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Osmotic Stress Tolerance Mechanisms in *Lactococcus lactis*

A Dissertation Presented For The Degree of Doctor of Philosophy

by

John O'Callaghan B.Sc

Department of Microbiology

University College Cork

Supervisor: Professor Séamus Condon.

November 1998



This Thesis is dedicated to my parents.

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Abstract.

The response of *Lactococcus lactis* subsp. cremoris NCDO 712 to low water activity (a_w) was investigated, both in relation to growth following moderate reductions in the a_w and in terms of survival following substantial reduction of the a_w with NaCl.

Lc. lactis NCDO 712 was capable of growth in the presence of ≤ 4% w/v NaCl and concentrations in excess of 4% w/v were lethal to the cells. The presence of magnesium ions significantly increased the resistance of NCDO 712 to challenge with NaCl and also to challenge with high temperature or low pH. Survival of *Lc. lactis* NCDO 712 exposed to high NaCl concentrations was growth phase dependent and cells were most sensitive in the early exponential phase of growth. Pre-exposure to 3% w/v NaCl induced limited protection against subsequent challenge with higher NaCl concentrations. The induction was inhibited by chloramphenicol and even when induced, the response did not protect against NaCl concentrations > 10% w/v.

When growing at low a_w, potassium was accumulated by *Lc. lactis* NCDO 712 growing at low a_w, if the a_w was reduced by glucose or fructose, but not by NaCl. Reducing the potassium concentration of chemically defined medium from 20 to 0.5 mM) produced a substantial reduction in the growth rate, if the a_w was reduced with NaCl, but not with glucose or fructose. The reduction of the growth rate correlated strongly with a reduction in the cytoplasmic potassium concentration and in cell volume. Addition of the compatible solute glycine betaine, partially reversed the inhibition of growth rate and partially restored the cell volume.

The potassium transport system was characterised in cells grown in medium at both high and low a_w. It appeared that a single system was present, which was induced approximately two-fold by growth at low a_w. Potassium transport was assayed *in vitro* using cells depleted of potassium; the assay was competitively inhibited by Na^+ and by the other monovalent cations NH_4^+, Li^+, and Cs^+.

There was a strong correlation between the ability of strains of *Lc. lactis* subsp. *lactis* and subsp. *cremoris* to grow at low a_w and their ability to accumulate the compatible solute glycine betaine. The *Lc. lactis* subsp. *cremoris* strains incapable of

growth at NaCl concentrations > 2% w/v did not accumulate glycine betaine when growing at low a_w, whereas strains capable of growth at NaCl concentrations up to 4% w/v did. A mutant, extremely sensitive to low a_w was isolated from the parent strain *Lc. lactis* subsp. *cremoris* MG 1363, a plasmid free derivative of NCDO 712. The parent strain tolerated up to 4% w/v NaCl and actively accumulated glycine betaine when challenged at low a_w. The mutant had lost the ability to accumulate glycine betaine and was incapable of growth at NaCl concentrations >2% w/v or the equivalent concentration of glucose. As no other compatible solute seemed capable of substitution for glycine betaine, the data suggest that the traditional; phenotypic speciation of strains on the basis of tolerance to 4% w/v NaCl can be explained as possession or lack of a glycine betaine transport system.

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Literature Review

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1. INTRODUCTION.

Bacteria exist in a wide range of natural environments, many of which involve either transient or continuous exposure to environmental stress(es). Osmotic stress, which can result from drying or increased extracellular solute concentrations is commonly encountered during growth in natural environments. The means by which the lactic acid bacteria (LAB) respond to osmotic stress is of interest as industrial processes involving these organisms often involve exposure to osmotic stress, e.g following salting during cheese production or during the production of lyophilised starter cultures. The osmotic tolerance response of the genus *Lactococcus* is of particular interest as the two most commonly used subspecies *Lc. lactis subsp. lactis* and *Lc. lactis* subsp. *cremoris* have traditionally been separated on the basis of their ability to grow in the presence of sodium chloride. Strains traditionally regarded as *lactis* strains are expected to grow in broth containing up to 4% w/v NaCl whereas the upper limit for growth of the *cremoris* subspecies is traditionally regarded as approximately 2% w/v. As the ability to grow in the presence of sodium chloride will influence the activity of a *Lactococcus* culture following salting of cheese, a characterisation of the osmotic tolerance response of this genus and elucidation of the mechanism(s) involved is of great interest to us.

This review is concerned with the general area of osmotic stress in bacteria and the various mechanisms by which bacteria adapt to osmotic stress are discussed. The osmoregulatory mechanisms of bacteria are outlined both at the genetic and physiological levels. In addition to describing the mechanisms which facilitate growth at high osmolarity, the mechanisms which protect the cell during lethal intensities of osmotic stress are examined. Where possible, the osmotolerance of Gram-positive bacteria, including *Lactococcus lactis* is described; however, study of the physiology and genetics of Gram-positive organisms is not as advanced as that in *Escherichia coli* and *Salmonella* and accordingly much of the literature reviewed concerns these two organisms. The susceptibility of bacteria to lethal levels of environmental stress is often growth phase dependent and in consequence, the phenomenon of growth phase

dependent gene expression and its influence on bacterial resistance to environmental stress is also reviewed.

2. OSMOTIC STRESS AND ITS EFFECTS ON THE CELL.

The bacterial cytoplasmic membrane is freely permeable to water while being impermeable to many solutes. The solute concentration of the bacterial cytoplasm is normally higher than that of the surrounding medium and therefore, water will tend to flow into the cell. The classic demonstration of this situation is with a model system consisting of two compartments open to the atmosphere and separated by a semi-permeable membrane. If the solute concentration of one of the compartments is increased, the influx of water required to adjust the solute concentration results in a difference in water level between the two compartments. This difference in level (hydrostatic pressure) is referred to as osmotic pressure. In the case of a bacterial cell, the volume cannot expand indefinitely therefore the pressure within the cell increases. This is known as turgor pressure and is proportional to the gradient of solute across the cell membrane.

The solute concentration of a solution can also be expressed as the activity (a) of the solvent. This may be expressed by the formula ($1 - {}^{\text{moles solute}}/_{\text{moles solvent}}$). In most biological systems the solvent in question is water and the osmolarity is referred to as the water activity (a_w).

The osmolarity of a solution can be expressed as the osmotic potential (π), which is a function of the water activity.

$$\pi = (RT/V) \ln a_w$$

Where V = partial molar volume of the solvent
R = the universal gas constant
T = the absolute temperature (K)

Turgor pressure is the difference between the osmotic potential of the cytoplasm and that of the the environment.

$$\text{Turgor pressure} = (RTV)(\ln a_{w\,(medium)} - \ln a_{w(cytoplasm)})$$

From this, an approximate expression for turgor pressure may be derived.

$$\text{Turgor pressure} \approx RT\,(C_{cells} - C_{medium})$$

Where C_{cells} and C_{medium} are the solute concentrations of the cell cytoplasm and the medium respectively (Csonka, 1989)

It follows that any increase in the extracellular solute concentration (decreased a_w) will lead to a decrease in turgor pressure. This may be achieved by addition of extra solutes such as salt or sugars, or by removal of water (drying). Turgor pressure has been suggested as the driving force required for expansion of the cell during growth (Koch *et al.*, 1981); if this is the case, then maintenance of turgor pressure is intrinsic to cell growth.

Hyperosmotic shock, i.e. a reduction in the a_w of the environment such that the transmembrane solute gradient decreases, causes a decrease in turgor pressure. This results in shrinkage of the cytoplasmic volume (plasmolysis). As there is a loss of water from the cytosol during plasmolysis its a_w decreases. Once the cytoplasmic a_w reaches a value outside its normal range it is likely that cellular activity will be impaired by a reduction in several enzyme activities. Loss of membrane tension may also affect the conformation of membrane bound proteins leading to a total or partial loss of activity. Carbohydrate uptake and glycolysis in *Escherichia coli* (Roth *et al.*, 1985a,b) and *Clostridium pasteurianum* (Walter *et al.*, 1987) were inhibited at low a_w. DNA replication and cell division in *E. coli* were also inhibited by osmotic stress. (Meury, 1988). Evidence that biosynthetic pathways are inhibited by loss of turgor pressure was provided by a report that intracellular ATP levels in *E. coli* are increased following osmotic stress (Ohwada and Sagisak 1988). This result suggests that ATP producing pathways are unaffected while pathways that consume ATP are inhibited and is in

conflict with the reports of inhibited glycolytic activity in *E. coli* and *C. pasteurianum* by Roth *et al.* (1985 a,b) and Walter *et al.* (1987) respectively.

If the a_w of the environment is reduced to a level outside the range that permits growth, cell death will probably occur. However, survival of cells under conditions of severe hyperosmotic stress has not been as extensively studied as the growth of cells under moderate osmotic stress.

3. THE RESPONSE OF BACTERIA TO OSMOTIC STRESS.

In order to cope with moderate fluctuations in the a_w of their environment, bacteria must possess a mechanism to maintain turgor pressure at, or close to, the optimal level. The simplest way of achieving this is to adjust the solute concentration of the cytoplasm such that the value $C_{cells} - C_{medium}$ remains at its optimal level. The cell therefore accumulates certain solutes in the cytoplasm in proportion to the increase in extracellular solute concentration. These solutes can be accumulated to relatively high levels without adverse effect on cellular metabolism and consequently, are known as compatible solutes (Brown and Simpson, 1972). Several compounds have been identified as compatible solutes. not alone for bacteria but also for a wide range of eukaryotic cells. The most important compatible solutes will be discussed below.

In contrast, when cells are subjected to more severe osmotic stress (i.e. that which may result in cell death) it is likely that maintenance of turgor may be less important. Under these conditions preservation of the integrity of cell constituents may be more important, as this will allow growth of the cell if conditions become more favourable. Protection against other lethal agents such as low pH or high temperature is achieved by synthesis of chaperone proteins whose function may be to stabilise cellular macromolecules (Minton *et al.*, 1982; Georgopoulos, 1992). There is a degree of cross-protection between the different stress responses, i.e. synthesis of some proteins is induced by more than one environmental stress. In addition to these general stress proteins there are some proteins which are induced by one specific stress e.g. acid shock proteins, or heat shock proteins.

4. COMPATIBLE SOLUTES.

Compatible solutes differ in their effects on the growth of cells under osmotic stress. Some compatible solutes are accumulated without greatly affecting the specific growth rate of the cell. Others, if present in the medium, will substantially stimulate the growth of osmotically stressed cells and are regarded as osmoprotectants. There are several reasons why compatible solutes differ in their ability to stimulate growth. Some compatible solutes may inhibit cellular processes if accumulated to high levels e.g. the addition of glycine betaine or proline had little effect on the activity of plant and vertebrate enzymes, even at relatively high concentrations (1 M), whereas ions such as sodium and potassium and some other amino acids did inhibit enzyme activity (Yancey *et al.*, 1982). Glycine betaine contains a trimethyl amino group (N,N,N-trimethyl glycine) and other osmoprotectants including carnitine also possess methylated amino groups. The extent of methylation appears to influence the ability of these compounds to act as osmoprotectants. Glycine betaine and dimethyl glycine reversed NaCl inhibition of barley malate dehydrogenase, with glycine betaine being the more effective of the two; in contrast, sarcosine (N-methylglycine) and glycine had no protective effect (Yancey *et al.*, 1982). In addition to the methylation of the amino group, the hydrocarbon chain length of the molecule is also a factor in determining its osmoprotective abilities. Fully N-methylated derivatives of propionate, butyrate and caproate differ in their osmoprotective abilities; the protective effect decreases with increasing chain length. Propionyl betaine is more stimulatory than butyrobetaine, which is in turn more stimulatory than caproic betaine (Peddie *et al.*, 1994).

4.1 GLYCINE BETAINE.

This compound (N,N,N-trimethyl glycine) is probably the most widely used osmoprotectant, not only in bacteria, but also in plants. Glycine betaine accumulation in bacteria can occur either by synthesis or by accumulation from the environment; however, not all bacteria can synthesise glycine betaine. The earliest reports of

osmoprotection of bacteria by glycine betaine were for *Pediococcus soyae* (Sakaguchi, 1960) and for an unnamed halotolerant bacterium Ba-1 (Rafael-Eshkol and Avi-Dor, 1968). Betaine accumulation in response to osmotic stress was first demonstrated in the halophilic species *Ectothiorhodospira halochloris* by Galinski and Truper (1983). These observations were extended to the enteric bacteria by Le Rudelier and Bouillard (1983) who observed stimulation of *E. coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* by glycine betaine. At the present time, there are numerous reports in the literature of the use of glycine betaine as an osmoprotectant by both Gram-positive and Gram-negative bacteria. One of the advantages of glycine betaine is that it is metabolically inert in most bacteria and therefore once accumulated, it is not degraded.

4.1.1 Synthesis of glycine betaine.

Few bacteria are capable of *de novo* synthesis of glycine betaine, this is confined to autotrophic bacteria such as cyanobacteria (Galinski and Truper, 1982; Reed and Stewart, 1985; Imhoff, 1986). Many bacteria incapable of *de novo* glycine betaine synthesis can convert choline (which contains a trimethylated amino group) to glycine betaine. It has also been reported that some pseudomonads can synthesise glycine betaine from carnitine (Kleber, 1997). In *E. coli*, glycine betaine is synthesised from choline in two steps. First choline is oxidised to glycine betaine aldehyde by the enzyme choline dehydrogenase; a second oxidation catalysed by either choline dehydrogenase, which is bifunctional, or glycine betaine aldehyde dehydrogenase, converts glycine betaine aldehyde to glycine betaine (Landfald and Strom, 1986). *Salmonella typhimurium* cannot oxidise choline to glycine betaine, as it lacks the genes encoding the pathway. (Styrvold *et al.*, 1986; Andresen *et al.*, 1988).

Choline is transported into the cytoplasm of *E. coli* by two transport systems, a high affinity system (Km, 8.0 μM) and a low affinity system (Km, 1.5 mM) (Styrvold *et al.*, 1986). The high affinity system was named BetT and the gene encoding this transporter was located close to an operon containing the genes encoding choline dehydrogenase and glycine betaine aldehyde dehydrogenase (Andresen *et al.*, 1988,

Lamark *et al.*, 1991). Mutants of *E. coli* in which the high affinity glycine betaine transport system ProU was inactivated did not display low affinity choline uptake (Lamark *et al.*, 1992). It is therefore likely that the low affinity transport of choline occurs via the ProU system.

The choline - glycine betaine pathway in *E. coli* is regulated by the osmotic strength of the medium, and maximal activity of choline dehydrogenase and glycine betaine aldehyde dehydrogenase was only observed in extracts from cells grown in medium of high osmolarity (Landfald and Strøm, 1986). Increased activity of both enzymes was observed in cells grown in medium of high osmolarity, even if choline was absent. However, inclusion of choline in the growth medium greatly increased the specific activity of both enzymes (Landfald and Strom, 1986). No stimulation of growth, or increased cytoplasmic levels of glycine betaine were observed in *E. coli* cells grown anaerobically in the presence of choline, indicating that the oxidation of choline only occurred under aerobic conditions. Conversion of glycine betaine aldehyde to glycine betaine was not oxygen dependent . Addition of glycine betaine aldehyde to anaerobic cultures both stimulated growth, and increased the cellular glycine betaine content. Growth of cultures in medium of low osmolarity followed by transfer to medium of high osmolarity in the presence of chloramphenicol did not result in the synthesis of glycine betaine, indicating that the increased enzyme levels resulted from *de novo* protein synthesis. Analysis of the choline dehydrogenase activity and glycine betaine aldehyde activities in subcellular fractions revealed that the former was membrane associated whereas the latter was a soluble enzyme indicating a cytoplasmic location (Landfald and Strom, 1986).

More recent work has focussed on the genetics of the choline-glycine betaine pathway. In *E. coli* the synthesis of glycine betaine from choline is under the control of the *bet* genes of which there are four, *bet* T which encodes the choline transport protein, *bet* B which encodes the betaine aldehyde dehydrogenase gene, *bet* A which encodes choline dehydrogenase, and *bet* I, a regulatory gene. The *bet* I, A, and B genes are located in an operon, while *bet* T is located upstream of *bet* IBA. The operons are

transcribed divergently under the control of separate, but partially overlapping promoters (Lamark *et al.*, 1991). Expression of *bet* A, *bet* B and *bet* T was increased seven to ten fold in response to increases in the osmotic strength of the medium. Addition of choline during osmotic stress further induced the expression of the *bet*T and *bet*A genes, while addition of glycine betaine prevented the induction by choline. Expression of *bet*A, *bet*B and *bet*T was reduced under anaerobic conditions (Eshoo *et al.*, 1988). Choline stimulated binding of the BetI regulatory protein to the promoter region (Røkenes *et al.*, 1996). Expression from both promoters was reduced under anaerobic conditions, by the ArcA regulatory protein (Lamark *et al.*, 1996). ArcA is the regulator protein of a two component regulatory system which controls the activity of *E. coli* genes repressed under anaerobic conditions. Osmotic induction of the *bet* genes occurred independently of *bet*I (Lamark *et al.*, 1996).

The Gram positive organism *Bacillus subtilis* is also capable of synthesising glycine betaine from choline (or glycine betaine aldehyde). In this organism, choline is transported by two transport systems, OpuB and OpuC. Both of these transport systems belong to the ATP binding cassette (ABC) superfamily of prokaryotic and eukaryotic transporters. OpuC also transports the compatible solutes glycine betaine and ectoine (Jebbar *et al.*, 1997). *B. subtilis* cells transported choline in a highly substrate specific manner and competition experiments indicated that a fully methylated quaternary ammonium group was intrinsic to substrate recognition (Boch *et al.*, 1994). A substantial amount of choline transport was observed even in cells grown at low osmolarity; however, a three- to four-fold increase in the rate of choline transport was observed in cells grown at high osmolarity. The Km value for choline transport remained approximately the same irrespective of the osmolarity of the growth medium (Boch *et al.*, 1994).

In *B. subtilis* the genes involved in the choline-glycine betaine pathway were cloned by functional complementation of *E. coli* mutants incapable of glycine betaine synthesis (Boch *et al.*, 1996). Two genes *gbs*A and *gbs*B were identified. Sequence analysis indicated that *gbs*A belonged to a superfamily of aldehyde dehydrogenases

which includes glycine betaine aldehyde dehydrogenases from plants and animals, and the *E. coli bet*B gene (39% homology) *gbs*B showed a significant similarity to the type III alcohol dehydrogenases. Over-expression of *gbs*A and *gbs*B revealed a 62 kDa and a 43 kDa protein respectively. Deletion of the *gbs*AB genes abolished the osmoprotective effect of choline indicating that the *gbs*AB genes encoded the only glycine betaine synthesis pathway in *B. subtilis*. There were significant differences between the *E. coli bet* genes and the *gbs*AB genes. *B subtilis* did not appear to have any equivalent to the gene for the membrane bound *bet*A protein from *E. coli* and in addition, although *gbs*AB appeared to be arranged in an operon, analysis of both upstream and downstream sequences did not reveal any homologues to the *E. coli* choline transporter *bet*T or the regulatory gene *bet*I. However, transcription of the *gbs*A and *gbs*B genes was increased in the presence of choline which suggests the presence of a regulatory protein in *B. subtilis* , similar to the BetI protein of *E. coli* (Boch *et al.*, 1996). It is clear that although *B. subtilis* can convert choline to the osmoprotectant glycine betaine, both the enzymes involved and the organisation of the genes encoding the pathway differ substantially from those that have been observed in *E. coli.*

The *gbs*A gene has been cloned and over-expressed in *E. coli* which allowed extensive *in vitro* studies of factors affecting GbsA activity. The effect of high concentrations of other osmoprotectants on the activity of GbsA was determined; 88% of the enzyme activity was retained in the presence of 2.4M potassium and 66% of its activity in 1M glycine betaine. Proline, which was accumulated to high levels by osmotically stressed *B. subtilis* cells (Whatmore *et al.*, 1990) was stimulatory to the enzyme, which increased its activity to 167% in 0.5 M proline (Boch *et al.*, 1997). The enzyme is therefore exceptionally well suited to its role under the conditions likely to be found in the cytoplasm of osmotically stressed *B. subtilis.*

4.1.2 Transport of glycine betaine.

4.1.2.1 Transport in *Escherichia coli* and *Salmonella.*

In general, if exogenous glycine betaine is present, expression of the *E. coli bet* genes is down-regulated. Under these conditions, glycine betaine transport becomes the major contributor to the cytoplasmic glycine betaine pool. Bacteria which cannot synthesise glycine betaine rely entirely on transport of glycine betaine to increase their intracellular glycine betaine content during osmotic stress.

