18 by using the SignalP-NN algorithm, with the most likely cleavage sites enclosed within red squares (c).

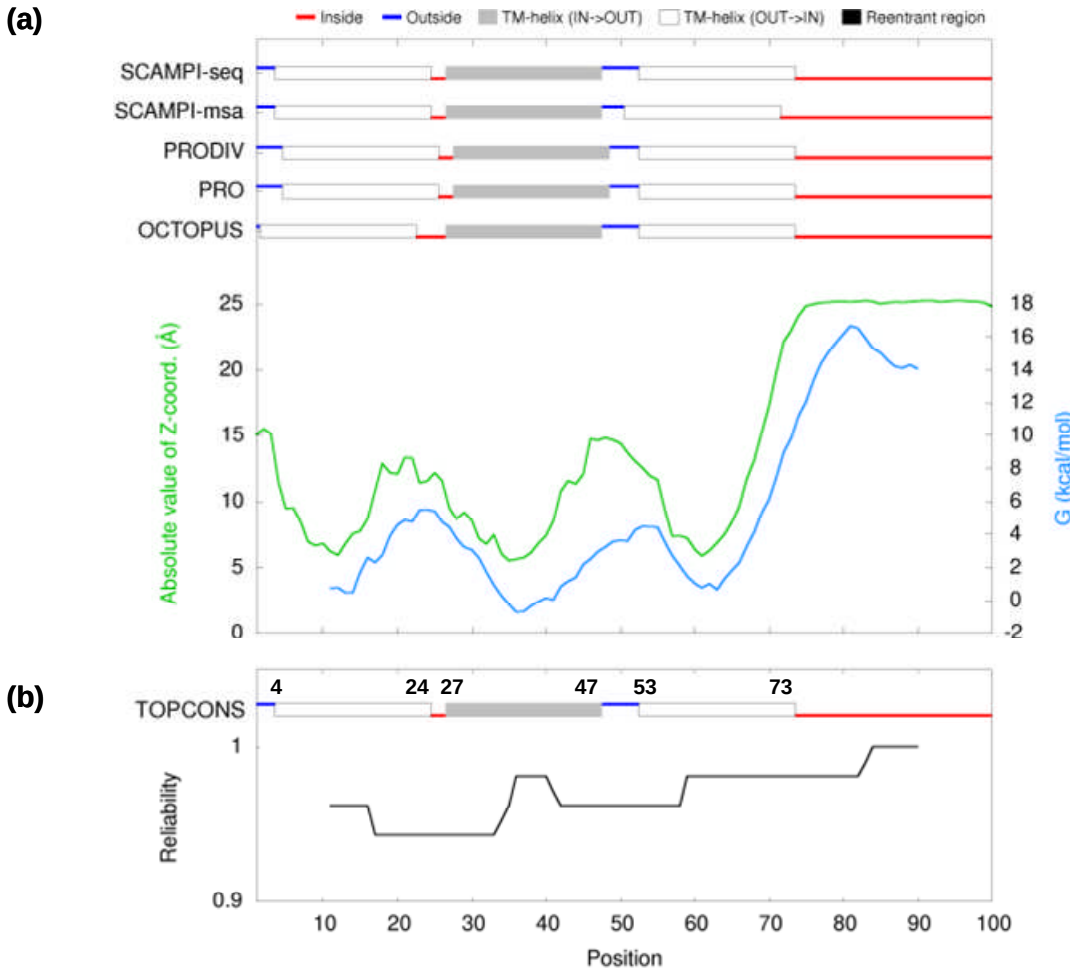


Figure 4. Topology prediction in ORF45 according to the algorithms SCAMPI-seq, SCAMPI-msa, PRODIV, PRO, and OCTOPUS (a), and determination of the consensus prediction for the protein by the TOPCONS hidden Markov model (b).

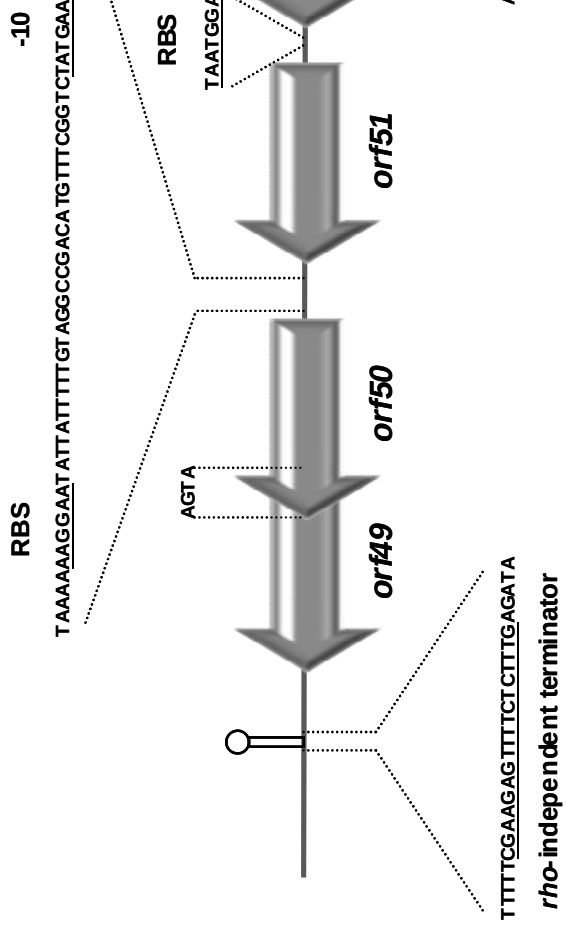


Figure 5. Diagramatic representation of the polycistronic operon *orf*s*52-49* and potential regulatory elements. Putative RBS, -10 promoter, and *rho*-independent terminator signals are underlined. The 4-bp overlap between *orf50* and *orf49* is also highlighted.