In *E. coli* and *S. typhimurium* transport of glycine betaine (and other compatible solutes) occurs via the ProU and ProP transport systems (Csonka and Hanson, 1991). ProU is a member of the ABC transporter family and belongs to a subgroup referred to as binding-protein dependent ABC transporters. Members of this family of transport systems share common structural features. In Gram negative bacteria a typical binding protein dependent ABC transporter contains a soluble periplasmic binding protein (PBP) which binds the substrate to be transported with high affinity, a permease composed of two membrane bound proteins which are not substrate specific and one or two hydrophilic polypeptides which have ATPase activity. The membrane bound permease proteins may be homo- or heterodimeric and can recognise more than one PBP, each with a different substrate specificity. In Gram-positive bacteria the periplasmic binding protein is replaced by an extracellular binding protein anchored to the cytoplasmic membrane.

ProU transports betaine with high affinity (Km, 1.3 μM) and transcription of *proU* is stimulated in proportion to the osmolarity of the medium, up to approx 100-fold (Cairney *et al.*, 1985b). Transport did not occur in low osmolarity medium even in cells pregrown in medium of high osmolarity (Cairney *et al.*, 1985b). ProP is a single membrane protein driven by cation symport. Transcription of *proP* was also induced (three to five fold) by increasing the osmolarity of the growth medium, and the rate of transport was higher in high osmolarity medium than in low osmolarity medium (Dunlap and Csonka, 1985; Cairney *et al.*, 1985a; Grothe *et al.*, 1986). ProP has a lower affinity for glycine betaine than does ProU (Km, 44 μM) but the Vmax value is greater (37 nmol/mg prt/min) as opposed to 12 nmol/mg prt/min for ProU. (Cairney *et al.*, 1985a, 1985b). The higher Vmax by ProP suggests that it is probably the major

contributor to the cytoplasmic pool of glycine betaine, when the external glycine betaine concentration is high (May *et al.*, 1986). Under limiting glycine betaine concentrations, cells with only ProU grew better than ProP$^+$ cells. At high glycine betaine concentrations, ProU$^+$ cells grew as well as ProP$^+$ cells (May *et al.*, 1986). Both the ProP and ProU systems were originally identified as proline transporters (Anderson *et al.*, 1980; Menzel and Roth, 1980). The affinity of these transport systems for glycine betaine was much higher than that for proline and in the presence of osmotic stress the affinity of ProP for proline decreased and that for glycine betaine increased (Cairney *et al.*, 1985a ,1985b). However, there has been a report from another laboratory in which it was claimed that the ProP system in *E. coli* had approximately equal affinities for glycine betaine and proline (Milner *et al.*, 1987).

Regulation of glycine betaine transport in *E. coli* is achieved both at the genetic and physiological levels. Both the ProP and ProU systems required increased osmolarity for maximal activity even with cells in which maximal expression of *proP* and *proU* was achieved, suggesting that the activity of the transport system was osmotically regulated (Cairney *et al.* 1985a; 1985b). The regulation of *proU* has been studied intensively by a number of laboratories. The components of the *proU* transport system are encoded by an operon containing three cistrons, *proV*, *proW* and *proX* (Gowrishankar, 1985). In *E. coli*, two promoters have been identified upstream of the operon, together with a negative regulatory sequence within the *proV* gene (Dattananda *et al.*, 1991). The major promoter is located 60 nucleotides upstream of the operon and the second promoter, which is dependent on the alternative sigma factor σ^S, is 250 nucleotides upstream (Dattananda *et al.*, 1991; Manna and Gowrishankar, 1994). In *S typhimurium* a negative regulator within *proU* was also identified but no σ^S dependent promoter was found (Overdier *et al.*, 1989; Overdier and Csonka, 1992). Deletion of the negative regulator increased expression of the *proU* operon in both *E. coli* and *S. typhimurium* by up to 40-fold in low osmolarity medium (Dattananda *et al.*, 1991; Overdier and Csonka, 1992). Partial osmotic control of the *proU* operon was retained even in the absence of the negative regulatory region (Dattananda *et al.*, 1991; Overdier

and Csonka, 1992; Owen-Hughes *et al.*, 1992). If the negative regulatory region was replaced with the luxAB region which is also AT rich, a significant degree of osmotic control was restored (Owen-Hughes *et al.*, 1992). Expression of *proU* at low osmolarity was repressed less effectively in mutants defective in the DNA binding protein H-NS (Dattananda *et al.*, 1991; Overdier and Csonka, 1992; Owen-Hughes *et al.*, 1992); this protein binds preferentially to curved DNA and the negative regulatory region is AT rich which favours DNA curving. There is evidence that another DNA binding protein HU activates *proU* expression in *E. coli* by interaction with the major promoter (Manna and Gowrishankar, 1994). H-NS/HU double mutants exhibited a level of expression equivalent to the effects of each as individual mutations (Manna and Gowrishankar, 1994).

Intracellular potassium concentration was suggested as a possible signal for *proU* expression by Sutherland *et al.* (1986) and later by Ramirez *et al.* (1989), who reported that expression of *proU in vitro* was increased in proportion to the potassium glutamate concentration in the assay buffer. Other workers have disputed this proposal and eliminated glutamate (but not potassium) as the inducing signal (Csonka *et al.*, 1994). Interaction between potassium and H-NS has been reported; high potassium concentrations prevented inhibition of *proU* expression by H-NS *in vitro*; this occurred irrespective of whether potassium was supplied as potassium glutamate or potassium chloride (Ueguchi and Mizuno, 1993).

DNA supercoiling has also been suggested as a regulator of *proU* expression in *S. typhimurium* (Higgins *et al.*, 1988; Ni Bhrían *et al.*, 1989); mutations that alter DNA supercoiling have been shown to alter the expression of *proU* and the extent of DNA supercoiling increased when *S. typhimurium* was grown in high osmolarity medium (Higgins *et al.*, 1988). DNA gyrase deficient mutants displayed reduced supercoiling and decreased *proU* expression. This effect was also observed in wild type cells treated with the DNA gyrase inhibitors novobiocin and nalidixic acid (Higgins *et al.*, 1988). In contrast to *S. typhimurium*, DNA gyrase mutants of *E. coli* did not display enhanced

proU expression and neither did wild type *E. coli* treated with DNA gyrase inhibitors (Ramirez and Villarejo, 1989).

In summary, it is not yet completely clear how expression of *proU* is regulated. There is a certain amount of evidence to support all the models outlined above, however no model has as yet gained universal acceptance. There seems to be strong evidence from several laboratories in support of the proposal that potassium accumulation stimulates *proU* expression. This hypothesis is attractive in that potassium accumulation is the primary response to osmotic upshock in *E. coli* and S. *typhimurium*, and regulation of *proU* by the cytoplasmic potassium concentration would facilitate integration of the different osmotic tolerance responses.

Activity of the *proP* system is also dependent on the osmolarity of the growth medium (Cairney *et al.*, 1985a). Expression of this system was stimulated only 2-3 fold by osmotic upshock (Cairney *et al.*, 1985a). Transport of both glycine betaine and proline by the system was dependent on external potassium; little uptake of either osmoprotectant was observed if potassium was absent (Koo *et al.*, 1991). In addition to osmotic stress, *proP* was inducible by amino acid starvation (Anderson *et al.*, 1980), indicating a probable role for ProP in supplying proline for nutritional purposes.

4.1.2.2 Transport in Gram-positive bacteria.

Investigation of glycine betaine transport in Gram-positives has been initiated relatively recently and while much work has been done on the physiology of the transport systems, genetic analysis is at a very early stage.

Staphylococcus aureus is the most osmotolerant of the non halophilic bacteria and will grow at a_w values as low as 0.86. *Staph. aureus* is a leading cause of food borne illness, particularly in high salt foods where competition from other bacteria is reduced. The osmotolerance mechanisms of *Staph. aureus* have been the subject of much investigation for these reasons.

Glycine betaine acts as an osmoprotectant in *Staph. aureus* and is accumulated to high levels following osmotic upshock (Miller *et al.*, 1991; Graham and Wilkinson

1992). Uptake of glycine betaine in *Staph. aureus* occurs via two transport systems, one of low affinity and the other of high affinity (Pourkomailan and Booth, 1992; Townsend and Wilkinson, 1994). Transport of glycine betaine is controlled by feedback regulation, the rate of uptake being inversely dependent on the cytoplasmic concentration of glycine betaine (Pourkomailan and Booth, 1994; Stimeling *et al.*, 1994). Cells preloaded with glycine betaine did not transport glycine betaine via the high affinity system, and activity of the low affinity system was greatly reduced (Pourkomailan and Booth, 1994). Both transport systems displayed only a low level of transport activity in the absence of sodium ions (Pourkomailan and Booth, 1992); the activity of the high affinity system was unaffected by the osmolarity of the assay or by the presence of proline in the assay. The low affinity system displayed increased activity with increasing osmolarity of the assay buffer, and transport was inhibited by proline at a concentration 10-fold higher than that of glycine betaine. This suggested that the low affinity system could also transport proline; further evidence to support this suggestion came from the observation that mutants defective in the low affinity glycine betaine transport system were also incapable of low affinity proline uptake (Pourkomailan and Booth, 1992; 1994).

As is the case with *Staph. aureus*, *Listeria monocytogenes* is a cause of foodborne illness, which has led to a significant amount of research into the mechanisms by which this organism adapts to environmental stresses, including osmotolerance. Glycine betaine stimulates growth of *L. monocytogenes* at high osmolarity (Patchett *et al.*, 1992; Ko *et al.*, 1994; Beumer *et al.*, 1994). Transport of glycine betaine was stimulated under conditions of high osmolarity, by cells grown on either low, or high osmolarity medium. The highest rate of transport was by cells grown and assayed at high osmolarity (Patchett *et al.*, 1994). Increased initial rates of glycine betaine transport, when assayed at high osmolarity, were also observed by Gerhardt *et al.* (1996) in membrane vesicles. Transport of glycine betaine by membrane vesicles was dependent on the trans-membrane electrical potential ($\Delta\psi$) and occurred with co-transport of sodium ions (Gerhardt *et al.*, 1996). There has been no evidence to

suggest the presence of more than one transport system in *L. monocytogenes*; the kinetics of uptake did not vary over a range of glycine betaine concentrations from 2 to 800 μM (Patchett *et al* ., 1994). Transport appeared to be regulated by intracellular glycine betaine concentration as transport by cells preloaded with glycine betaine occurred at a rate inversely proportional to the cytoplasmic concentration of glycine betaine (Verheul *et al.*, 1997). Verheul *et al.* (1997) also reported that glycine betaine uptake by cells grown in the absence of glycine betaine was unaffected by the osmolarity of the assay buffer or the osmolarity of the medium in which the cells had been grown. This observation was in conflict with that of Patchett *et al.*, (1994); the differences were ascribed to the fact that Patchett *et al.* (1994) had grown the cells in complex medium (which contained glycine betaine) rather than the glycine betaine-free defined medium of Verheul *et al.* (1997). Cells preloaded with glycine betaine prior to adding radiolabelled glycine betaine, accumulated glycine betaine at rates dependent on the osmolarity of the assay buffer (Verheul *et al.*, 1997) and this was in agreement with the results of Patchett *et al.* (1994) assuming that pregrowth in complex medium had resulted in some accumulation of glycine betaine. However, the results of Gerhardt *et al.* (1996) who also reported stimulation of uptake by the osmolarity of the medium in which the cells were grown prior to preparation of membrane vesicles and by the osmolarity of the assay buffer, still suggest that both the level of the permease and its activity are controlled by osmolarity. As do the results of Ko *et al.* (1994) who reported that cells grown in medium of elevated osmotic strength accumulated betaine at higher rates than those grown in low osmolarity medium. Glycine betaine is also reported to enhance cryotolerance in *L. monocytogenes* (Ko *et al.*, 1994)..

Growth of *Lactobacillus acidophilus* (Hutkins *et al.*, 1987) and *Lb. plantarum* (Glaasker *et al.*, 1996a) was stimulated by glycine betaine in medium of high osmolarity. The rate of glycine betaine transport in *Lb. plantarum* was increased three-fold by pregrowth of the cells in high osmolarity medium and was increased even further if the osmolarity of the assay buffer was raised, even in the presence of chloramphenicol (Glaasker *et al.*,1996a). Activation of glycine betaine transport was

independent of the extent of the osmotic upshock, but the amount of glycine betaine accumulated was proportional to the extent of upshock. The time interval between upshock and glycine betaine addition did not influence the rate of accumulation by *Lb. plantarum* but there was a slight decrease in the final level accumulated, suggesting that osmotic equilibrium was partially restored before addition of glycine betaine, possibly by potassium uptake (Glaasker *et al.*, 1996a). A rapid efflux of glycine betaine resulted when *Lb. plantarum* cells were subjected to hypo-osmotic stress. The efflux ocurred at a more rapid rate than was observed for uptake and the extent of glycine betaine loss was dependent on the extent of downshock (Glaasker *et al.*, 1996a). Further examination of glycine betaine fluxes in *Lb. plantarum* revealed that steady state glycine betaine concentrations were maintained by the combined action of influx and efflux systems. Osmotic upshock resulted in stimulation of glycine betaine uptake and inhibition of efflux. Osmotic down shock resulted in a brief inhibition of influx together with a rapid efflux. The efflux was not due to cell lysis and the influx rate began to return to the steady state level approximately two minutes following downshock probably due to restoration of osmotic equilibrium (Glaasker *et al.*, 1996b). Efflux of glycine betaine appeared to occur in two distinct phases, an initial extremely rapid phase and a second slower phase. The rapid phase occurred too rapidly to permit analysis, but the secondary phase appeared to be dependent on the internal glycine betaine concentration at the end of the rapid phase, i.e. higher cytoplasmic glycine betaine concentrations resulted in a faster rate of efflux. The fact that activation of uptake following osmotic stress and the efflux following downshock occurred at rates independent of extracellular osmolarity suggests that these mechanisms are regulated by an on/off type mechanism, possibly related to turgor pressure/membrane tension (Glaasker *et al.*, 1996b).

Bacillus subtilis is the only Gram-positive bacterium in which genetic studies of glycine betaine synthesis and transport are well advanced. These studies were facilitated by the construction of an *E. coli* strain with mutations in all the genes essential for glycine betaine transport and synthesis, which facilitated the cloning of the

corresponding *B. subtilis* genes by functional complementation. *B. subtilis* has three known glycine betaine uptake systems (Kappes *et al.*, 1996). One of the systems, OpuC, is an ABC transporter similar to the ProU system of *E. coli* and *S. typhimurium* (Lin and Hansen, 1995). Another of the systems, OpuA, is also an ABC transporter, with similarities to the ProU system (Kempf and Bremer, 1996). The third system, OpuD, does not share any characteristics with previously identified glycine betaine transport systems but does share similarities with the BetT (choline) and the CaiT (carnitine) transport systems in *E. coli*. The substrates for these three systems are all trimethylammonium compounds; however, OpuD does not display any choline or carnitine transport activity (Kappes *et al.*, 1996). Mutant strains were constructed which possessed each of the three systems alone, which allowed the kinetic parameters of each to be determined, along with the contribution of each to osmoregulation. $OpuC^+$ or $OpuD^+$ strains were stimulated by glycine betaine in high osmolarity medium but not to the same extent as the $OpuA^+$ strain. This was reflected in the Vmax values for OpuA, OpuC and OpuD of 282, 65 and 61 nmol/min/mg protein, respectively. The Km values for all three strains were in the range 2.4 to 13 μM, demonstrating that all three strains had a high affinity for glycine betaine.

Lactococcus lactis, the subject of this thesis, was stimulated by glycine betaine in medium of elevated osmolarity when the osmolarity was increased by addition of NaCl or KCl. Interestingly however, no stimulation was observed when the osmolarity was increased by addition of sorbitol or sucrose (Molenaar *et al.*, 1993). Glycine betaine appears to be the preferred osmoprotectant in *Lc. lactis*, as proline was only accumulated in medium from which glycine betaine was absent. Transport of glycine betaine in *Lc. lactis* occurred via a high affinity transport system with a Km value of 1.5 μM; neither the rate of transport nor the Km was affected by pregrowth in complex medium of increased osmolarity. This high affinity system was energy dependent but independent of the proton-motive force. Growth in defined medium with high osmolarity however, resulted in the induction of very rapid glycine betaine transport that was independent of an energy source. It is interesting that this high velocity

transport of glycine betaine only occurred when proline transport was also stimulated (i.e in high osmolarity chemically defined medium), and that proline transport was inhibited by glycine betaine (Molenaar *et al.*, 1993). These data suggested to the authors that in addition to the high affinity non-inducible uptake system observed in cells grown in complex medium, there is a second, inducible system, which also transports proline. A proline auxotrophic mutant of *Lc. lactis* was isolated, which was unable to grow in the absence of exogenous proline, this mutant was capable of growth in the presence of 0.5 mM KCl in the absence of glycine betaine but was inhibited if glycine betaine was added. Growth of the mutant was not inhibited in low osmolarity medium in the presence of glycine betaine, which suggested that inhibition of proline uptake by glycine betaine may depend on the osmolarity of the growth medium (Molenaar *et al.*, 1993). The data of Molenaar *et al.* (1993) suggest that the the uptake system(s) for glycine betaine may be similar to those reported for *Staph. aureus* by Pourkomailan and Booth (1992; 1994) and Stimeling *et al.* (1994).

4.2 PROLINE.

Proline was first reported as an osmoprotectant in *S. oranienburg* by Christian (1955). The effect of increased osmolarity on the amino acid content of a number of Gram-positive and -negative bacteria was investigated by Measures (1975) who reported that proline and glutamate were the amino acids whose intracellular levels were most influenced by the osmolarity of the growth medium. Csonka (1981) observed a doubling of the specific growth rate of *S. typhimurium* when proline was added to high osmolarity medium. *S. typhimurium* mutants which overproduced proline were more osmotolerant than the wild type on proline free medium (Csonka, 1981). In general, accumulation of proline as a compatible solute by Gram-negative bacteria occurs as a result of transport, while some Gram-positive bacteria are capable of osmotically induced synthesis of proline (Csonka, 1989). In *B. subtilis*, proline synthesis was responsible for its accumulation following osmotic stress (Whatmore *et al.*, 1990). Unlike glycine betaine, proline can be used by some bacteria as a carbon or nitrogen

source, in addition to its incorporation into proteins. This creates a situation whereby accumulated free proline levels decrease due to catabolism and macromolecule synthesis.

4.2.1 Proline transport in *E. coli* and *S. typhimurium*.

In *E. coli* and *S. typhimurium* proline uptake in response to osmotic stress occurs via the transport systems ProU and ProP. Another proline transport system PutP transports proline for use as a carbon and/or nitrogen source and has no role in osmoprotection as PutP$^-$ mutants of both *E. coli* and *S. typhimurium* were stimulated by proline in high osmolarity medium (Csonka, 1982; Gowrishankar, 1985). ProP was identified first by Menzel and Roth (1980) in *S. typhimurium* and ProU by Csonka (1982), also in *S. typhimurium*. The regulation of these systems has already been outlined above in relation to the role of these systems in glycine betaine uptake. Measurement of growth in high osmolarity medium by mutants deficient in either ProU or ProP revealed that ProP was the major contributor to osmoprotection by proline (Csonka *et al.*, 1982). The affinity of these systems for glycine betaine was reported to be greater than for proline (Cairney *et al.*,1985a); however, other workers claimed that ProP has approximately similar affinities for both substrates (Milner *et al.*, 1987). In any event, it is not disputed that ProU preferentially transports glycine betaine, as the Km value for glycine betaine is 44 μM (Cairney *et al* 1985a) and that for proline is 340 μM (Anderson *et al.*, 1980); therefore, the net effect is a higher rate of glycine betaine uptake when both osmoprotectants are present.