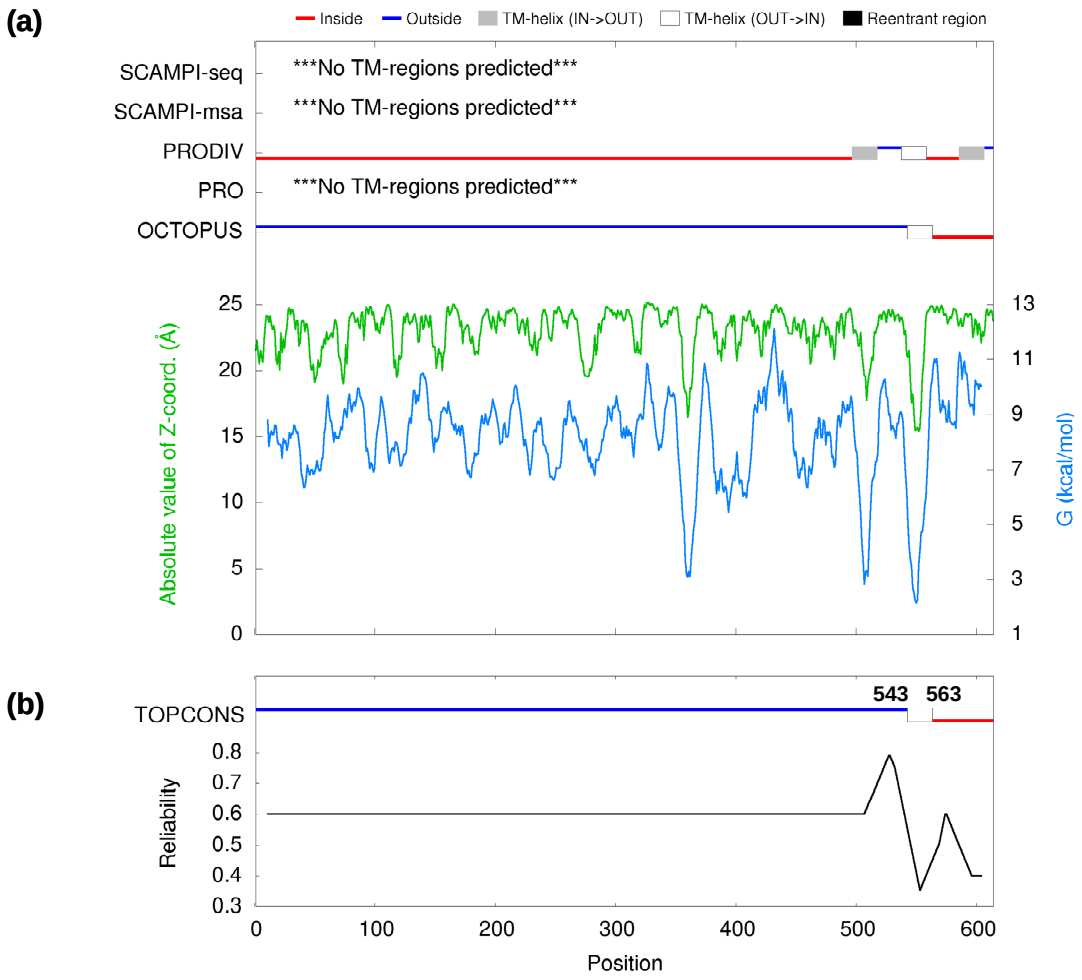


Figure 6. Topology prediction in ORF50 according to the algorithms SCAMPI-seq, SCAMPI-msa, PRODIV, PRO, and OCTOPUS (a), and determination of the consensus prediction for the protein by the TOPCONS hidden Markov model (b).