4.2.2 Proline transport in Gram positive bacteria.

Among the Gram-positive bacteria, osmoprotection by proline has been best studied in *Staph. aureus*.; the first reports were by Measures (1975) and Koujima *et al.* (1978). The total amino acid pool increased 6-fold when the NaCl concentration of the medium was increased from 0.1 to 1.8M, practically all of the increase being due to proline accumulation. Uptake of proline from the medium increased immediately

following upshock (Koujima *et al.*, 1978). These results were confirmed by Anderson and Witter (1982), who also reported that proline accumulation was entirely due to accumulation of exogenous proline rather than by synthesis. These workers also reported an increase in the intracellular glutamic acid pool by synthesis in high osmolarity medium. Measures *et al.* (1975), Koujima *et al.* (1978) and Anderson and Witter (1982) relied on amino acid analysis to determine the cytoplasmic pools of amino acids. Miller *et al.* (1991) used natural abundance ^{13}C nuclear magnetic resonance spectroscopy to analyse the cytoplasmic content of *Staph. aureus* under conditions of osmotic stress. This analysis identified glycine betaine as another osmoprotectant. The effect of adding proline and glycine betaine to defined medium with high osmolarity was then examined, and proline was found to substantially stimulate growth, but not to the same extent as glycine betaine (Miller *et al.*, 1991). *Staph. aureus* was found to possess two proline transport systems, one being a high affinity system with a Km value of 7μM and the other of a low affinity with a Km value of 420 μM (Bae and Miller, 1992). The Vmax for the high affinity system was 10 nmol/min/mg prt. whereas the low affinity system had a much higher Vmax of 110 nmol/min/mg prt. These results were similar to those of Townsend and Wilkinson (1992) who also reported the presence of high- and low-affinity proline uptake systems in *Staph. aureus*. Both laboratories reported that sodium ions were essential for the activity of both systems and that activity of the low affinity system was stimulated by high osmolarity. Inhibition of proline uptake by glycine betaine was not observed for either system (Bae and Miller, 1992; Townsend and Wilkinson 1992). Conversely, the results presented by Pourkomailan and Booth (1992; 1994) in relation to glycine betaine transport, seem to suggest that the low affinity uptake of proline and glycine betaine by *Staph. aureus* is due to a single system. The gene for the high affinity proline uptake system from *Staph. aureus* has been cloned and the DNA sequence shared 49% homology with the PutP proline transport gene from *E. coli*. This observation, together with the lack of any significant osmotic stimulation of the *Staph. aureus* high affinity system, suggests its role may be to transport proline for use as a carbon or nitrogen source i.e. a similar

function to that of PutP (Wengender and Miller, 1995). In summary, proline transport in *Staph. aureus* is due to two systems, only one of which appears to be responsible for osmotic-stress related proline transport. It is probable that this system also transports glycine betaine.

Proline accumulation in response to osmotic stress has been reported for *Lc. lactis* (Molenaar *et al.*, 1993). Stimulation of growth was not investigated by these workers as the strain used in their study was partially auxotrophic for proline and it would not have been possible to separate stimulation due to a nutritional requirement from that due to osmoprotection. Proline accumulation by *Lc. lactis* in high osmolarity medium only occurred in the absence of glycine betaine. The proline transport system was inducible by pregrowth in defined medium at high osmolarity and in addition, high osmolarity during the assay was also necessary for maximal uptake. Proline uptake was not induced by growth in high-osmolarity complex medium. As outlined above, induction of the proline transport system coincided with induction of extremely rapid glycine betaine uptake, and proline transport was strongly inhibited by glycine betaine which suggested that the proline uptake system also transported betaine. The proline transport system had an extremely low affinity for proline (Km ≈ 5 mM), it had previously been thought that proline entered *Lc. lactis* by passive diffusion and not by active transport (Smid and Konings, 1990). This low affinity for proline and repression of the proline transport system may reflect the use of *Lc. lactis* as a dairy starter culture. The milk protein casein is particularly rich in proline and regular growth in milk would have led to *Lc. lactis* becoming more reliant on proline-containing peptides, than on free proline.

Lb. plantarum also accumulates proline in response to osmotic stress (Glaasker *et al.*, 1996a); accumulation only occurred if glycine betaine was absent. Induction of proline uptake was observed following growth in high osmolarity medium and activation of proline transport by the osmolarity of the assay buffer was also observed. This occurred in a very similar manner to that observed for the induction and activation of glycine betaine transport as outlined above for *Lc. lactis*. A rapid efflux of proline

occurred following osmotic downshock; again this was similar to that observed for glycine betaine, in that the extent of the efflux was dependent on the magnitude of the downshock (Glaasker *et al.*, 1996a).

4.3 POTASSIUM.

Potassium is accumulated by a wide range of bacteria in response to osmotic stress. Potassium accumulation usually occurs as a primary response to increases in osmolarity by which cells maintain turgor pressure until other osmoprotectants have been accumulated. Potassium accumulation in response to osmotic upshock has been reported for both Gram-negative and Gram-positive bacteria. Potassium is essential for bacterial growth and is the most prevalent intracellular cation, which suggests that accumulation of potassium is possibly less deleterious to the cell than other cations. As with most other aspects of bacterial physiology, the accumulation of potassium as a compatible solute has been best studied in *E. coli.*

4.3.1 Potassium accumulation by *E. coli* in response to osmotic stress.

In growing *E. coli* cells, an increase in the extracellular osmolarity from 0.1 to 1.2 osM resulted in an increase in the cytoplasmic potassium concentration from 0.15 to 0.55 M. This increase was only observed when the osmolarity was increased with membrane-impermeant solutes such as NaCl or sucrose, rather than glycerol which being membrane permeant is not osmotically active (Epstein and Schultz, 1966). Sudden osmotic upshock resulted in an immediate influx of potassium and osmotic downshock caused an efflux of potassium (Epstein and Schultz, 1966). The immediate influx of potassium indicates that activation of existing systems rather than protein synthesis is involved. The accumulation of potassium imposes a requirement for a negatively charged counter-ion. In a number of bacteria, including *E. coli,* glutamate is thought to fulfil this role (Measures, 1975). Accumulation of glutamate ocurred in conjunction with potassium accumulation in *E. coli* (McLaggan *et al.*, 1994), the glutamate concentration reaching approximately 70% of the potassium concentration.

Decreased glutamate catabolism was responsible for the accumulation of glutamate as accumulation appeared to be independent of the pathway by which glutamate synthesis occurred (McLaggan *et al.*, 1994) The cytoplasmic glutamate pool began to increase within one minute of osmotic upshock. *E. coli* possesses four constitutive low affinity transport systems for potassium, TrkG, TrkH, Kup and TrkF and an inducible high affinity system Kdp.

The high affinity Kdp system has a Km value for potassium of 2μM, a maximal rate of 150 μmol/g cells/min (Rhoads *et al.*, 1976; Epstein *et al.*, 1978) and is extremely specific for potassium in that it does not transport the potassium analogue, rubidium. The driving force for potassium transport by the Kdp system comes from ATP hydrolysis (Epstein *et al.*, 1978). The Kdp system is a member of a family called the P-type ATPases, which are characterised by the formation of a phosphorylated intermediate during catalysis and sensitivity to vanadate (Siebers and Altendorf, 1992). The Kdp transport complex consists of three cytoplasmic membrane proteins KdpA, KdpB and KdpC (Laimins *et al.*, 1978). The genes for the structural proteins *kdpA*, *-B* and *-C* are arranged as an operon (Laimins *et al.*, 1978), which also contains *kdpD* and *-E*, genes which positively regulate *kdpABC* and are located at the promoter-distal end of the operon (Rhoads *et al.*, 1978; Polarek *et al.*,1992). The *kdpDE* genes are expressed as an operon from a promoter located within *kdpC; kdpDE* is expressed at high levels along with *kdpABC*. The *kdpE* and *-D* gene products are members of the two component sensor-effector class of regulators (Walderhaug *et al.*,1992). The *kdpD* gene encodes a membrane bound sensor while *kdp*E is a soluble transcriptional effector.

Expression of the Kdp system is inducible by conditions of potassium limitation, and also by hyperosmotic stress (Laimans *et al.*, 1981). Potassium concentrations had to be reduced to a level which reduced the growth rate before induction occurred. Increasing the potassium concentration during osmotic stress repressed *kdp* expression (Sutherland *et al.*, 1986; Gowrishankar, 1985) and non-ionic solutes did not induce Kdp expression as effectively as did ionic solutes. It was

proposed by Laimins *et al.*, (1981) that regulation of *kdp* expression was by the turgor pressure of the cell. The model assumed that low internal K^+ concentrations resulting from low transport rates by the low affinity uptake systems led to decreased turgor pressure. Changes in membrane tension in response to a reduction in turgor were thought to affect conformation of the membrane bound sensor. The external or internal potassium concentrations did not directly affect transcription from the *kdp* promoter and so it appeared that turgor was the most likely signal for de-repression of *kdp*. Hyper-osmotic stress would also reduce turgor pressure, which would therefore be a common consequence of both inducing conditions. More recently however, evidence has been presented which implies that turgor pressure does not regulate *kdp* expression (Asha and Gowrishankar, 1993). These workers presented data which indicated that expression of *kdp* remained dependent on the extracellular potassium concentration in cells grown in the presence of glycine betaine. The added glycine betaine restored the specific growth rate to its normal level; therefore, it was reasonable to assume that the turgor pressure had been restored as well. In *S. typhimurium*, mutants in *sap*G (the equivalent of the *E. coli* Trk system) in which the Kdp system was functional, became progressively more inhibited as the extracellular potassium concentration increased. It appears from this result that high extracellular potassium levels decreased *kdp* expression, and in the absence of the low affinity system (SapG), potassium starvation occurred and the growth rate decreased (Frymier *et al.*, 1997). These results suggest that the regulation of the *kdp* genes is more complex than previously thought and support a model proposed by Sugiura *et al.* (1994), in which potassium acts as a negative regulator of *kdp* and osmotic stress acts as an inducer. The positive effect of osmotic stress in this model may still be due to reduced turgor pressure, but the negative regulation by potassium is clearly not due to restoration of turgor (Asha and Gowrishankar, 1993; Frymier *et al.*, 1997).

The low-affinity Trk systems are constitutive in *E. coli*; however, the activities of the systems are regulated by turgor pressure and the Vmax values in K^+ replete cells are much lower than in K^+ depleted cells. The rate of potassium influx is independent

of the extent of osmotic upshock, but is dependent on the potassium concentration of the cytoplasm (Meury *et al.*, 1985). It was once thought that the major low affinity transport system was TrkA; however, more recent work has revealed that the potassium uptake attributed to TrkA was in fact due to the action of two transport systems TrkG and TrkH (Dosch *et al.*, 1991). The TrkG and TrkH proteins are thought to be membrane proteins with up to twelve membrane-spanning domains. The DNA sequences were 49% identical at the nucleotide level and the gene products were 40% identical in their amino acid sequences and a further 18% are conservative substitutions. The *trkA* gene product was involved in both systems and was thought to mediate activation by ATP or act as a protein kinase (Bossemeyer *et al.*, 1989a; Bakker, 1993). The product of another gene, *trkE*, was essential for TrkH activity but TrkG was capable of a reduced level of activity in its absence (Dosch *et al.*, 1991). The Km values for potassium of TrkG and TrkH were 0.3-1 mM and 2.2-3.0 mM respectively and the Vmax values were >200 nmol/mg prt /min (Trk G) and >300 nmol/mg prt /min (TrkH) (Bossemeyer *et al.*, 1989a; Dosch *et al.*, 1991).

The Kup system (formerly TrkD) was differentiated from the other low affinity systems on the basis of its additional ability to transport cesium (Bossemeyer *et al.*, 1989b). This system has a Km value of 0.3-0.4 mM and a Vmax of approx 30 nmol/mg prt /min (Bossemeyer *et al.*, 1989a; Dosch *et al.*, 1991) which is only approx. 10% of the uptake rate of the TrkG and TrkH systems. Kup does not participate in osmoadaptation of *E. coli* as Kdp, Trk mutants accumulated little potassium following osmotic upshock (Dinnbier *et al.*, 1988; Schleyer *et al.*, 1993) and were inhibited completely by 0.3M sodium chloride (Bakker, 1993). The inhibition of Kup by sodium chloride was not due to reduced turgor, as cells which had restored turgor pressure did not display any Kup activity (Bakker, 1993).

The final potassium transport system in *E. coli* TrkF, has a very low affinity for potassium. Mutant strains in which TrkF is the only potassium transport system require potassium concentrations >15 mM for growth (Epstein and Kim, 1971; Rhoads *et al.*, 1976). Little is known about this transport system as no gene has been linked to its

activity. The Km value is between 20 and 30 mM and the Vmax extremely low (<15 nmol/min/mg prt).

Potassium transport in *E. coli* is therefore chiefly attributable to the activity of the TrkG/H systems along with Kdp. This combination of transport systems allows optimal growth at a range of potassium concentrations. Osmoadaptation is facilitated by the rapid activation of the Trk systems along with activation of Kdp at lower potassium concentrations.

4.3.2 Potassium as a compatible solute in Gram-positive bacteria.

Potassium accumulation by Gram-positive bacteria following osmotic upshock has been observed but little is known about the transport systems involved. *Staph. aureus* is extremely osmotolerant; however, no significant changes in its cytoplasmic potassium content under osmotic stress have been observed (Graham and Wilkinson, 1992; Kunin and Rudy, 1993). This may be due to the fact that even in the absence of osmotic stress,the potassium content of *Staph. aureus* was quite high (≈1.0 M), which may account for the extremely high turgor pressure maintained by this species.

Potassium accumulation in response to osmotic stress has been reported for *B. subtilis* (Whatmore *et al.*, 1990) and *B. acidocaldarius* (Michels and Bakker, 1987). *B. subtilis* accumulated potassium to approximately double the concentration (350mM increasing to 750 mM) observed in non-stressed cells (Whatmore *et al.*, 1990). The concentration of glutamate increased by approximately 60% following osmotic upshock; however, the major organic compound accumulated was proline. The accumulation of potassium was also observed when glycine betaine was present in the growth medium, although the initial and final levels (135 mM and 350 mM respectively) were lower than those of cells grown in the absence of glycine betaine (Whatmore *et al.*, 1990). Recovery of turgor pressure following osmotic upshock was dependent on potassium accumulation which in turn was essential for the secondary stage of osmoadaptation, as proline synthesis did not occur following osmotic stress unless potassium was present (Whatmore *et al.*, 1990). Potassium transport in the

genus *Bacillus* has been investigated, not in *B. subtilis*, but in the acidophilic, moderate thermophile *B. acidocaldarius* (Michels and Bakker, 1987; Bakker *et al.*, 1987). Two potassium transport systems were identified, one being of low affinity and the other of high affinity for potassium. The high affinity system was very similar to the *E. coli* Kdp system, even to the extent of immunological crossreactivity with the KdpB subunit from *E. coli* but no immunoprecipitation of KdpA and -C homologues from *B. acidocaldarius* protein was observed. A more detailed examination has subsequently revealed that *B. acidocaldarius* does possess subunits almost identical in size to the *E. coli* KdpA and KdpC subunits (Hafer *et al.*, 1989). The low affinity system had a Km of 1.0 mM and a Vmax of 50-80 μmol/min/g prt for potassium, and transported rubidium with the same Vmax but with a Km of 6 mM. The kinetics and substrate specificity of this system are similar to those of the *E. coli* TrkG and -H systems (Michels and Bakker, 1987).

The intracellular solute content of *L. monocytogenes* grown under osmotic stress was determined by Patchett *et al.* (1992). The cytoplasmic potassium content increased from 0.163 M in medium without added sodium chloride to 0.319 M in medium containing 7.5% NaCl. The cytoplasmic potassium concentration of *Enterococcus faecalis* doubled when grown in medium containing 0.9 M NaCl (Kunin and Rudy, 1993). The potassium content of *Lb. plantarum* increased almost two-fold over that observed in the absence of osmotic stress when grown in medium containing 0.8 M potassium chloride, but not when grown in medium containing sodium chloride (Glaasker *et al.*, 1993). This somewhat unusual observation was further investigated by measuring potassium transport immediately following osmotic upshock; a rapid increase in the potassium content of the cytoplasm was observed when upshocked with potassium chloride but not if upshocked with sodium chloride. Glaasker *et al.*, (1993) concluded that the increase in potassium content observed following upshock with potassium chloride was not due to accumulation as a compatible solute, but was possibly due to non-specific binding of potassium to the cell surface. Another possible explanation, not considered by Glaasker *et al.* (1993), is the phenomenon of 'ion

antagonism' reported almost 50 years ago by MacLeod and Snell (1948). These workers observed that the potassium requirement of the lactic acid bacteria *Lb. arabinosus*, *Lb. casei*, *Ent. faecalis* and *Leuconostoc mesenteroides* was dependent on the concentration of other monovalent cations such as sodium or ammonium. Ammonium ions were more toxic than sodium ions, and the toxic effects of either ion were relieved by increasing the potassium concentration of the medium, indicating some form of competition for uptake of potassium ions by the bacterial cells. Inhibition of potassium transport seems to be the most likely reason for 'ion-antagonism' and the observation that potassium transport by *Ent. hirae* was competitively inhibited by sodium ions (Harold and Baarda, 1967) seems to support this assumption. The failure of Glaasker *et al.* (1993) to observe potassium transport/accumulation in the presence of NaCl may be due to 'ion-antagonism'. If *Lb. plantarum* cells were upshocked with 0.85 M potassium chloride 10 min before addition of glycine betaine, less glycine betaine was accumulated compared to cells which had glycine betaine present at the time of upshock (Glaasker *et al.*, 1993). This suggested that partial restoration of turgor had occurred before the glycine betaine was added. The assays were performed in potassium phosphate buffer, in the absence of any other osmoprotectant; therefore, potassium was the sole means of turgor restoration until glycine betaine was added. It is therefore possible, that the accumulation of potassium by the lactic acid bacteria in response to osmotic stress may be dependent on the solute added to impose the osmotic stress. The report by Kunin and Rudy (1991) of potassium accumulation by *Ent. faecalis* in medium containing added NaCl may reflect the known ability of enterococci to resist higher NaCl concentrations than lactococci.

4.3.3 Potassium efflux systems.

Potassium accumulation is the primary response of many bacteria to osmotic stress; subsequent stages of the response in such bacteria involve the accumulation of other compatible solutes and the requirement for potassium accumulation is therefore

reduced. Similarly osmotic downshock (hypo-osmotic stress) makes it necessary for the cell to release some or all of the compatible solutes accumulated in response to osmotic upshock. The most rapid method is probably the use of stretch activated channels. As the name implies, an increase in turgor pressure causes these channels to open due to stretching of the cell membrane and results in an efflux of small cytoplasmic molecules. Osmotic downshock of *E. coli* cells which had previously accumulated potassium, resulted in an extremely rapid loss of potassium ions (Epstein and Schultz, 1965; Meury *et al.*, 1985; Bakker, 1993; Schleyer *et al.*, 1993). Loss of potassium occurred to the extent that the potassium content of downshocked cells decreased below that observed in steady state conditions at the lower osmolarity (Bakker *et al.*, 1993; Schleyer *et al.*, 1993). The extent of potassium loss was dependent on the magnitude of the downshock. Cells upshocked by addition of 0.2 M NaCl to the basal medium followed by a downshock to 0.1 M sodium chloride lost only 25% of their potassium, compared to cells downshocked from 0.6 M sodium chloride to 0.3 M which lost >80% of their potassium (Schleyer *et al.*, 1993). The rate of efflux was also reported to be dependent on the osmolarity prior to downshock, but independent of the extent of downshock (Meury *et al.*, 1985). Almost immediately following downshock, there was a certain amount of potassium re-accumulation, presumably to stabilise the cellular potassium content at the required level for the new osmolarity (Schleyer *et al.*, 1993). The efflux of solutes was quite selective in that the compatible solutes potassium, glutamate and trehalose were lost by the cells in large quantities; yet only 5% of cellular ATP was lost (Schleyer *et al.*, 1993). In addition to stretch activated channels, *E. coli* has at least two K^+/H^+ antiporters KefB and KefC which are not thought to have a role in turgor regulation (Booth *et al.*, 1993); efflux of potassium following osmotic downshock occurred even in mutants with inactive KefB and -C systems. There is evidence that a third system, referred to as KefA, is the predominant efflux mechanism which contributes to turgor but it is difficult to quantify the contribution of a particular efflux system to turgor regulation as the number of systems is not known for certain (Booth *et al.*, 1993).