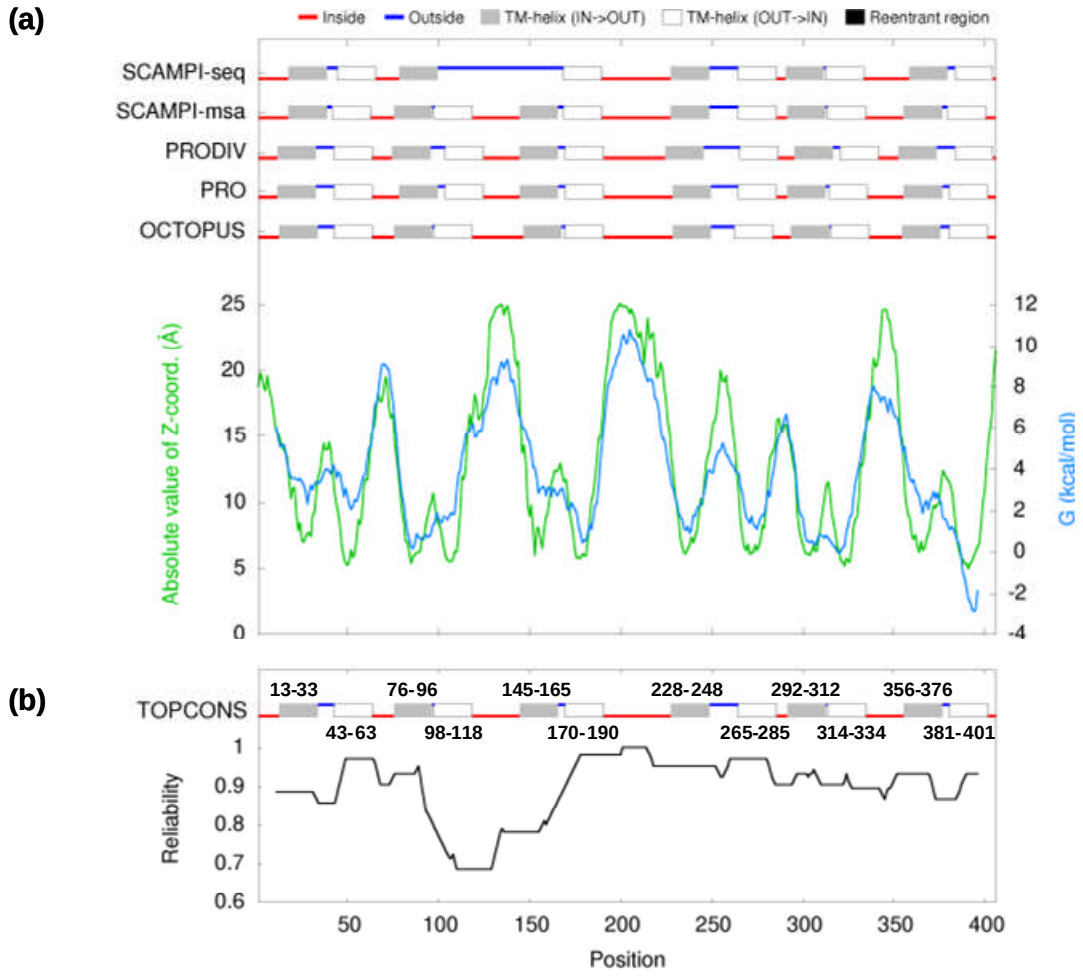


Figure 7. Topology prediction in ORF49 according to the algorithms SCAMPI-seq, SCAMPI-msa, PRODIV, PRO, and OCTOPUS (a), and determination of the consensus prediction for the protein by the TOPCONS hidden Markov model (b).

Chapter VII

General Conclusions

Lactococcus lactis strains are essential components of modern industrial starters and as such their sensitivity to infection by virulent homologous phages calls for the adoption of adequate measures to protect these cultures and their technological efficiencies. The combination of Good Manufacturing Practices and rotation of cultures carrying multiple natural anti-phage defence mechanisms has greatly improved the reliability and lifespan of industrial lactococcal starters, but the phage capacity to evolve and circumvent the resistance mechanisms requires the continuous devise of alternative solutions to counter the phage threat. In recent years, the sequencing of many phage genomes has provided a better understanding of phage-host interactions in *Lactococcus* by revealing insights into the life cycles and adaptive mechanisms of these viruses, and allowing in some cases the identification and structural elucidation of the phage components required for specific adsorption to and penetration of the host cell membrane (Emond and Moineau, 2007, Spinelli *et al.*, 2006, Tremblay *et al.*, 2006). Much less attention has been reserved for the analysis of the molecular response of the host to these early steps of phage infection, which might allow the identification of potential gene targets for the rational design of novel and sophisticated phage resistance and control strategies.

Chapter II of this thesis attempts to bridge this knowledge gap by providing for the first time in *L. lactis* a holistic view of the host genetic reprogramming at the onset of a lytic phage challenge. It shows that the cell senses the phage presence as a cell envelope stress and responds in a targeted manner by activating stress membrane signalling and regulatory elements, collectively redefined as the CesSR stimulon, that are also stimulated by other membrane stressors (i.e. lysozyme and antimicrobial peptides) in *L. lactis* (Martinez *et al.*, 2007, Veiga *et al.*, 2007). Interestingly, a further link between phage invasion, membrane stress and host response is provided by a recent study in *E. coli* showing that clustered, regularly interspaced short palindromic repeats (CRISPRs)-mediated immunity to foreign invading DNA requires the presence of the BaeSR two-component system involved in envelope stress response (Perez-Rodriguez *et al.*, 2011). These findings suggest that the bacterial systems responsive to cell envelope stress are not merely involved in maintaining membrane integrity but ultimately committed to the wider role of preserving cell viability. The transcriptional data of Chapter II also contribute to describing the putative molecular mechanisms adopted by the cell to restore the PMF