4.3.4 Glutamate.

The accumulation of glutamate following osmotic stress has been reported for a wide range of both Gram-negative and -positive bacteria (Measures, 1975). In general the greatest increases in the cytoplasmic glutamate concentration were observed in Gram-negative bacteria. Gram-positive bacteria maintain a higher amino acid pool under non-stressed conditions and while some accumulation of glutamate occurs following osmotic stress the increases are generally lower than in Gram-negatives (Measures, 1975). Glutamate is negatively charged at neutral pH (i.e the situation that prevails in the cell cytoplasm) and therefore, to maintain electroneutrality a counterion must also be accumulated. Accumulation of glutamate by *E. coli* is usually associated with potassium uptake, and usually occurs as a result of synthesis rather than transport (McLaggan *et al.*, 1994). Following osmotic upshock, the immediate increase in the cytoplasmic potassium concentration is accompanied by alkalinisation of the cytoplasm, presumably as a result of proton extrusion. Accumulation of glutamate by *E. coli* co-incides with a reduction in the cytoplasmic pH, indicating that glutamate accumulation allows re-accumulation of protons and restoration of the pH and electroneutrality to their normal levels (Dinnbier *et al.*, 1988). The effect of limiting potassium accumulation on glutamate accumulation and vice versa was examined by McLaggan *et al.* (1994). Glutamate accumulation did not occur in a K^+ free medium, but accumulated when potassium was added 10 minutes after upshock. Prevention of glutamate accumulation by using ammonium-free medium did not prevent potassium uptake; however, the level of potassium uptake was slightly lower. These results appear to indicate that glutamate accumulation is a secondary event following potassium accumulation. *S. typhimurium* mutants which lacked the glutamine synthase-GOGAT system had much lower cytoplasmic glutamate pools than did the wild type,when grown in medium with sub-optimal levels of ammonia. These mutants were osmotically sensitive and addition of glycine betaine or proline did not reverse the growth inhibition (Csonka *et al.*, 1995). Yan *et al.* (1996) showed that growth of glutamine synthase-GOGAT mutant strains

was inhibited even at low osmolarity when ammonium concentrations were low, and further inhibition was observed when 0.5 M sodium chloride was added to the growth medium. Glutamate accumulation following osmotic upshock was much lower than in the wild-type; potassium accumulation occurred in the mutant strains; however, the concentration soon declined to the level prior to upshock. In contrast, the wild type strain maintained a higher cytoplasmic potassium concentration at high osmolarity. These results suggest that the role of glutamate in osmoprotection is to act as a counterion to maintain electroneutrality following potassium uptake.

5. OTHER COMPATIBLE SOLUTES.

In addition to the aforementioned compatible solutes, there are others which because of their relatively recent discovery or because they are only utilised by a narrow range of bacterial species, have been less well studied. A brief summary of the most significant of these is given below.

5.1 CARNITINE.

The importance of carnitine as a compatible solute has only come to be recognised relatively recently. Carnitine, in common with glycine betaine has a trimethyl-amino group, a feature seemingly important in compatible solutes. The first reports of osmoprotection by carnitine was by Kets *et al.* (1994), following NMR spectroscopy of cell extracts from *Lb. plantarum* grown in medium containing added NaCl and by Beumer *et al.* (1994) who reported stimulation of *L. monocytogenes* by carnitine in medium containing NaCl. Addition of D or L carnitine to chemically defined medium containing 0.5 M NaCl produced an approximate doubling of both the specific growth rate and the final cell numbers of *Lb. plantarum* (Kets *et al.*, 1994). Carnitine was also found to protect *Lb. plantarum* and other lactic acid bacteria during drying (Kets *et al.*, 1996). The carnitine analogues acetyl carnitine and propionyl

carnitine also stimulated growth of *Lb. plantarum*, not however, to the same extent as did carnitine (Kets and de Bont, 1997).

The level of stimulation of growth of *L. monocytogenes* by carnitine in medium containing high concentrations of NaCl was less than that observed when glycine betaine was added (Beumer *et al.*,1994). Tombras-Smith (1996) observed that carnitine protected *L. monocytogenes* cells against low temperature, high osmolarity or a combination of both; however, the extent of stimulation was less than that observed for glycine betaine.

Transport of carnitine by *L. monocytogenes* was via a high affinity system (Km of 10μM) and appeared to be ATP dependent (Verheul *et al.*, 1995). Addition of glycine betaine and proline at a 100-fold excess did not inhibit carnitine transport, demonstrating that carnitine uptake was independent of the glycine betaine transport system. The kinetics of carnitine transport were not altered by pregrowth in medium of increased osmolarity (Verheul *et al.*, 1995). Accumulation of betaine by *L. monocytogenes* occurred in preference to carnitine, where both were available at equal concentrations. As with glycine betaine uptake, transport of carnitine was regulated by feedback inhibition and efflux of carnitine occurred rapidly when cells were subjected to an osmotic downshock (Verheul *et al.*, 1997). *Bacillus subtilis* was also stimulated by carnitine in high osmolarity medium; the structurally related compounds crotonobetaine and γ-butyrobetaine had a similar effect to that of carnitine. Transport of carnitine, crotonobetaine and γ-butyrobetaine was via the OpuC transport system (Kappes and Bremer, 1998), which also transports glycine betaine, ectoine and choline (Jebbar *et al.*, 1997). Addition of carnitine to medium containing 2.5% sodium chloride stimulated the growth of the Gram-negative *Yersinia enterocolitica*; but not as well as did glycine betaine (2-fold stimulation with carnitine compared to a 4-fold stimulation with glycine betaine).

5.2 ECTOINE.

Ectoine belongs to a relatively novel family of osmoprotectants, the 1,4,5,6 - tetrahydro-2-methyl-4-pyrimidine carboxylic acids. Osmoprotection by ectoine and hydroxy-ectoine was first reported for halophilic bacteria by Galinski *et al.* (1985) and subsequently by Severin *et al.* (1992). In high osmolarity medium, addition of exogenous ectoine stimulated growth of *E. coli* , the cytoplasmic ectoine concentration increasing in proportion to the osmolarity of the medium (Jebbar *et al.*, 1992). Ectoine was as effective as glycine betaine as an osmoprotectant (Jebbar *et al.*, 1992). Both the ProU and ProP systems were involved in ectoine transport, but with lower affinities than for glycine betaine and ProU was the more significant of the two. Analysis of the cytoplasmic content of *Streptomyces* strains grown under osmotic stress revealed the presence of tetrahydropyrimidine derivatives similar in structure to ectoine. These compounds stimulated the growth of *E. coli* in medium of high osmolarity and also at high temperatures (Malin and Lapidot, 1996). The genes for ectoine synthesis from the Gram-positive halophile *Marinococcus halophilus* were cloned by functional expression in *E. coli*.. The cytoplasmic ectoine content of *E. coli* cells containing the cloned genes increased in proportion to the extracellular osmolarity, demonstrating that osmotic control of these genes occurred in *E. coli.* (Louis and Galinski, 1997). The halophile *Brevibacterium linens* accumulated ectoine when grown in high osmolarity medium. The cytoplasmic concentrations of other compatible solutes such as glycine betaine did not increase to the same extent as did ectoine indicating that ectoine may be the major osmoprotectant in *Brevibacterium linens* (Bernard *et al.*, 1993; Nagata *et al.*, 1996). The plant pathogen *Erwinia chrysanthemi* accumulated ectoine by two transport systems one of which was similar in many respects to the ProP systems of *E. coli/S. typhimurium* (Gousbet *et al.*, 1996). Ectoine also functions as an osmoprotectant in *B. subtilis* and is accumulated by the OpuC uptake system (Jebbar *et al.*, 1997), which also transports glycine betaine, carnitine and choline (Kappes and Bremer, 1998)and appears to have a role as a multipurpose osmoprotectant uptake system. Ectoine was not as effective as glycine betaine in stimulating the growth of *B. subtilis* at high

osmolarity. Another soil bacterium, *Rhizobium meliloti,* possesses a high affinity binding protein dependent transport system for ectoine (Talibart *et al.*, 1994). The evolution of such a system indicates that ectoine is probably available in soil at levels which allow its regular use as a compatible solute.

5.3 TREHALOSE.

The cytoplasmic concentration of trehalose in *E. coli* increased in proportion to the osmolarity of the growth medium, when the growth medium did not contain glycine betaine. Trehalose accumulation was the result of synthesis rather than accumulation as exogenous trehalose was not supplied (Larsen *et al.*, 1987). Mutants unable to synthesise trehalose were osmotically sensitive in glucose mineral salts medium (Giæver *et al.*, 1988). Two genes, *otsA* and *otsB*, are involved in trehalose synthesis in *E. coli*. Expression of these genes was dependent on the osmolarity of the growth medium; increasing the sodium chloride concentration from 0 to 0.3 M produced a 6-fold increase in expression (Giæver *et al.*, 1988). The *otsA* gene encodes trehalose-6-phosphate synthase which catalyses the condensation of glucose-6-phosphate and UDP glucose; *otsB* encodes trehalose-6-phosphate phosphatase which generates free trehalose (Giæver *et al.*, 1988). Trehalose accumulation did not occur when an *otsAB* constitutive strain was grown at low osmolarity, demonstrating that trehalose synthesis was controlled both at the genetic and protein levels (Giæver *et al.*, 1988). Expression of *otsAB* was RpoS dependent; the level of expression was increased 4 to 5-fold from mid exponential phase to stationary phase. Growth phase dependent expression was not observed in *rpoS* mutants (Hengge-Aronis *et al.*, 1991). In addition to abolishing growth phase dependent expression, the inactivation of the *rpoS* gene also prevented osmotic induction of *otsA* and *otsB* (Hengge-Aronis *et al.*, 1991).

6. INTEGRATION OF OSMOTIC STRESS RESPONSES

Maintenance of the cytoplasmic solute concentration at the optimum level so as to maintain turgor pressure requires the integration of the activities of multiple systems

for compatible solute accumulation/efflux. In *E. coli* the primary signal of osmotic stress is thought to be loss of turgor pressure. The activity of the constitutively expressed KdpG and-H systems is <5% of the maximum rate in the absence of osmotic stress and potassium transport occurs almost instantaneously following osmotic upshock (Meury *et al.*, 1985). The transport of potassium is mirrored by accumulation of glutamate so as to maintain electroneutrality (Dinnbier *et al.*, 1988; McLaggan *et al.*, 1994; Yan *et al.*, 1995). Because of the charges on potassium and glutamate, accumulation of these solutes results in an increase in the ionic strength of the cytoplasm. The second stage of adaptation to increased osmotic stress entails replacement of potassium/glutamate with neutral compatible solutes. In the absence of exogenous compatible solutes, synthesis of trehalose occurs, which allows the cell to reduce the cytoplasmic potassium/glutamate concentration (Dinnbier *et al.*, 1988). The accumulation of trehalose was inhibited by addition of chloramphenicol at the time of upshock, indicating that trehalose synthesis was inducible (Giæver *et al.*, 1988). The activity of trehalose-6-phosphate synthase was stimulated by potassium and other monovalent cations (Giæver *et al.*, 1988); therefore, the bacterial cytoplasm immediately following osmotic upshock provides a stimulatory milieu for the enzyme. The high cytoplasmic potassium concentration is thought to enhance the expression of some genes involved in osmotolerance but this means of stimulation is unlikely to be involved in trehalose synthesis, as trehalose is accumulated normally by *kdp/trk* double mutants that accumulate potassium at very low rates (Dinnbier *et al.*, 1988). Trehalose accumulation is usually only observed in minimal medium, from which other osmoprotectants are absent (Larsen *et al.*, 1987). In complex medium the predominant solutes accumulated are glycine betaine and proline, with glycine betaine being preferred. In *E. coli* and *S. typhimurium* the ProU and ProP systems are the means of glycine betaine uptake (Cairney *et al.*, 1985a; 1985b). The ProU system is detected only in cells grown at high osmolarity i.e cells that have a high cytoplasmic potassium concentration. Sutherland *et al.* (1986) proposed that ProU is induced by the potassium content of the cytoplasm. This was also suggested by Ramirez *et al.*, (1989) who

observed that transcription of *proU in vitro* was stimulated by potassium glutamate. An alternative theory is that DNA supercoiling varies with osmolarity, and that expression of genes involved in osmoregulation is stimulated by changes in the level of supercoiling (Higgins *et al.*, 1988; Ní Bhrían *et al.*, 1989). Another process that must be controlled in bacteria in which it is present, is the synthesis of glycine betaine. Expression of the *bet* genes was reduced by the presence of glycine betaine or proline in the growth medium (Eshoo, 1988). Whether this was due to repression by glycine betaine, or increased turgor as a consequence of glycine betaine uptake is not clear. Choline acted as an inducer of glycine betaine synthesis, and this effect was repressed by glycine betaine (Eshoo, 1988).

The control of the response to increased osmolarity, is relatively complex. In addition to the possible role of potassium ions on transcription, there is also the effect of the DNA binding proteins such as HU or H-NS on proU transcription (Dattananda *et al.*, 1991; Overdier and Csonka 1992; Owen-Hughes *et al.*, 1992; Manna and Gowrishankar 1994) particularly as the potassium concentration influences interaction between the *proU* negative regulator and H-NS *in vitro* (Ueguchi and Mizuno, 1993). In addition to control at the genetic level, the uptake systems for most osmoprotectants are subject to control at the enzyme level as well. The ProU and ProP systems of *E. coli* and *S. typhimurium* require high osmolarity for maximal activity (Cairney *et al.*, 1985a; 1985b). Membrane tension which is directly affected by turgor pressure is a likely effector in regulation at this level. In contrast, efflux of solutes can occur very rapidly by stretch activated channels. This is important, as without a rapid solute efflux system, Gram-negative cells in particular would be liable to burst following osmotic downshock. In the Gram-positive bacteria, there has been little or no study at the genetic level but control at the enzyme level has received some attention. In *L. monocytogenes*, accumulation of glycine betaine and carnitine appears to be regulated by feedback inhibition (Verheul *et al.*, 1997). A similar observation has been reported for *Staph. aureus* by Pourkomailan and Booth (1994) and Stimeling *et al.* (1994). Glycine betaine accumulation by *Lb. plantarum* was finely controlled by a combination

of influx and efflux systems (Glaasker *et al.*, 1996b). Another important aspect in the regulation of compatible solute uptake is the fact that while a fall in turgor pressure is the obvious trigger for the induction of these systems, this signal will be diminished following restoration of turgor by the primary response. Cells growing at high osmolarity must continue to express the genes encoding compatible solute uptake systems even after turgor has been restored (Csonka and Hanson, 1991).

7 PROTEIN SYNTHESIS DURING OSMOTIC STRESS.

It has been reported that synthesis of some proteins in bacteria is regulated by osmotic stress. It is obvious that the induction of any genes required for compatible solute synthesis or uptake will be accompanied by synthesis of the corresponding protein. There is also evidence of some overlap between the proteins synthesised during different environmental stresses.

Proteins induced by osmotic stress in *E. coli* were analysed using two dimensional polyacrylamide gel electrophoresis. Three proteins were identified which were absent from cells grown in low osmolarity medium, but which were induced following osmotic upshock and remained present during growth at high osmolarity (Clark and Parker, 1984). Another, more comprehensive study of osmotically induced proteins in *E. coli* revealed that at least 41 proteins were induced by osmotic stress. In cultures grown in the absence of NaCl these proteins comprised 0.26% of the total cell protein whereas in cultures grown in 0.6 M NaCl they accounted for 23% of total cell protein (Botsford, 1990). A detailed analysis of osmotically induced proteins has been performed in *B. subtilis*. Twenty three proteins were induced by high osmolarity, three of which were exclusively induced by osmotic stress; the remaining 20 proteins were also inducible by heat-shock or other environmental stresses (Hecker *et al.*, 1988). From this study it is clear that osmotic stress is a good inducer of general stress proteins as well as those specifically associated with osmotic stress. A similar study of proteins induced in *Ent. faecalis* by osmotic stress identified 20 proteins induced more than 10-fold following a 2 hour incubation in 6.5% w/v NaCl (Flahaut *et al.*, 1996). Osmotic

upshock with 52% sucrose produced an identical pattern of protein synthesis, demonstrating that the induction was due to increased osmolarity rather than a specific effect of NaCl. Osmotic upshock (2.5% w/v sodium chloride) of *Lc. lactis* subsp. *cremoris* MG1363 resulted in the induction of at least 12 proteins, among which were the well known heat shock proteins DnaK, GroEL and GroES; no proteins specific to salt stress were detected and all the heat-shock proteins were induced to lower levels by NaCl than by heat (Kilstrup *et al.*, 1997).

The disadvantage of 2-D gel electrophoresis is that apart from demonstrating the presence of proteins and the rate of induction, little other information on the physiological function is provided. Analysis of mutants with impaired growth in high osmolarity medium is probably a better approach. It is obvious that mutants in compatible solute uptake systems are likely to display osmo-sensitive phenotypes; however, other mutations may be in the regulatory mechanisms of the osmotolerance response and the activity of numerous systems may be affected. A membrane protein which appears to be essential for the growth of *Lc. lactis* subsp. *cremoris* MG1363 in medium of elevated osmolarity has been identified. The deduced amino acid sequence of this protein was homologous to the essential *E. coli* protein Ftsh (Nilsson *et al.*, 1994). *Lc. lactis ftsH* mutants grew in medium without added sodium chloride but were incapable of growth on 4% w/v sodium chloride. In addition to being osmotically sensitive, *ftsH* mutants were incapable of growth at 38°C or 16°C. A gene homologous to the *E. coli* Ftsh has also been identified in *B. subtilis*. Inactivation of this gene by antisense RNA resulted in impaired growth at high osmolarity; however, stimulation by the osmoprotectants glycine betaine and proline was still observed, (Deuerling *et al.*, 1995). The *B. subtilis ftsH* gene was transiently induced by osmotic or heat stress, implying that it is a general stress gene rather than being induced specifically by one stress. It was also classified as being a class three heat shock gene, *ie* one that is not regulated by the upstream heat shock regulatory element CIRCE (controlling inverted repeat of chaperone expression) or by the alternative sigma factor σ^B. The regulator of class III heat shock genes is not known (Deuerling *et al.*, 1995). The phenotypic effects

of *ftsH* inactivation in *B. subtilis* are pleomorphic; *ftsH* mutants were incapable of growth in minimal medium, were twice as long as wild type cells, did not sporulate, had altered levels of penicillin-binding proteins and were unable to recover from osmotic upshock (Lysenko *et al.*, 1997). The expression of *ftsH* was not affected by SpoA and was induced by osmotic stress. Cultures subjected to osmotic upshock (7% w/v NaCl) entered a transient lag phase, after which growth resumed. Expression of *ftsH* increased immediately following upshock and reached a peak value at approximately the same time that growth resumed, after which expression decreased to the normal level (Lysenko *et al.*, 1997). This suggests that *ftsH* was required for recovery from osmotic upshock, but not for growth at the higher osmolarity. The FtsH protein belongs to the AAA-protein family of ATPases. Members of this family are found both in prokaryotes and eukaryotes; they are involved in a range of activities, ranging from protein assembly to cell cycle control . The *E. coli* FtsH protein was the first AAA-protein identified in bacteria (Tomoyasu *et al.*, 1993).

8. SURVIVAL OF LETHAL OSMOTIC STRESS.

8.1 INDUCED STRESS TOLERANCE IN BACTERIA.

The function of many proteins synthesised in response to adverse environmental conditions extends to protecting the cell against lethal intensities of environmental stress(es) rather than merely facilitating growth at more moderate levels of a particular stress. In recent years, the phenomenon of induced stress resistance has been investigated in many bacteria. The best examples of induced stress tolerance are the heat shock (HSR) and the acid tolerance (ATR) responses. The former response has been observed in all organisms examined to date. Pre incubation of bacteria at a temperature in excess of the optimal growth temperature results in greater resistance to a subsequent, lethal, temperature challenge (Mackey and Derrick, 1986). The first report of an ATR was for *E. coli* by Goodson and Rowbury (1989); since then the presence of an ATR has been reported in a number of bacteria, including *Lc. lactis* (O' Sullivan and

Condon, 1997), *L. monocytogenes* (O' Driscoll *et al.*, 1996) and *S. typhimurium* (Foster and Hall, 1990). Inhibition of *de novo* protein synthesis during the adaptation step prevents induction of the tolerance response.