physiological value, which is usually disrupted following phage infection in both gram-positive and gram-negative bacteria (Labedan and Letellier, 1981). Finally, this study creates the premise for the design of novel anti-phage approaches by identifying the cell-wall TAs modifying *dlt* operon as a target of further investigations regarding its putative role in phage resistance. It is certainly open to different interpretations whether the results of Chapter II are indicative of host intelligence or simply a metabolic response to the phage threat. In the opinion of the author of this thesis, the articulation of such a complex strategy, which putatively aims at evading phage invasion, optimizing energy resources and recovering full fitness, is strongly indicative of a planned, optimal selection and use of the genetic assets by the host. It suggests a degree of host intelligence till now undiscovered in the simplest of living organisms and that could correspond to the same one triggering bacterial evolution where the strategy that succeeds in providing a competitive advantage becomes a learnt behaviour.

In order to widen the repertoire of solutions available to combat the phage threat, researchers have recently searched for novel *L. lactis* strains or genetic traits from natural ecological niches and good quality artisanal products. These natural strains are expected to possess a larger, and mostly plasmid-encoded, genomic diversity compared to industrial strains and therefore can be exploited to replace, complement or genetically improve the starter cultures currently used in dairy industry (Cogan *et al.*, 1997, Delgado and Mayo, 2004, Salama *et al.*, 1995). Chapter III confirms the value of the approach targeting natural isolates as it shows an intriguing abundance and diversity of plasmids in a collection of raw milk cheese isolates, as well as a wealth of genetic traits in the 8-plasmid complement of *L. lactis* subsp. *cremoris* DPC3758 with the potential to improve the technological properties of dairy starters. By carrying multiple anti-phage systems, food-grade selectable markers and functional conjugative/mobilization regions, these natural plasmids are a tremendous addition to the range of genetic tools available to researchers to obtain novel starter formulations with enhanced robustness and long-term efficiency (Coffey and Ross, 2002, Mills *et al.*, 2006). Chapter III provides the gene knowledge base for their rational exploitation, and it also reaffirms the major role played by plasmids in contributing to host fitness and preservation within its ecological niche.

The plasmid complement is probably the major reservoir of genotypes specifically linking a strain to a particular niche, and can therefore be used as a sort of bar-coding system containing traceability markers of the organisms' origin. Examples of such plasmid gene markers are those promoting efficient utilization of milk substrates and a competitive advantage in dairy strains, or those contributing to the growth on plant cell walls substrates and to a greater tolerance of the numerous environmental stresses in natural isolates (Mills *et al.*, 2006, Rodionov *et al.*, 2006, Siezen *et al.*, 2005, Siezen *et al.*, 2008, Teuber *et al.*, 1999). Based on these considerations, it was very intriguing to find a sort of dairy-natural hybrid plasmid complement in the raw milk cheese isolate *L. lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901 (Chapter IV), which includes not only a milk-specialized plasmid, but also plasmids carrying numerous genotypes novel to *Lactococcus* and typical of bacteria associated with the plant ecosystem, as well as rare genes previously exclusive to plant-derived lactococcal plasmids (Tanous *et al.*, 2007, Tanous *et al.*, 2002). The presence of these traits in a dairy strain can be reasonably inferred as genetic traces of a previous plant niche inhabited by the organism where they served a real colonization advantage. This hypothesis is highly feasible as plant-associated strains are considered the original source of dairy strains (Salama *et al.*, 1995, Siezen *et al.*, 2008) and can be theoretically retrieved from raw milk cheeses where the absence of pasteurization preserves the natural milk microflora. No clear evidence of such an origin has ever been documented in *Lactococcus* but the numerous plant gene markers found in *L. lactis* DPC3901 plasmids almost certainly suggest a plant origin for the raw milk cheese isolate and provide for the first time the genetic basis to support the concept of the plant-milk transition for *Lactococcus* strains. The interesting findings of Chapter IV deserve further investigations, such as the sequencing of the entire genome of *L. lactis* DPC3901, which might provide the definitive genetic basis to further support or confute the plant-origin hypothesis.