Pre-exposure of *E. coli* to 0.35 M NaCl produced increased resistance to subsequent challenge with 2.5 M NaCl (Jenkins *et al.*, 1990). A similar observation was made for *B. subtilis* (Völker *et al.*, 1992) and a slight increase in the osmotolerance of *Ent. faecalis* was observed following pre-incubation at 6.5% w/v NaCl, although the equivalent concentration of sucrose resulted in increased sensitivity (Flahaut *et al.*, 1996). Comparison of the data obtained on stress-induced protein synthesis outlined above, shows that there is a certain level of overlap between the proteins induced by different stresses. This suggests that pre-exposure to a sublethal intensity of a specific environmental stress might also protect against other stresses. This has proved to be the case and tolerance to multiple environmental stresses was induced in a number of bacteria. Tolerance to osmotic stress has been induced in *E. coli* by nutrient starvation (Jenkins *et al.*, 1990) and exposure to sublethal pH (Leyer and Johnson, 1993). In addition, *E. coli* mutants defective in the DnaK protein which is induced by heat shock, were unable to accumulate potassium in response to osmotic upshock (Meury and Kohiyama, 1991). Overlap between stress responses has been quite well studied in the lactic acid bacteria. Carbohydrate starved *Lc. lactis* cultures showed increased resistance to challenge with 3.5 M NaCl, and also to challenge with heat, ethanol, low pH and oxidative stresses (Hartke *et al.*, 1994). Induction of the acid tolerance response in *Lc. lactis* also induced resistance to 20% w/v NaCl, along with tolerance to heat (42°C), ethanol and hydrogen peroxide (O' Sullivan and Condon, 1997). Osmotic stress was also a potent inducer of tolerance to other stresses; pre-exposure to 6.5% w/v NaCl enhanced the resistance of *Ent. faecalis* to heat (62°C), ethanol, bile salts, hydrogen peroxide and sodium dodecyl-sulphate. Interestingly though, only a relatively minor increase in tolerance to 28.5% NaCl (20% survival following 24h exposure for the control and 45% survival for the adapted cells) was observed. (Flahaut *et al.*, 1996). In *Lc. lactis*, pre-exposure to 2.5% w/v NaCl induced the heat shock proteins DnaK,

GroEL and GroES (Kilstrup *et al.*, 1997), In *Listeria monocytogenes* thermotolerance was enhanced by pre-exposure to osmotic stress (Jørgenson *et al.*, 1995). Induction of DnaK by osmotic shock in *Lc. lactis* was notable because of the role this protein is thought to have in osmotically induced potassium transport in *E. coli* (Meury and Kohiyama, 1991). There appears to be a difference in the inducing efficiency of different environmental stresses; for example, acid adaptation conferred resistance to a number of other stresses on *Lc. lactis*, but pre-exposure to these stresses did not necessarily result in acid tolerance (O' Sullivan and Condon, 1997). It is also interesting that pre-exposure to osmotic stress only induced a relatively minor degree of osmotolerance in *Ent. faecalis*, but induced much more significant protection against other stresses (Flahaut *et al.*, 1996). Up until recently, little was known about the mechanisms by which stress responses were induced in bacteria; however, the pH of the cytoplasm (pHi) is thought to be important in the induction of the ATR in *Lc. lactis* (O' Sullivan and Condon, 1997). In the light of this report it may well be worth examining the induction of stress responses for a correlation between the effect of the inducing stress on pHi and the level of induction observed.

8.2 GROWTH PHASE DEPENDENT RESPONSES OF BACTERIA.

In recent years it has become apparent that many bacterial genes are regulated in a growth phase dependent manner. Survival of bacteria subjected to lethal stress challenges appears to be one example of this. The tolerance of bacteria to environmental stresses is usually dependent on the phase of growth from which the cells were taken. The tolerance of *Lc, lactis* to acid challenge undergoes quite considerable variation during growth from inoculation to stationary phase, and this will be discussed in more detail later. Growth phase dependent tolerance to environmental stress has been reported for other bacteria also. The acid tolerance of *L. monocytogenes* varied during growth, with mid-exponential phase cells being most sensitive (O' Driscoll *et al.*, 1996; Davis *et al.*, 1996). The thermotolerance of *L. monocytogenes* was reported to follow a similar pattern during growth (Lou and Yousef, 1996). Growth phase dependent acid

tolerance has also reported for *E. coli* O157:H7 (Benjamin and Datta, 1995; Arnold and Kaspar, 1995). Stationary phase or starved *E. coli* cells were more resistant to osmotic, heat or oxidative stress than were exponentially growing cells (Jenkins *et al.*, 1988; 1990).

8.2.1 Growth phase dependent stress tolerance in *Lc. lactis.*

Growth phase dependent variation in the tolerance of *Lc. lactis* to various environmental stresses was reported by Hartke *et al.* (1994). This response has been studied in more detail for acid tolerance in *Lc. lactis* by O' Sullivan (1996), the sensitivity of *Lc. lactis* to acetic acid varied quite dramatically during growth from inoculation to stationary phase. Immediately following inoculation the cells were quite resistant to acid challenge, this resistance was gradually lost during growth until a period of maximal sensitivity was reached in early to mid exponential phase of growth. Following this period of sensitivity, the resistance of the cells gradually increased until stationary phase was reached, at this point almost all the cells survived challenge at pH 4.0 for two hours. These cultures were grown at constant pH 7.0 so as to eliminate induction by the decrease in pH of the growth medium that would result from lactic acid production during growth. A decrease in cytoplasmic pH has been reported to induce acid tolerance in *Lc. lactis* (O' Sullivan and Condon, 1997) but measurement of the cytoplasmic pH of the cultures grown at constant pH 7.0 revealed no variation in the cytoplasmic pH that would have induced an acid tolerance response (O' Sullivan, 1996; Alemayehu, 1997). Inoculation of cells from the sensitive point in the growth curve into medium harvested from late exponential phase cultures led to rapid acquisition of acid tolerance. Inoculation of sensitive cells into medium supplemented with yeast extract or tryptone delayed acquisition of acid tolerance (Alemayehu, 1997). These results suggest that growth phase dependent acid tolerance observed in *Lc. lactis* resulted from some form of starvation stress response.

8.2.2 Growth phase regulation of gene expression in *E. coli*: the effect of *rpoS*.

Expression of many genes in *E. coli* is under the control of the stationary-phase sigma factor σ^S encoded by the *rpoS* gene (Lange and Hengge-Aronis, 1992). The cellular content of σ^S varies in a growth-phase dependent manner; levels are low during exponential phase, but increase substantially on entry to the stationary phase (Yamashino *et al.*, 1995). σ^S is essential for the stationary phase stress tolerance of *E. coli*, *Yersinia enterocolitica*, and *Shigella flexneri* (Cheville *et al.*, 1996; Badger and Miller, 1995; Small *et al.*, 1994). The growth phase control of σ^S levels was dependent on the DNA-binding nucleoprotein H-NS. Mutants which do not produce H-NS have constitutively high levels of σ^S. The effect of H-NS on σ^S levels was post-transcriptional, with both the rate of translation and protein stability increasing in *hns* mutants (Lange and Hengge-Aronis, 1994; Yamashino et al., 1995)

There has also been a report of positive regulation of *rpoS*; guanosine 3',5'-bispyrophosphate (ppGpp) levels increase in *E. coli* following starvation for amino acids, nitrogen source or an energy source, the so-called stringent response. Because of the accumulation of ppGpp under conditions known to induce σ^S production, the effect of ppGpp on expression of *rpoS* was examined (Gentry *et al.*, 1993). Mutants deficient in ppGpp produced little σ^S in response to starvation for glucose, phosphate or amino acids. Mutants in which ppGpp was over-produced had higher levels of σ^S than the wild-type culture and gratuitous induction of ppGpp led to greatly increased σ^S levels (Gentry *et al.*, 1993). Gentry *et al.* (1993) measured σ^S levels using western blots but transcription of *rpoS* was not measured. It was therefore not clear where ppGpp acted to increase σ^S levels, *ie.* did regulation occur at the transcriptional, translational or post-translational levels. More recent work has indicated that the initiation of *rpoS* transcription was not affected in ppGpp deficient mutants, but that uncoupling of transcription and translation occurred, leading to a decreased level of transcript (Lange *et al.*, 1995).

transcription and translation occurred, leading to a decreased level of transcript (Lange *et al.*, 1995).

Other factors thought to play a role in regulation of σ^S levels are UDP-glucose, which negatively regulated σ^S levels. Mutants deficient in UDP-glucose had increased σ^S levels and increased *rpoS* transcription (Bohringer *et al.*,1995). Cyclic-AMP also appeared to function as a negative regulator of *rpoS*; mutants with deletions in the *cya* and *crp* loci were unable to produce cyclic-AMP and displayed increased transcription of *rpoS* (Lange and Hengge-Aronis, 1994). It has been proposed that negative regulation by cAMP acts to prevent induction of *rpoS* during glucose starvation and allows the cell to switch to another energy source following exhaustion of available glucose (Lange and Hengge-Aronis, 1994).

The increase in σ^S levels observed in late exponential phase/early stationary phase appeared to be due in part to the cell density of the culture. The induction of translational *rpoS::lacZ* fusions was observed when the growth rate of the culture had not yet decreased. Induction of these translational fusions was not observed when grown in medium containing a lower glucose concentration, in which the cells entered stationary phase at a lower cell density (Lange and Hengge-Aronis, 1994). Cell density mediated induction of bacterial genes is normally due to the production of signal molecules by the cells. The basic mechanism entails low level production of the signal molecule by the cells during growth. When cell density increases, so does the concentration of the inducer in the medium. Increasing inducer concentration results in autoinduction of its synthesis, leading to a rapid rise in the level of the signal molecule, which above a threshold value activates a particular cellular target.

The expression of *rpoS* was increased by a dialysable heat-stable factor present in medium harvested from stationary phase cultures (Mulvey *et al.*, 1990). Mutants in the threonine biosynthesis pathway of *E. coli* which could not produce homoserine and homoserine phosphate produced little or no σ^S unless homoserine lactone was added (Huisman and Kolter, 1994) Homoserine lactones are well known as autoinducers of gene expression. For example, expression of virulence genes and secondary metabolite

production by *Pseudomonas aeruginosa* are also regulated by homoserine lactones (Winson *et al.*, 1995) These reports appear to suggest that there may be a cell density dependent signal for σ^S induction.

8.3 COMPOUNDS WHICH PROTECT AGAINST LETHAL STRESS.

The effect of compatible solutes on the growth of bacteria in an environment with elevated osmolarity has already been discussed. In this section the effect of the extracellular milieu on the survival of bacteria subjected to lethal levels of osmotic stress is discussed. This aspect of the survival of bacteria has not been well studied and there is little published information available. The availability of magnesium seems to be an important factor in the survival of *Lc. lactis*; during starvation in phosphate buffer survival was increased if magnesium was present in the buffer. The presence of arginine further increased survival during starvation (Thomas and Batt, 1968). The thermotolerance of *S. typhimurium* was also greatly increased by the presence of magnesium during the challenge. The stability of ribosmes was increased in the cells to which magnesium had been added. A considerable reduction in 16S rRNA was observed in cells heat-shocked in the absence of magnesium which demonstrated a link between magnesium induced ribosomal stability and increased survival of a lethal challenge (Tolker-Nielsen and Molin, 1996).

Magnesium is essential for the activity of the heat-shock protein DnaK; this protein belongs to the hsp70 family of heat-shock proteins which prevent aggregation of heat-damaged proteins. DnaK has ATPase activity which is dependent on the free magnesium content of the assay buffer (Skowyra and Wickner, 1995). The importance of this protein in the heat-shock response suggests another target on which magnesium could act to protect cells during stress challenge. The thermal stability of the *E. coli* ribonuclease H1 was also markedly increased by the presence of magnesium and a similar degree of stability was provided by calcium and manganese (Kanaya *et al.*, 1996). Manganese could only partially replace magnesium as a stimulator of RNAase activity (Berkower *et al.*, 1973). RNase proteins may also stabilise other proteins; denaturation of bovine serum albumin during heat treatment was reduced in the presence of heat-stable RNase (Minton *et al.*, 1982).

8.4 THE EFFECT OF COMPATIBLE SOLUTES ON SURVIVAL AT HIGH OSMOLARITY.

Survival during osmotic stress/drying was reported to be increased when *Lb. plantarum* cells were dried in the presence of the osmoprotectant glycine betaine (Kets *et al.*, 1994; 1996). A related effect was observed for the survival of *E. coli* during incubation in seawater. Pre-incubation in high osmolarity medium, which caused the cells to accumulate potassium and glutamate, decreased the extent of the loss of viability observed when the cells were incubated in seawater. The presence of glycine betaine in the pre-incubation medium provided protection even if the pre-incubation was at low osmolarity (Munro *et al.*, 1989). Mutations in specific compatible solute transporters (*proU, proP, trk and kdp*) reduced or eliminated the protection resulting from preincubation in high osmolarity medium (Munro *et al.*, 1989) Osmotic downshock prior to inoculation into seawater (which caused loss of pre-accumulated compatible solutes), abolished the protective effect (Gauthier *et al.*, 1991). These reports suggest that it was the accumulation of the compatible solutes that was osmoprotective rather than synthesis of other protective mechanisms. The presence of glycine betaine during the challenge did not protect the cells, providing further evidence that it was accumulation of the compatible solutes during the preincubation that provided protection (Munro *et al.*, 1989).

9. THE GENUS *LACTOCOCCUS*.

The lactococci are Gram-positive catalase negative coccoid bacteria which produce L (+) lactic acid during the fermentation of carbohydrates. The first description of a pure bacterial culture was of an organism named *Bacterium lactis* by Lister in 1873 which was later to be named *Streptococcus lactis*. An investigation of spontaneous fermentation of sour cream, sour milk and cheese by workers in Denmark (Storch) and Germany (Weigmann) led to the isolation of the lactic streptococci responsible for these fermentations. In 1909 the genus was renamed as *Streptococcus lactis* and by 1919 the

mesophilic lactic streptococci had been subdivided into two species namely *S. lactis* and *S. cremoris* (Orla-Jensen). Serological classification by Lancefield in 1933 assigned the lactic streptococci to group N which was serologically distinct from groups A, B and C which contained the pathogenic streptococci (for review see Teuber, 1995).

In 1981 the *cremoris* and *lactis* species were reclassified as subspecies of *S. lactis* (Garvie, 1981). The classification of the genus *Streptococcus* was altered greatly in the mid 1980's when a new genus, *Lactococcus* was created which contained what had previously been referred to as the mesophilic dairy streptococci. The faecal streptococci were placed in the new genus *Enterococcus* leaving only the pathogenic streptococci in the genus *Streptococcus* (Schleifer *et al.*, 1985; Schleifer and Kilpper-Balz, 1987).

9.1 *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* AND *L. LACTIS* SUBSP. *CREMORIS.*

The traditional means of differentiating *Lc. lactis* subsp. *lactis* from *Lc. lactis* subsp. *cremoris* is by means of biochemical tests, many of which are based on the ability to grow during various environmental stresses. One differentiating characteristic was that the *lactis* subspecies strains were capable of growth at 4% w/v sodium chloride while the *cremoris* subspecies had an upper limit of 2% w/v. Growth at 40 °C was another characteristic used to distinguish between the *lactis* and *cremoris* subspecies as most *lactis* strains grew at this temperature while *cremoris* strains did not. In recent years many of the strains classified as belonging to either the *lactis* or *cremoris* subspecies based on phenotypic characteristics have been reclassified on the basis of nucleic acid hybridisation and 16s rRNA sequences. Comparison of 16s rRNA sequences indicated that one *lactis* strain examined was more homologous to the *cremoris* strains examined than to other *lactis* strains (Salama *et al.*, 1991). DNA hybridisation experiments using cloned chromosomal genes as probes, led to the conclusion that certain members of the *lactis* subspecies were misclassified and belonged in the *cremoris* subspecies and *vice versa* (Godon *et al.*, 1992). As a result,

the subspecies *cremoris* now contains some strains which are phenotypically similar to the traditional *lactis* subspecies. Another report on the chromosomal organisation of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* revealed the presence of a large chromosomal inversion in the *cremoris* subspecies, compared to the *lactis* subspecies, (Le Bourgeois *et al.*, 1995). It has been reported that the chromosome of *Lc. lactis* subsp. *cremoris* has a mosaic structure and contains regions in which the G+C content and codon usage are atypical of the genus *Lactococcus* (Delorme *et al.*, 1994). It is not clear how the evolutionary divergence between the *cremoris* and *lactis* subspecies came about; however it is almost impossible to isolate *cremoris* strains from natural sources other than dairy environments while the *lactis* subspecies has been isolated readily from plant material (Sandine *et al.*, 1972). This suggests that the *cremoris* subspecies may have arisen as a result of the use of naturally occurring lactococci in dairy fermentations. Another reason why *cremoris* strains are difficult to isolate may be that they are present in low numbers and the absence of a selective medium for *cremoris* strains renders it impossible to distinguish them from the more abundant *lactis* strains. As mentioned earlier, *cremoris* strains may be distinguished from *lactis* strains by the use of 16s rRNA probes (Salama *et al.*, 1991). This approach has been used to identify new *cremoris* strains by colony blotting of *Lactococcus* isolates. It was notable that all the new *cremoris* strains isolated by this technique originated from raw milk samples from Morocco and China, while raw milk originating in the U.S.A did not yield any *cremoris* isolates (Salama *et al.*, 1993).

9.2 *LACTOCOCCUS LACTIS* AND ENVIRONMENTAL STRESS.

The response of *L. lactis* to environmental stress has been quite well studied in recent years. An inducible heatshock response was triggered by incubation at 42 °C which conferred protection against a subsequent challenge at 52 °C. The synthesis of approximately 13 proteins was enhanced following incubation at 42 °C; among these proteins were homologs of the GroEL and DnaK, chaperone proteins of *E. coli*. (Whitaker and Batt, 1991). The GroEL chaperon requires the co-chaperonin GroES for

folding of newly synthesised polypeptides (Zeilstra-Ryalls and Georgeopoulos, 1991) and the genes for GroES and GroEL are arranged in an operon. Unlike the organisation in Gram-negative bacteria the heat-shock genes in Gram-positive bacteria are not under the control of an alternative sigma-factor, but appear to be negatively regulated at an inverted repeat sequence (IR) upstream of the genes (Hecker *et al.*, 1996). In *B. subtilis* this IR has been named CIRCE (controlling inverted repeat of chaperone expression). The *groESL* operon of *Lc. lactis* has been cloned and was found to be preceded by a CIRCE element and the expression of *groESL* was derepressed by heat shock (Kim and Batt, 1993). Synthesis of the GroES and GroEL proteins was induced 12 and 5 fold respectively by heat shock at 42 or 50 °C. In addition to heat shock, GroES and GroEL were induced to a lesser extent by a pH shift from 7.0 to 5.5 and by UV irradiation (Hartke *et al.*, 1997). GroES, GroEL and DnaK were induced by incubation of *Lc. lactis* subsp. *cremoris* MG1363 in 2.5% w/v NaCl. It was also noted that all NaCl induced proteins were inducible by heat shock indicating that there is a substantial overlap between heat shock and salt stress responses in *Lc. lactis* (Kilstrup *et al.*, 1997).

Acid stress resulting from lactic acid production by the cells themselves is probably the most common stress encountered by the lactococci during milk fermentation. *Lc. lactis* possesses an inducible acid tolerance response (ATR) that was induced by exposure to sublethal pH values and was dependent on *de novo* protein synthesis (O'Sullivan, 1996; Rallu *et al.*, 1996). Another report claimed that the induced ATR in *Lc. lactis* did not require protein synthesis as the induction by acid was not prevented by chloramphenicol (Hartke *et al.*, 1996) This may be discounted as the chloramphenicol addition was at the time of the pH downshift and it has been demonstrated that for total inhibition of the ATR, chloramphenicol must be added 30 minutes prior to the pH shift (O'Sullivan and Condon, 1997). Induction of the ATR in *Lc. lactis* also conferred tolerance to other environmental stresses, heat, NaCl, H_2O_2 and ethanol (O'Sullivan and Condon, 1997). Cross protection induced by carbohydrate starvation (Hartke *et al.*, 1994) and UV irradiation (Hartke *et al.*, 1995) has also been

reported in *Lc. lactis*. Little is known about the mechanism by which stress tolerance responses are induced in *Lc. lactis*, or how the cells sense environmental stress. The cytoplasmic pH appeared to be an important factor in the induction of the ATR in *Lc. lactis* with concomitant induction of tolerance to other environmental stresses (O'Sullivan and Condon, 1997), indicating that at least in the case of acid stress, internal pH is an important signal in induction of the response. It is not known if cross-protection results from one signal (e.g internal pH) inducing multiple responses, or multiple signals, each specific to a particular stress and capable of inducing more than one stress response.

9.3 OSMOTIC TOLERANCE IN *LACTOCOCCUS LACTIS*.

Little work has been done on the osmotolerance of *L lactis*. As discussed already, proline and glycine betaine are accumulated in response to osmotic stress (Molenaar *et al.*, 1993) but apart from this, nothing is known of the role of other potential compatible solutes such as potassium. Another feature of interest in the case of *Lc. lactis* is the fact that some members of the species are unable to grow at NaCl concentrations in excess of 2% w/v. These strains comprise the 'traditional' *cremoris* subspecies some of which are now reclassified as *Lc. lactis* subsp *lactis* (Godon *et al.*, 1992). Other strains are capable of growth on 4% w/v NaCl and these include some *Lc. lactis* subsp. *cremoris* strains reclassified from the 'traditional' phenotypically classified *lactis* subspecies. Each subspecies of *Lc. lactis* therefore, contains strains that differ quite considerably in their tolerance to NaCl. It is not unreasonable to assume that the differences in salt tolerance are related to the osmotolerance mechanisms of these strains. Investigation of the differences in salt tolerance between different strains would be of value given the economic importance of these strains in dairy manufacture.

During the manufacture of cheese, salting of the curd is a part of the manufacturing process. The method of salt addition differs, depending on the cheese type and is an important determinant of the rate of lactose fermentation (Cogan, 1995). The salt in moisture (s/m) ratio is the important parameter and obviously depends on

both the amount of salt added and the moisture content of the curd. The lactose content of cheddar cheese during ripening is strongly affected by the s/m ratio. A s/m content of 4.1% w/v allows rapid metabolism of lactose and practically all available lactose is exhausted within the first 10 days of ripening. Increased s/m content results in greatly reduced lactose metabolism and lactose levels remain high for over 20 days (Turner and Thomas, 1980). The s/m ratio is important in determining the quality of cheddar cheese, as a low (< 4.5%) s/m value results in high starter numbers following salting, which can favour the development of bitter flavour. In general, a s/m value of 4.5-5.5% will ensure good cheddar quality cheese, provided that other parameters affecting quality are also in the acceptable range.

9.4 INSERTIONAL MUTAGENESIS IN *LACTOCOCCUS LACTIS*.

The isolation of mutants is important both in the laboratory study of bacterial metabolism and in the manipulation of strains for industrial purposes. Transposon mutagenesis is a particularly useful means of generating mutants and transposable genetic elements are present in the lactococci. The development of a reliable transposition system for generating chromosomal mutations in *Lc. lactis* has been reported relatively recently. Tn*919* which originated in *Streptococcus sanguis* was capable of random insertion in the chromosome of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* (Hill *et al.*, 1987); insertion of Tn*919* was not random in all strains however, which limited its use to those strains in which insertion was random. A high frequency conjugation system was also necessary for efficient transposition. Tn*919* was however used to isolate mutants incapable of citrate and maltose metabolism (Hill *et al.*, 1991).

The insertion sequence IS*946* was used to construct a suicide integration vector (pTRK145) capable of insertion into the *Lc. lactis* chromosome; insertion appeared to be random and the system was used to isolate mutants deficient in maltose utilisation (Romero and Klaenhammer, 1992; Dinsmore *et al.*, 1993). A similar system was developed using the insertion sequence IS*S1* and the temperature sensitive replicon

pG$^+$host containing an antibiotic resistance gene. The advantage of this system is that the vector replicates at 30 but not at 37 °C, and by manipulating the incubation temperature of the culture it is possible to eliminate unintegrated plasmid from the host cell. This allows selection of cells containing insertions by means of the antibiotic resistance marker on the integrated vector. Excision of the vector is possible which leaves a copy of IS*S1* at the integration site, thus generating a food grade insertional mutant (Maguin *et al.*, 1996). This system has been used to generate mutants defective in DNA repair (Maguin *et al.*, 1996) and also to isolate acid resistant mutants (Rallu *et al.*, 1996). The frequency of transposition observed was high and transposition was observed in *Ent. faecalis* and *Strep. thermophilus* as well as in *Lc. lactis*. This transposition system therefore appears to hold great promise as a means of generating chromosomal mutants of *Lc. lactis*.

10.CONCLUDING REMARKS.

A comprehensive understanding of the means by which bacteria adapt to changing environmental conditions is of great benefit in the use of microorganisms in industry. The growth and/or metabolism of industrially important microorganisms may then be manipulated by adjustment of the conditions under which they are grown. *Lc. lactis* is extremely important in the dairy industry, and in recent years the responses of *Lc. lactis* to various stresses has been investigated in some detail. To our knowlege however, there has only been one publication to date in the area of osmotolerance in *Lactococcus* (Molenaar et al., 1993). There are several aspects of the response of *Lc. lactis* to osmotic stress which warrant investigation. The difference in NaCl tolerance between *Lc. lactis* subsp *lactis* and *Lc. lactis* subsp. *cremoris* suggests that there may well be a difference in the osmotic tolerance of these subspecies. The role of potassium in the adaptation of *Lc. lactis* to osmotic stress also needs to be clarified. The results of MacLeod and Snell (1948) and others, suggest that NaCl may reduce the ability of *Lc. lactis* and other lactic acid bacteria to accumulate potassium. In addition, the factors

which facilitate survival during exposure to a_w values less than those that permit growth are of interest.

Chapter II
Materials and Methods

2.1 Bacterial strains, plasmids and media.

The bacterial strains and plasmids used in this study are listed in Table 2.1. *Lactococcus lactis* strains were routinely grown in the M-17 medium of Terzaghi and Sandine (1975) modified by using glucose (0.5% w/v) as the energy source (GM-17), Two chemically defined media were used. The first was that of Thomas *et al.* (1979) modified by replacement of Na_2HPO_4 and KH_2PO_4 with disodium ß-glycerophosphate (7.2 g/l) and $NH_4H_2PO_4$ (1.0 g/l) (MTEL medium); potassium was added to this medium as KCl to the required concentration. In some experiments MTEL medium was buffered with 0.01M 3-[N-Morpholino]propane-sulfonic acid (MOPS). The second chemically defined medium (CDM) was that of Molenaar *et al.* (1993). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth (Sambrook et al., 1989). The antibiotics erythromycin and chloramphenicol were added where necessary at the concentrations specified in the text. Culture stocks were maintained in Protect™ vials (Technical service consultants Ltd, Lancashire, UK) at -20 °C. Some lactococcal cultures were stocked in 10% reconstituted skim milk at -20 °C. The water activity of medium containing added solutes was determined using a water activity meter (Labcell).

2.2 Growth conditions.

Unless otherwise specified, all *Lactococcal* cultures were grown without aeration at 30°C in plastic universal containers containing 20ml of growth medium. *E. coli* cultures were grown at 37 °C with vigorous shaking to ensure aeration. Growth was measured as OD_{580} in a Beckman DU 640 UV / Vis spectrophotometer. The specific growth rates (μ, h^{-1}) of cultures were calculated from semi-logarithmic plots of OD_{580} against time using the formula : $\mu = \ln X_2 - \ln X_1 / t_2 - t_1$ where X_2 and X_1 were OD_{580} values of the cultures at times t_2 and t_1 respectively. Cultures at constant pH were grown in a 1.5 l fermenter (Braun Biolabs) under a nitrogen head. The pH was continuously adjusted with 1N NaOH.

Table 2.1 Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source and/or reference
Strain		
L. lactis subsp. *lactis*		
C10 (NCDO 509)		UCC*
BA1		UCC
BA2		UCC
L. lactis subsp. *cremoris*		
NCDO 712		UCC (Davies *et al.*, 1981))
MG1363	Plasmid free derivative of NCDO 712	UCC (Gasson, 1983))
HP (NCDO 607)		UCC
US3 (NCDO 1197)		UCC
BK5		UCC
OSM2X	NaCl sensitive mutant of MG1363	This study
Plasmids		
pGhost9:ISS1		Maguin *et al.* (1996)
pCPL 1	2.5kb Sau3A fragment from *Listeria monocytogenes* cloned in BamH1 digested pUC 18	R. Sleator U.C.C
pCPL 3	2.5 kb *Xma*1 - *Xba*1 fragment from pCPL 1 cloned in pCI 372.	This study
pCI 372	*Escherichia coli* - *Lc. lactis* shuttle vector Em^r	

* UCC culture collection

2.3 Challenge with lethal NaCl concentrations.

Aliquots of the culture to be challenged were diluted 10-fold into medium or buffer as appropriate containing X/0.9% w/v NaCl, where X was the desired sodium chloride concentration. This resulted in the correct sodium chloride concentration following the addition of the cells. Viable cell numbers were determined using the spot-plate technique described by O'Sullivan and Condon (1997). Survival was expressed as a percentage of the initial cell numbers. Death rates were calculated from the equation

$$\text{Death rate} = \ln X_2 - \ln X_1 / t_2 - t_1$$

where X_1 and X_2 are the cell numbers at times t_1 and t_2 respectively.

2.4 Determination of intracellular potassium concentration.

3.0 ml of exponentially growing culture was filtered through a 0.45 μm membrane filter (Millipore) and washed through with 5.0 ml 0.01 M MOPS-Tris pH 7.0 buffer containing NaCl or fructose at identical concentrations to those of the growth medium. The filters were placed in 10 ml of 0.5 M perchloric acid to precipitate cell protein. The K^+ content of the resulting extracts was determined using a PFP 2 flame photometer (Jenway).

2.5 Transport assays.

Overnight cultures were depleted of potassium by loading with either Na^+ or choline (in 0.1M sodium phosphate buffer pH 7.0 or 0.05 M choline citrate adjusted with choline to pH 7.0 respectively) in the presence of 2,4-dinitrophenol (5mM) and 25 mM glucose as described by Bakker and Harold (1980). The Na^+ loaded cells were resuspended in 0.01 M MOPS-Tris buffer (MTB) at pH 7.0; glucose was added to a concentration of 25 mM and after 10 minutes incubation at 30°C KCl was added to start the assay. Samples were taken at intervals for determination of K^+ content as outlined above.

2.6 Glycine betaine transport assays.

Exponential phase cells were harvested, washed in assay buffer (0.05M K_2HPO_4,-0.05M NaH_2PO_4,-2mM $MgSO_4$ at pH 6.0), resuspended to a high cell density in the same buffer and stored on ice. When assays were being started 0.15 ml of cell suspension was added to 1.35 ml of assay buffer at 30°C and incubated for 5 minutes. Glucose was added to a concentration of 25 mM (30µl of a 1.25 M stock) and incubation continued for a further 5 minutes after which ^{14}C labelled glycine betaine was added to start the assay, to a final concentration of 10 µM (except for experiments designed to measure Km). Samples were removed at the required intervals, filtered through a pre-wetted 0.45 µM polyethersulfone filter (Gelman Sciences, Ann Arbor Michigan, USA) and washed with 3 ml of assay buffer. The filters were placed in 3 ml scintillation fluid (Ultima Gold, Packard), and the radioactivity counted in a scintillation counter (LS 6500, Beckman).

2.7 Glycine betaine accumulation measurements.

4 ml of chemically defined medium (Molenaar *et al.*, 1993) containing 1 mM unlabelled glycine betaine was inoculated from an overnight culture. A 2 ml aliquot of culture was removed and added to a tube containing 20µl of a 100mM solution of ^{14}C labelled glycine betaine (22.7 µCi/mM). The cultures were grown to mid exponential phase at which time 1ml aliquots were filtered through 0.45µM filters, washed twice with 3 ml volumes of isotonic medium and the radioactivity per filter determined by scintillation counting. The protein content was determined from the remaining 2ml aliquot of the culture that did not contain radiolabelled betaine.

2.8 Measurement of cell protein and dry weight.

Exponentially growing cells were harvested, washed three times with distilled water and resuspended in distilled water. A series of dilutions was then prepared, each at a different optical density. The cellular protein content of the suspensions was determined using a commercial protein assay (Biorad, Laboratories GmbH, München, Germany)

using bovine serum albumin as a standard. A standard curve relating protein concentration (mg / ml) to O.D.$_{580nm}$ was prepared. The protein content of a culture at an O.D.$_{580nm}$ of 1.0 was 0.16 mg / ml. The dry weight was estimated by drying 10 ml aliquots of the cell suspensions on aluminium foil dishes at 105°C and measuring the mass of the dishes before and after drying. A standard curve relating dry weight to OD$_{580nm}$ was prepared from which the dry weight of a culture with an OD580 of 1.0 was determined to be 0.33 mg/ml.

2.9 Measurement of cytoplasmic volume.

Cytoplasmic volume was determined by measuring the difference in accumulation of the cytoplasmic impermeable marker ^{14}C sorbitol and the permeable marker $^{3}H_2O$, using the method of Patchett *et al.* (1992).

2.10 Measurement of cytoplasmic pH and $\Delta\Psi$.

The cytoplasmic ΔpH and $\Delta\Psi$ were determined simultaneously by measuring accumulation of ^{14}C benzoic acid and ^{3}H tetra-phenyl-phosphonium (TPP^+). Cultures were grown in MTEL medium until the pH approached 5.8 at which point the culture was concentrated to an OD 580 of approximately 2.0 and the pH was maintained at 5.8 by automatic titration with NaOH. An aliquot of culture was removed for determination of the cytoplasmic volume, as described previously. ^{14}C benzoate and ^{3}H TPP^+ were added (approximately 0.2µCi/ml of each) and the cells separated from the medium by centrifugation through 1-bromododecane. The cell pellets were resuspended in 200µl MTEL medium; 100µl aliquots were added to 3ml scintillation cocktail (Ultima Gold, Packard) and the ^{14}C and ^{3}H radioactivity measured in a scintillation counter using full spectrum counting (Beckman LS 6500). 100µl aliquots of the supernatant were similarly counted. The resulting data were corrected for spillover from the ^{14}C channel, for non specific binding of TPP^+ by measuring the TPP+ accumulated in toluenised cells, and for ^{14}C and ^{3}H counts due to medium present in the interstitial space. The ΔpH was calculated as described by O'Sullivan and Condon (1997) and the $\Delta\Psi$

determined using the formula $\Delta\Psi = Z. [TPP^+]_{in}/[TPP^+]_{out}$. [TPP+]in and [TPP+]out are the internal and extracellular TPP^+ concentrations respectively while Z = 2.3RT/F where F= 96.519 J mV^{-1} eq^{-1}, R = 8.314 J mol^{-1} K^{-1} and T = culture temperature (Z = 60 at 30°C/303K).

2.11 Insertional mutagenesis of *L. lactis* and isolation of osmotic stress-sensitive mutants.

Insertional mutagenesis of *L. lactis* subsp. *cremoris* MG 1363 was carried out using the insertion sequence IS*S1* contained in the temperature sensitive vector pVE6156 (pGhost 9) as described by Maguin *et al.* (1996). The procedure of Maguin *et al* was modified by using 35 °C as the non permissive temperature for plasmid replication instead of 37 °C as the plating efficiency of MG 1363 was only ≈ 10% at 37 °C while it was ≈100% at 35 °C. Following an overnight incubation at 30 °C to allow transposition of the vector, the culture was inoculated into fresh medium and grown at 35 °C which inhibited plasmid replication and caused elimination of non integrated plasmid from the cells. Cells containing pGhost 9:IS*S1* insertions were erythromycin resistant and following plating on GM-17 agar with 2 μg erythromycin/ml 1000 resistant colonies were replica plated on GM-17 agar and GM-17 agar containing 3.5% NaCl. Colonies unable to produce growth on 3.5% NaCl were screened by two further replica platings in the presence and absence of 3.5% NaCl and colonies retaining the NaCl sensitive phenotype were taken to be osmotic stress sensitive mutants.

2.12 Isolation of DNA.

Chromosomal DNA from lactococcal cells was prepared using the method of Leenhouts *et al.* (1989) except that mutanolysin was omitted during the lysis step. Plasmid DNA from *Lactococcal* cells was prepared by the method of Anderson and McKay (1983). Plasmid DNA from *E. coli* was prepared either using the alkaline lysis mini-prep procedure described in Sambrook *et al.* (1989) or using a plasmid DNA isolation kit (Qiagen spin-prep, Qiagen GmBH).

2.13 DNA manipulation.

Restriction enzyme digests and ligation reactions were performed as described in Sambrook *et al* (1989), all molecular biology enzymes were obtained from Promega (Madison WI, U.S.A). DNA extraction from agarose gels was performed using the GeneClean™ kit (Bio 101 CA. U.S.A). DNA fragments were labelled with ^{32}P using the Prime-A-Gene kit (Promega) and the ^{32}P-ATP was obtained from Amersham Ltd (United Kingdom). Inverse PCR reactions were performed using a long template PCR kit (Boehringer Mannheim GmbH, Germany).

2.14 Statistical treatment of results.

All data presented are the means ± standard deviations of triplicate experiments. Where error bars are not shown, the extent of the errors is smaller than the data point. In some cases the data presented was representative of triplicate experiments rather than being the mean and this is indicated in the legends where appropriate.

Chapter III

Factors influencing the survival of *Lactococcus lactis* cells exposed to high NaCl concentrations.

Abstract.

The survival of *Lc. lactis* subsp. *cremoris* NCDO 712 during challenge with 20% w/v NaCl was strongly influenced by the growth phase of the culture, with mid-exponential cells being extremely sensitive to the challenge. NaCl concentrations as low as 5% w/v were lethal to exponential phase cells. It was possible to induce tolerance to the NaCl challenge by pre-exposure to 3% w/v NaCl for one hour. Induction of the tolerance response was protein synthesis dependent. The protection afforded by the induced response depended on the intensity of the challenge conditions; at NaCl concentrations >10% no protection was observed. The sensitivity of the cells to NaCl, as well as other stresses such as low pH and high temperature was dependent on the presence of magnesium ions. Unlike NCDO 712 most strains of *Lc. lactis* subsp. *cremoris* are incapable of growth at 4% w/v NaCl; these strains were more sensitive to challenge at 20% w/v NaCl than was NCDO 712.

3.1 Introduction.

While the responses involved in maintaining the ability of bacteria to grow when subjected to relatively mild osmotic stress, have been extensively studied, much less is known about the response(s) necessary to ensure survival during exposure to more extreme osmotic stress. The response of bacteria to other lethal environmental stresses, in particular heat shock and acid stress have been the subject of intensive research. In many bacteria the induction of stress-tolerance responses, whereby pre-exposure to non-lethal intensities of environmental stress induces tolerance to a subsequent lethal challenge, has been reported. In addition, the induced tolerance is sometimes not specific to the stress causing induction; induction of tolerance to acid, for example, may also result in enhanced thermotolerance. The response of *Lc lactis* to environmental stress has been quite well studied recently. An inducible acid tolerance response (ATR) which also conferred protection against other stresses, including 20% w/v NaCl was reported by O' Sullivan and Condon (1997). A starvation-induced stress response which increased survival during nutrient starvation, with concomitant resistance to a number of other stresses, including NaCl has also been described for *Lc. lactis* (Hartke *et al.*, 1994). In many bacteria, induction of stress tolerance is associated with the synthesis of stress proteins, the best known being DnaK, GroES and GroEL. Synthesis of GroES and GroEL was induced by exposure of *Lc. lactis* to high temperature (Kim and Batt, 1993) and by acid stress (Hartke *et al.*, 1997). Exposure of *Lc lactis* to 2.5% w/v NaCl resulted in the induction of GroES, GroEL and DnaK (Kilstrup *et al.*, 1997). Twelve proteins in all were induced by exposing *Lc. lactis* to 2.5% NaCl and it was notable that all of these proteins were also inducible by heat (Kilstrup *et al.*, 1997).It appears from the results outlined above, that as with most other bacteria, there is a considerable overlap between the responses induced by different stresses in *Lc. lactis*. What is also clear is that induction of protection against lethal concentrations of NaCl is possible in *Lc lactis*. However, to our knowlege there are no reports of protection against higher concentrations of NaCl in *Lc. lactis*, by

lower concentrations of NaCl although such induced tolerance to NaCl has been reported for *Escherichia coli* (Jenkins *et al.*, 1990).

A major factor to be considered in determining the resistance of bacteria to a particular environmental stress, is that the lethality of the stress is often dependent on the growth phase of the culture. This has been reported for acid tolerance in *Lc. lactis* by O'Sullivan (1996) and also for acid tolerance in *Listeria monocytogenes* (O'Driscoll *et al.*,1996) and *E. coli* (Benjamin and Datta, 1995; Arnold and Kaspar, 1995). These reports indicate that to make valid comparisons between different experiments it is necessary to ensure that the culture is always at a similarly sensitive point when the sample is taken for challenge.

The composition of the medium in which cells are challenged may have a bearing on their sensitivity to the challenge. It has been reported that the presence of compatible solutes such as glycine betaine and carnitine protected several lactic acid bacteria against loss of viability during drying (Kets *et al.*, 1996). In addition, there is a certain amount of evidence in the literature to suggest that magnesium may be an important protective compound during exposure to lethal stress. The heat resistance of *Salmonella typhimurium* (Tolker-Nielsen *et al.*, 1996) and the resistance of *Lc. lactis* to starvation (Thomas and Batt, 1968) were both increased in the presence of magnesium ions.