Following the isolation of novel plasmids carrying traits of technological interest, improved derivatives of lactococcal starters can be generated by using a food-grade delivery approach (Coffey and Ross, 2002). However, this process is likely to impose a burden on regular cell metabolism and may affect the growth characteristics of the derivative strain (Kobayashi *et al.*, 2002, Lee and Moon, 2003). Chapter V reveals that the acquisition of pMRC01, a large 60-kb plasmid carrying anti-phage system

and bacteriocin production genotypes, does not significantly affect the acidification capacities of lactococcal starters. This finding is very important as implies that plasmid-improved derivatives of starter cultures can be efficiently used in dairy industry. The metabolic burden placed by pMRC01 on the host results in lower growth rate and increased cell permeability and autolysis, which, again, does not preclude its industrial application. Indeed, strains prone to lysis are particularly sought and utilized as components of dairy starters due to the early release of intracellular enzymes that contributes to accelerate cheese ripening and flavour development (Beresford and Williams, 2004).

Plasmid pMRC01 has been extensively exploited to enhance the phage resistance of lactococcal strains as it harbours conjugative functions and a potent Abi mechanism. Despite the plasmid's complete sequence has been recently obtained, the Abi genetic determinant(s) are yet to be identified (Coakley *et al.*, 1997, Dougherty *et al.*, 1998, Mills *et al.*, 2006). In Chapter VI, pMRC01-based microarrays were used to detect the putative activation of the Abi system, or elements of the system, during the early and/or late stage of a lytic infection. Results suggest that the functioning of the Abi system could be subjected to a complex regulatory control by the bivalent Rgg-like ORF51 and CopG-like ORF58 proteins. These regulators are likely to modulate the activity of the putative Abi effectors, which might be the constitutive proteins ORF50 and ORF49 exhibiting topology and functional similarities to the two-component Rex system that aborts phage λ lytic growth via membrane depolarization (Parma *et al.*, 1992). Chapter VI does not provide a definitive identification of pMRC01 Abi system but identifies potential regulators of its activity, which might be targeted by using a gene knock-out approach in order to finally elucidate the elements and mechanisms conferring the Abi phenotype to pMRC01-carrying cells.

In addition to phage protection, pMRC01 also confers the host with the ability to produce a ribosomally-synthesized peptide or bacteriocin named lacticin 3147. This broad spectrum antimicrobial has proved to be very effective in increasing the safety of fermented dairy foods (McAuliffe *et al.*, 1999, O'Sullivan *et al.*, 2006, Scannell *et al.*, 2000b) as well as in contributing to enhance cheese quality and aroma (Fenelon *et al.*, 1999, Martinez-Cuesta *et al.*, 2006, Ryan *et al.*, 2001). Similarly to lacticin 3147, many other bacteriocins (i.e. enterocin AS-48, lacticin 481, variacin, and sakacin P) produced by different LAB hold great potential for bio-preservation and shelf-life

extension of food products. These antimicrobials have been successfully used for the selective inhibition of certain food pathogens, without affecting the natural beneficial microflora, or for wider preservative function (Galvez *et al.*, 2008), and are therefore the primary candidates for future commercial exploitation in food preservation. However, the outcomes of most recent research have shown that the effectiveness of bacteriocins can decrease in response to a number of physicochemical and microbiological factors related to the particular food matrix (Schillinger *et al.*, 1996), whereas the combination of bacteriocins with selected hurdles often results in a more effective form of preservation (Galvez *et al.*, 2008). It is likely therefore that one of the major directions of future research on food protection will be the exploitation of bacteriocinogenic cultures or their pure bacteriocins within a hurdle technology program. In particular, the combination of bacteriocin-activated packaging films and traditional hurdles holds promise for the extension of shelf-life and improvement of microbiological safety of food products.

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