In this chapter, we report the results of an investigation into factors which may affect the survival of *Lc. lactis* during challenge with high concentrations of NaCl. As a preliminary experiment, the sensitivity of *Lc. lactis* NCDO 712 to different NaCl concentrations was assessed and whether or not NaCl tolerance was growth phase dependent was determined. It was then attempted to induce tolerance to NaCl by pre-exposure to sublethal concentrations of NaCl. The influence of the challenge medium on survival during osmotic stress was examined, with an emphasis on the role of magnesium. In the final section of the chapter, The abilities of strains from the subspecies *lactis* and *cremoris* to survive challenge with high concentrations of NaCl were compared.

3.2 RESULTS.

3.2.1 Examination of *Lc. lactis* subsp. *cremoris* for growth phase dependent sensitivity to osmotic stress.

To eliminate the possibility that a cross-protective response could be induced by a decrease in the pH of the growth medium (due to acid production during growth), *Lc lactis* NCDO 712 was grown at constant pH 7.0 in complex broth (GM-17) in a 1.5L fermenter. Samples were removed at intervals and survival was determined following 2 hours exposure to 20% w/v NaCl in GM-17 broth. The overnight culture was quite resistant to NaCl, but this resistance was lost rapidly following inoculation into the fresh medium. The culture reached a point of maximal sensitivity in early exponential phase (OD 580, 0.2-0.3), after which it became progressively more resistant until stationary phase was reached (Fig. 3.1). In all further experiments, cultures were grown to an OD 580 of 0.2-0.3 before being challenged.

3.2.2 The effect of NaCl concentration on growth and survival.

In the initial experiment (3.2.1), 20% w/v NaCl was selected for the challenge in order that cell death would occur rapidly and hence shorten the duration of the experiment. Once the point in the growth cycle at which maximal sensitivity occurred was identified, we then sought to determine the threshold NaCl concentration at which cell death occurred. NaCl concentrations between 5 to 20% w/v, added to GM-17 were lethal to NCDO 712 cells. The rate of killing was proportional to the NaCl concentration at concentrations $\leq$ 10% w/v (Fig. 3.2). Addition of NaCl to the growth medium at concentrations between 1 and 4% w/v caused a reduction in the rate of growth. It is obvious from Fig. 3.2 that NaCl concentrations even slightly in excess of those that permit growth (4% w/v) were lethal to NCDO 712. Increasing the NaCl concentration above 10% w/v did not greatly increase the rate of killing.

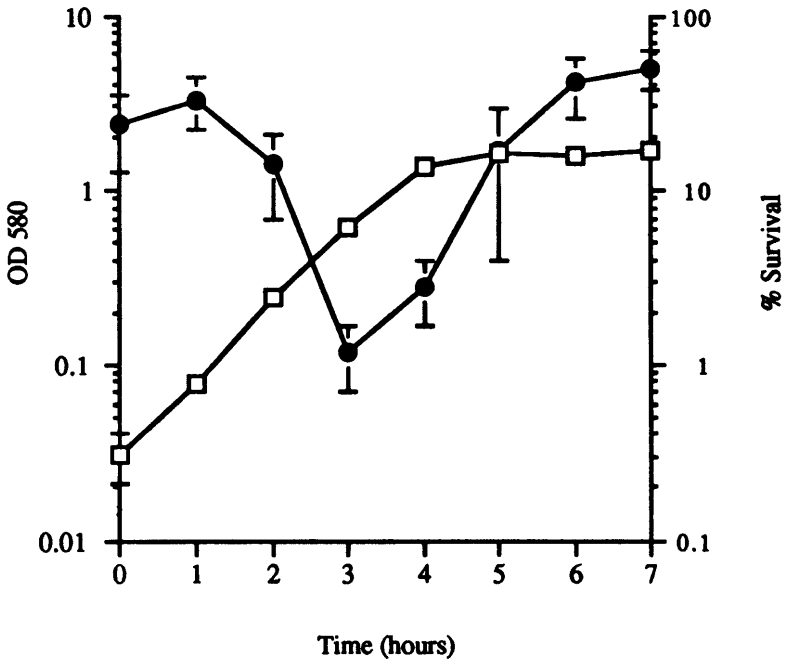


Fig. 3.1 The influence of growth phase on the survival of *Lc. lactis* subsp. *cremoris* NCDO 712 cells exposed to 20% w/v NaCl. The culture was grown in GM-17 medium at constant pH 7.0, anaerobically. Samples were assayed for optical density (□) and survival following 2 hours exposure to 20% NaCl at pH 7.0 in GM-17 (●).

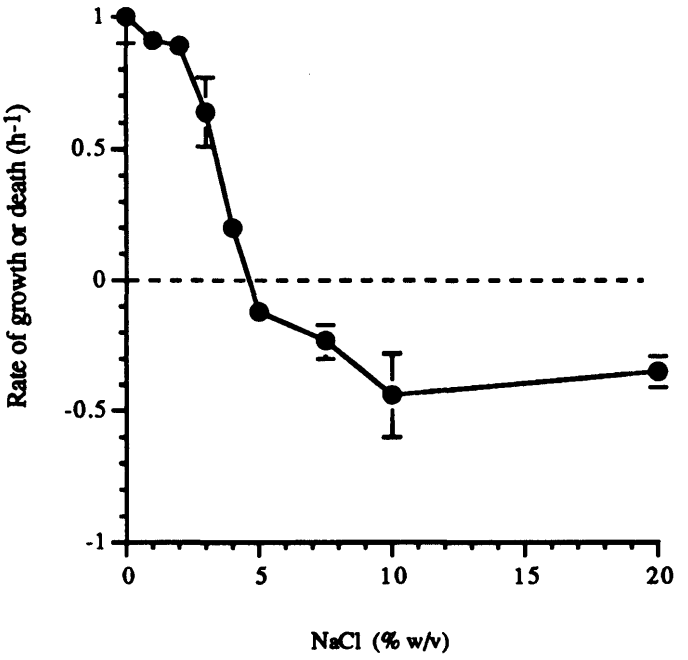


Fig. 3.2 The influence of NaCl concentration in GM-17 medium on the specific growth rate and death rate of *Lc. lactis* subsp. *cremoris* NCDO 712. The growth rates were calculated from optical density measurements of unaerated cultures at 30°C. Death rates were calculated from viable cell counts of similar cultures.

3.2.3 Examination of NCDO 712 for an inducible osmotic stress tolerance response.

Exponentially growing cells in GM-17 medium were removed at an OD 580 of 0.2-0.4 and exposed to 3% w/v NaCl in fresh GM-17 for 1 hour, prior to being challenged at various NaCl concentrations. The survival of these cells was compared to that of cells which had not been pre-exposed to 3% w/v NaCl. A substantial level of tolerance was induced by exposure to 3% w/v NaCl for one hour (Fig. 3.3). The induced tolerance appeared to be only effective at challenge conditions of up to 10% w/v NaCl (Fig. 3.3). When challenged at 20% w/v NaCl the prior exposure to 3% w/v NaCl sensitised the cells to the lethal salt challenge. The induced response appeared to be protein synthesis dependent, as induction was prevented by addition of chloramphenicol to the growth medium during the adaptation step (Fig. 3.4).

3.2.4 The effect of magnesium on survival during challenge with NaCl and other environmental stresses.

For these experiments we switched to HEPES buffer or a chemically defined medium in order to control the Mg^{2+} concentration during the NaCl challenge. The effect of adding an energy source (glucose) and $MgSO_4$ to HEPES buffer containing 20% w/v NaCl on survival was examined, Addition of glucose did not significantly enhance the tolerance of NCDO 712 to high NaCl concentrations, whereas addition of $MgSO_4$ led to a significant increase in the tolerance of the cells. The protective effect of magnesium was neither enhanced nor diminished by the presence of glucose in the buffer (Fig. 3.5). The protective effect of magnesium was concentration dependent and the maximum degree of protection was provided by 10 mM Mg^{2+} (Fig. 3.6). The protective effect appeared to be specific to magnesium; calcium had no effect on survival while manganese rendered the cells slightly more sensitive than the control (Fig. 3.7). Magnesium chloride was as effective as magnesium sulphate in protecting the cells (Fig. 3.7). To be effective in protecting the cells, it appeared that magnesium had to be present throughout the challenge. Addition of magnesium prior to challenge

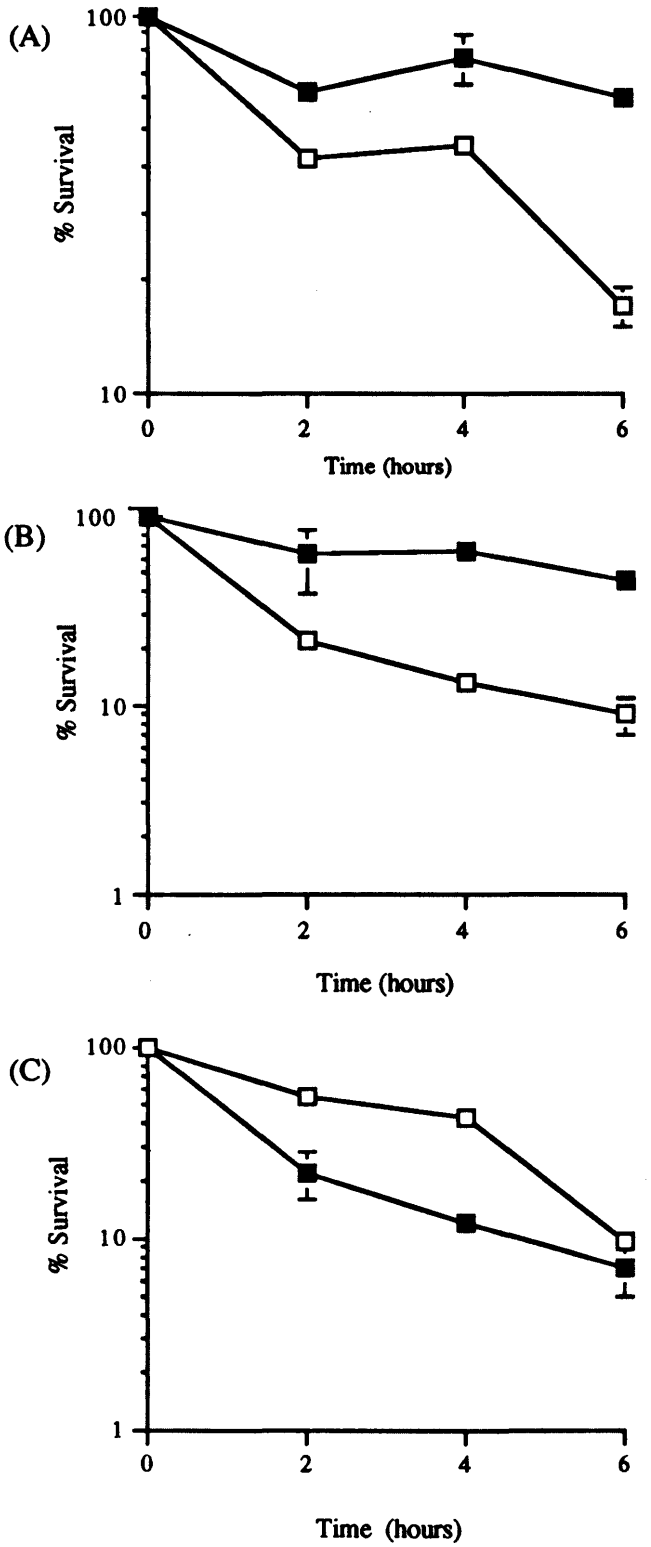


Fig. 3.3 Induction of tolerance to high concentrations of NaCl by pre-exposure to 3% w/v NaCl. *Lc. lactis* subsp. *cremoris* NCDO 712 was grown in GM-17 medium and either challenged with 7.5% (A), 10% (B) or 20% w/v NaCl (C) directly (□), or following exposure to 3% w/v NaCl in GM-17 for 1 hour (■).

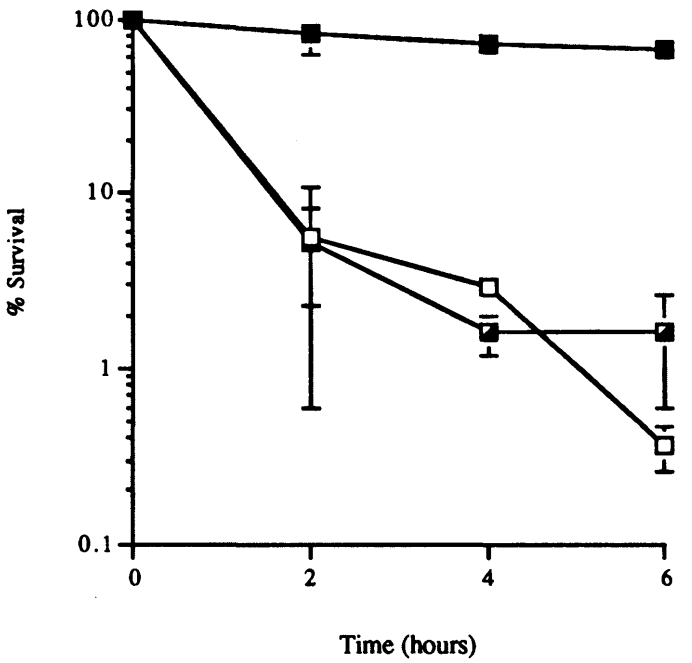


Fig. 3.4 The effect of 100μg chloramphenicol/ml on the induction of tolerance to lethal NaCl concentrations. *Lc. lactis* subsp. *cremoris* NCDO 712 was grown in GM-17 medium followed by challenge with 10% w/v NaCl in GM-17. The cells were challenged directly (□), or following exposure to 3% w/v NaCl for 1 hour(■) or following exposure to 100 μg/ml chloramphenicol for 30 minutes prior to being exposed to 3% w/v NaCl in GM-17 for 1 hour (◪).

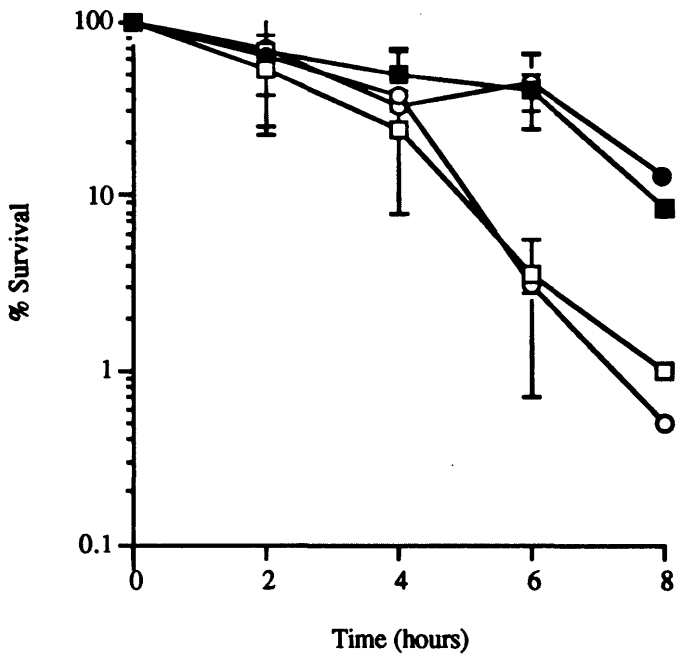


Fig. 3.5 The influence of magnesium with or without glucose on the survival of *Lc. lactis* subsp. *cremoris* NCDO 712 in HEPES buffer pH 7.0 containing 20% w/v NaCl. Exponential phase cells were challenged with 20% w/v NaCl without additions (□),with 25 mM glucose (○), with 10 mM $MgSO_4$ (■) or with 10 mM $MgSO_4$ plus 25 mM glucose (●).

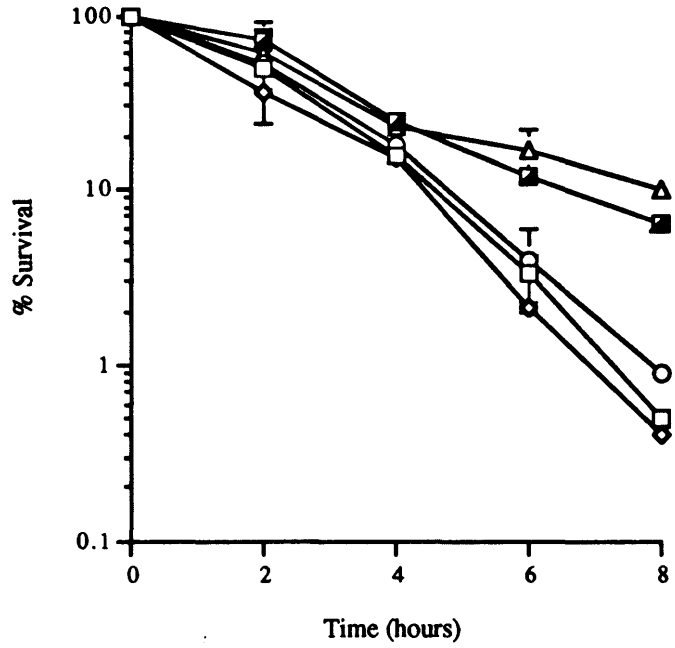


Fig. 3.6 The influence of the magnesium concentration on the survival of *Lc. lactis* subsp. *cremoris* NCDO 712 in HEPES buffer containing 20% w/v NaCl. The exponential phase cells grown in GM-17 broth were challenged with 20% NaCl in the absence of magnesium (□) and in the presence of 0.1 mM (◇), 1 mM (○), 10 mM (■), or 100 mM (▲) magnesium sulphate.

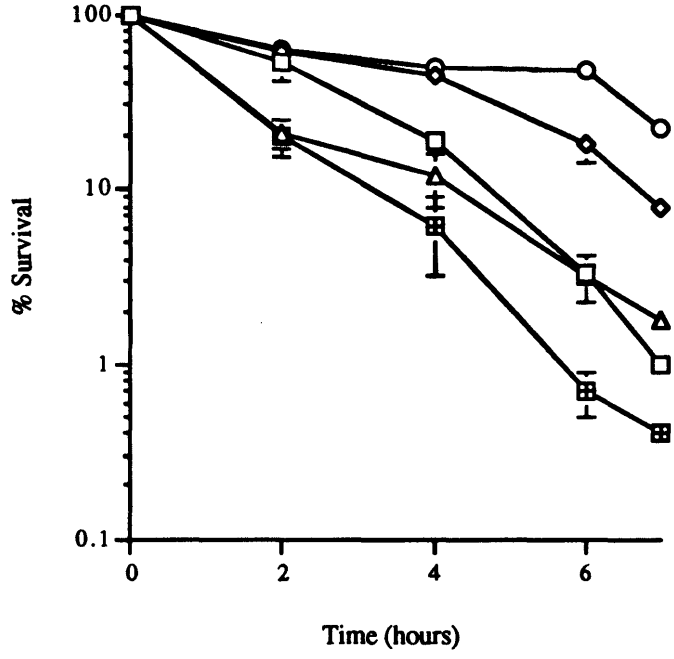


Fig. 3.7 Survival of exponential phase *Lc. lactis* subsp. *cremoris* NCDO712 cells grown in GM-17 medium when exposed to 20% NaCl in HEPES buffer containing 20% w/v NaCl alone (□) or in HEPES buffer with 10 mM MgCl (◇), 10 mM $MgSO_4$ (○), 10 mM $CaCl_2$ (△) or 10 mM $MnCl_2$ (⊞).

and its removal at 30 or 60 minutes did not protect the cells beyond the time of removal (Fig. 3.8). Similarly, if magnesium was not present at the initiation of the challenge but was added during the challenge, resistance to the high salt was gained from the time of magnesium addition (Fig. 3.9a). The degree of protection decreased as the interval between exposure to the challenge and addition of magnesium, increased (3.9b) The protective effect of magnesium was not a phenomenon unique to the HEPES buffer system. When the cells were challenged in the chemically defined medium MTEL omission of magnesium also resulted in increased sensitivity to the NaCl challenge; however, the difference between survival of cells challenged in the presence of magnesium and those challenged in its absence was less marked than in the HEPES buffer (Fig. 3.10). Protection by magnesium was not confined to NaCl stress; its presence also resulted in increased tolerance to acid stress (200 mM acetate, pH 3.7) or heat stress (42°C) (Fig. 3.11a and b).

3.2.5 A comparison of the sensitivities to NaCl of *Lc lactis* subsp. *cremoris* strains capable of growth at 4% w/v NaCl with those of *Lc. lactis* subsp.*cremoris* inhibited by 4% w/v NaCl.

This experiment investigated the possibility that there may be a link between the ability to grow at relatively high concentrations of NaCl and survival of a lethal NaCl challenge. NCDO 712 is capable of growth at 4% w/v NaCl whereas strains HP, US3 and BK5 are inhibited by NaCl concentrations >2% w/v. When challenged with 20% w/v NaCl in GM-17 broth, NCDO 712 was noticeably more resistant, particularly during the first 6 hours of challenge than the other *cremoris* strains (Fig. 3.12). An interesting feature of this result was that the loss of viability in NCDO 712 was biphasic, in that initially the cell numbers decreased slowly followed by a more rapid decline after 6 hours of challenge. This was noticeable in this experiment in which the the challenge with 20% NaCl was continued for 9 hours rather than the shorter times in earlier experiments.

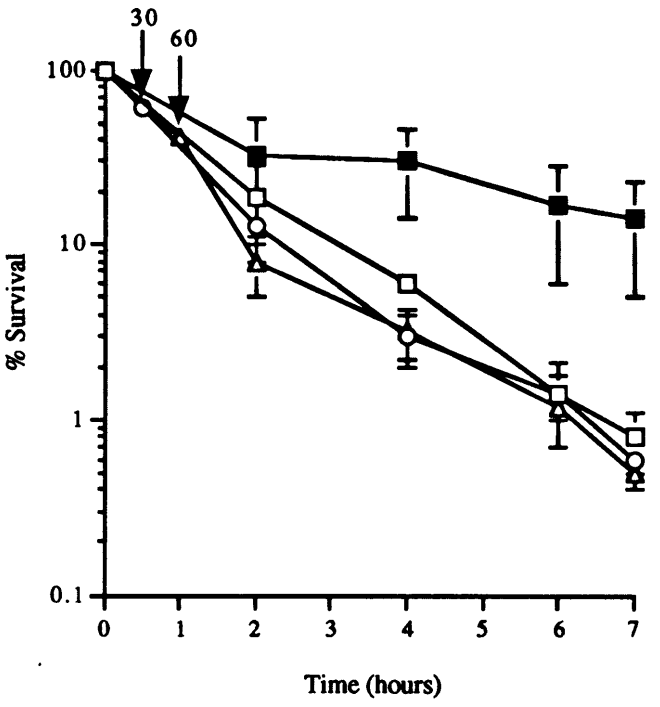


Fig. 3.8 The effect of magnesium removal during challenge with 20% w/v NaCl on the survival of *Lc. lactis* subsp. *cremoris* NCDO 712. The cells were challenged in HEPES buffer with 20% NaCl in the presence (■) or absence (□) of 10 mM magnesium sulphate. At 30 (○) and 60 (△) minutes after initiation of the challenge aliquots of the cells challenged in HEPES buffer containing magnesium were removed, centrifuged and washed twice with magnesium free HEPES buffer and resuspended in HEPES with 20% w/v NaCl and without magnesium.

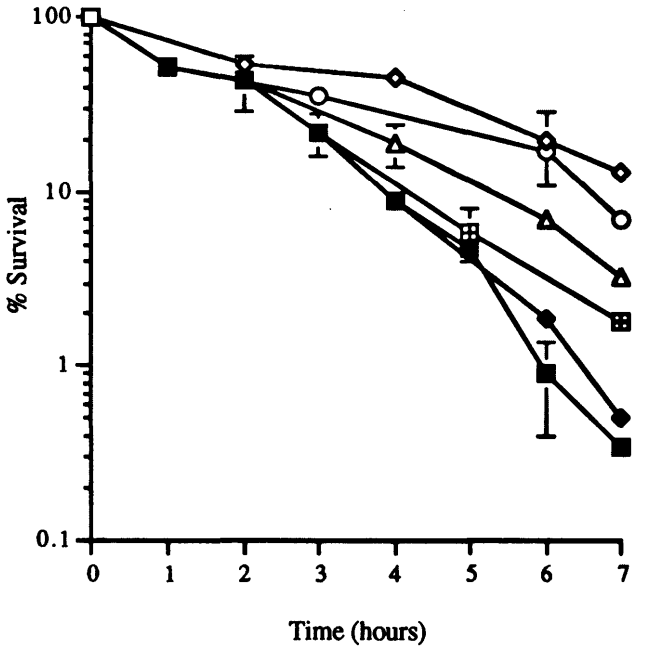


Fig. 3.9a The effect of delaying the addition of magnesium on the survival of exponential phase *Lc. lactis* subsp. *cremoris* NCDO 712 cells in HEPES buffer containing 20% w/v NaCl. 10 mM magnesium sulphate was added at the start (◇) at 1 (○), 2 (△), 3 (⊞), or 4 (◆) hours after addition of the cells to the buffer. Magnesium was completely omitted from the control sample (■).

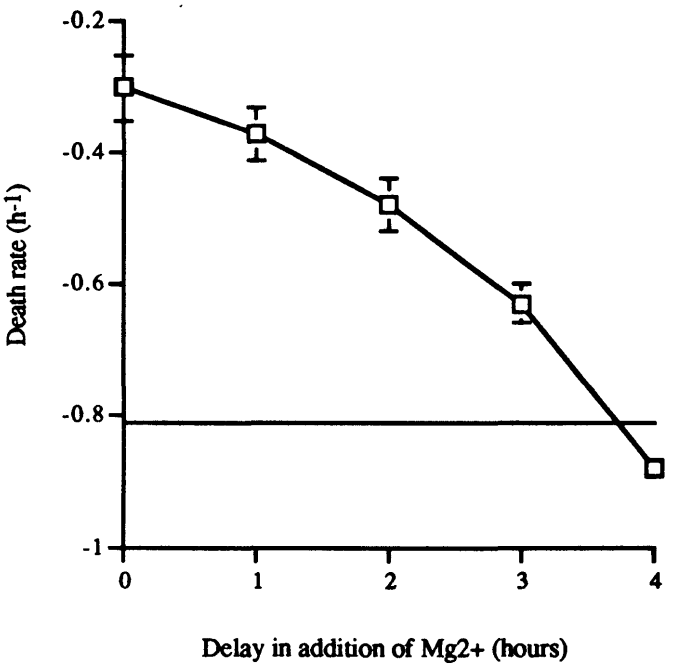


Fig. 3.9b The influence of delaying the addition of Mg2+ to the HEPES buffer with 20% NaCl challenge system on the subsequent death rate of NCDO 712 cells. The death rates were calculated from the data shown in Fig. 3.9a. The horizontal line indicates the death rate of cells challenged in the absence of magnesium.

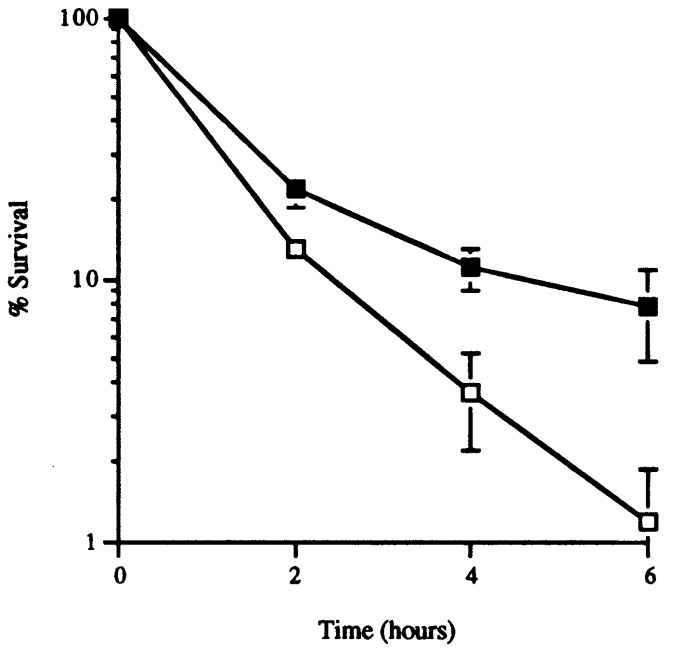


Fig. 3.10 Survival of exponential phase *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in MTEL medium when challenged in MTEL medium containing 20% w/v NaCl in the presence (■) and absence (□) of 10 mM $MgSO_4$.

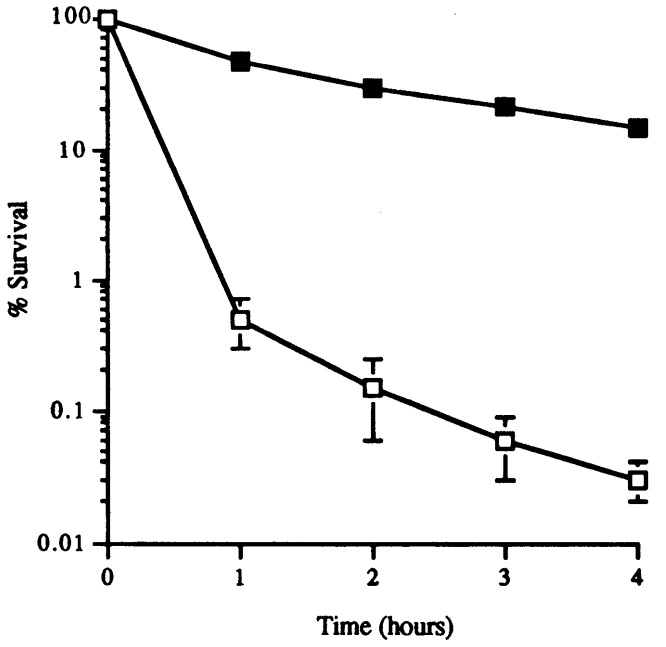


Fig. 3.11a Survival of exponential phase *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in MTEL medium during challenge in MTEL medium containing 200 mM acetic acid (pH 3.7) in the presence (■) and absence (□) of 10 mM $MgSO_4$.

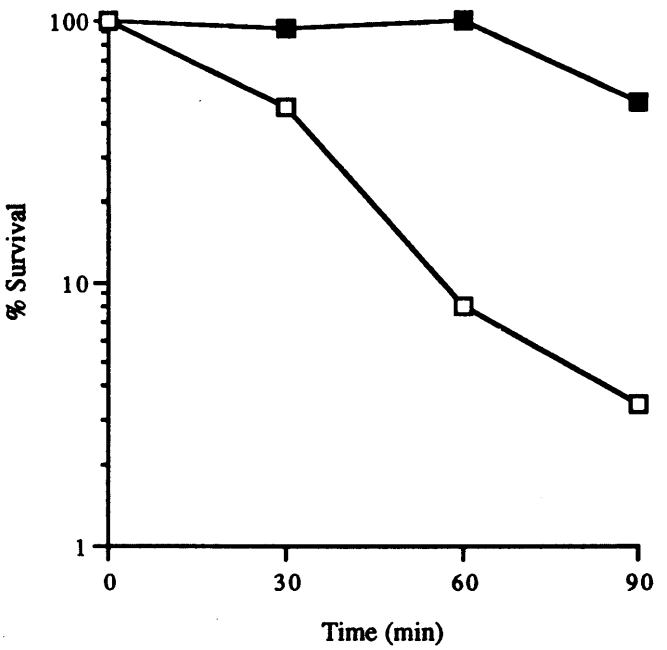


Fig. 3.11b Survival of exponential phase *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in MTEL medium when challenged in MTEL medium at 42°C in the presence (■) and absence (□) of 10 mM magnesium sulphate. The data presented are representative of triplicate experiments

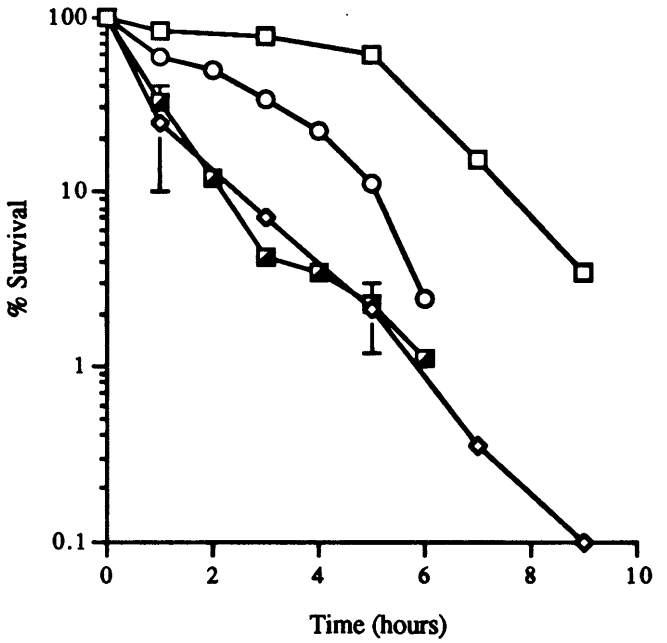


Fig. 3.12 Comparison of the survival of exponential phase cells of *Lc. lactis* subsp. *cremoris* strains NCDO 712 (□), HP (◇), US3 (○). and BK5 (◪) during challenge in GM-17 broth containing 20% w/v NaCl.

3.3. Discussion.

In this chapter, the survival of *Lc. lactis* subsp. *cremoris* during exposure to high concentratons of NaCl was examined. The resistance of NCDO 712 to 20% w/v NaCl was growth phase dependent. This observation was not unexpected as the resistance of NCDO 712 to low pH is also growth phase dependent (O'Sullivan, 1996). Growth phase dependent tolerance of *Lc. lactis* to a number of other stresses was also reported by Hartke *et al.* (1994). Growth phase dependent acid tolerance has also been reported in *L. monocytogenes* (O'Driscoll *et al.*, 1996;; Davis *et al.*, 1996), and *E. coli* O157:H7 (Benjamin and Datta, 1995; Arnold and Kaspar, 1995). In *E. coli* many genes are under the control of the stationary phase sigma factor σ^S encoded by the *rpoS* gene (Hengge-Aronis, 1995) and some of these genes are osmotically induced (Hengge-Aronis *et al.*, 1993). The *rpoS* gene product has a role in the resistance of *E. coli* and *Yersinia enterocolitica* to a number of environmental stresses, including osmotic stress (Badger and Miller, 1995; Cheville *et al.*,, 1996). In the Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *L. monocytogenes* an alternative sigma factor (σ^B) has been identified (Kullik and Giachino, 1997; Becker *et al.*, 1998). It is possible that growth phase dependent stress tolerance in *Lc. lactis* is due to a similar sigma factor or a similar transcriptional activator, repressed in early exponential phase and expressed in late exponential phase.

Incubation of *Lc. lactis* subsp. *cremoris* NCDO 712 in growth medium with 3% w/v NaCl for one hour resulted in induced tolerance to subsequent challenge with higher NaCl concentrations. Induced tolerance to NaCl by pre-exposure to non-lethal concentrations has been reported for *E. coli* (Jenkins *et al.*, 1990), *B. subtilis* (Volker *et al.*, 1992) and *Enterococcus faecalis* (Flahaut *et al.*, 1996). In addition, tolerance to 20% w/v NaCl in *Lc. lactis* subsp. *cremoris* NCDO 712 by pre-exposure to pH 5 has also been reported (O'Sullivan and Condon, 1997). Overlap between responses to different stresses is common in bacteria and there have been reports of this in *Lc. lactis* (Hartke *et al.*, 1994; Hartke *et al.*, 1995). The induction of NaCl tolerance was prevented by the protein synthesis inhibitor, chloramphenicol which has been a feature

of most of the induced tolerance systems reported in bacteria. Kilstrup *et al.* (1997) identified at least 12 proteins induced by exposure of the NCDO 712 derivative strain, MG1363 to 2.5% w/v NaCl; the proteins included the well known heat shock proteins DnaK, GroEL and GroES. It has been proposed that DnaK has a role in *E. coli* in maintaining potassium accumulation during osmotic stress and in renaturing proteins following plasmolysis (Meury and Kohiyama, 1991).

The induced tolerance response was only effective at the less severe challenge conditions, in contrast to the acid-induced response reported by O'Sullivan and Condon (1997), which did protect against 20% NaCl. This suggests that the mechanisms may be different or that 3% w/v NaCl is a poor inducer of stress tolerance and elicits a response that is insufficient to protect against higher NaCl concentrations. There is some evidence to suggest that, in general, a sublethal NaCl concentration may not be a good inducer of stress tolerance, in that 4% NaCl did not induce tolerance to acid stress (O'Sullivan and Condon, 1997). In addition, some proteins (including GroEL, GroES, and DnaK) are induced to higher levels in *Lc. lactis* subsp. *cremoris* by heat shock than by exposure to 2.5% w/v NaCl (Kilstrup *et al.*, 1997). In contrast, NaCl induces a number of proteins to high levels in *Ent. faecalis* (Flahaut *et al.*, 1996) and *B. subtilis* (Hecker *et al.*, 1988). The induction of stress proteins in *Ent. faecalis* correlates with induction of a multi-stress tolerance response (Flahaut *et al.*, 1997).

The sensitivity of *Lc. lactis* subsp. *cremoris* to 20% w/v NaCl, as well as low pH and heat shock was substantially influenced by the availability of magnesium. To our knowlege the only previous report of magnesium influencing tolerance to stress in *Lc. lactis* is that of Thomas and Batt (1967), in which magnesium increased the resistance of cells to starvation. It has long been recognised that magnesium stabilises ribosomes (Hui Bon Hoa *et al.*, 1980). The heat-resistance and ribosome stability of *Salmonella typhimurium* were increased by the presence of 100 mM $MgCl_2$ during challenge at 52°C (Tolker-Nielsen and Molin, 1996). In addition to its stabilising effect on ribosomes, magnesium is also required for optimal activity of the chaperone DnaK (Skowyra and Wikner, 1995). Enhanced DnaK function could also explain the effect of

at least some of the contribution of magnesium to the acid and heat tolerance of NCDO 712, as DnaK is well known as a heat shock protein, and is also induced by low pH in *Lc. lactis* (Hartke *et al.*, 1996). The protective effect of magnesium was concentration dependent and appeared to be specific to magnesium, as neither manganese nor calcium could substitute for it. This is in agreement with available data on the interaction between magnesium and DnaK, as the ATPase activity of the DnaK protein is dependent on the free magnesium concentration and the stimulation appears to be magnesium-specific (Skowrya and Wickner, 1995). The loss of protection by removing the magnesium during challenge suggests that the protective effect of magnesium does not involve irreversible binding of magnesium within the cell. The protective effect of magnesium diminished as the interval between initiation of the challenge and magnesium addition increased. This presumably reflects the fact that cell damage increases in proportion to the time of challenge in the absence of magnesium. Addition of magnesium to the damaged cells is then less effective in preventing further loss of viability. The greater resistance of cells challenged in GM-17 medium reflects the presence of $MgSO_4$ (1 mM) in this medium.

NCDO 712, which is capable of growth at 4% w/v NaCl, was more tolerant to NaCl than the three strains incapable of growth at 4% NaCl. The classical phenotype of the subspecies *cremoris* included the inability to grow at 40°C, as well as NaCl sensitivity. Strains with the classical *cremoris* phenotype seem to be less tolerant of environmental stress than NCDO 712 which for many years was phenotypically classified as a *lactis* strain. The limited data available to us do not allow any conclusions to be drawn at present, however the superior performance of the traditional type *cremoris* strains as dairy starters (Urbach *et al.*, 1997) might warrant investigation of stress tolerance in such strains and its possible contribution to starter performance.

The major conclusions to be drawn from the data presented in this chapter are the presence in *Lc. lactis* subsp. *cremoris* NCDO 712 of an inducible NaCl tolerance mechanism and that magnesium has been identified as having a major role to play in the survival of a number of environmental stresses.

Chapter IV

The role of potassium in the adaptation of *Lactococcus lactis* subsp. *cremoris* NCDO 712 to low water activity.

Abstract.

Accumulation of potassium was shown to be an important component of the response of *Lactococcus lactis* subsp. *cremoris* NCDO 712 to a decrease in the water activity (a_w) of the medium. Accumulation of potassium was dependent on the nature of the solute used to adjust the a_w of the growth medium. If the external solute was NaCl accumulation of K^+ did not occur whereas solutes such as glucose or fructose resulted in a doubling of the cytoplasmic potassium content. The specific growth rate of NCDO 712 was substantially reduced in a chemically defined medium (MTEL) with its K^+ concentration reduced to 0.5 mM. Increasing the K^+ content of the medium to 20 mM abolished the growth inhibition. In contrast, if the a_w was reduced by addition of fructose, there was no difference between the growth rates of cultures in MTEL medium containing 0.5 mM K^+ or 20 mM K^+. The inhibition of the growth rate in medium containing NaCl at low K^+ concentrations correlated strongly with a reduction in the cytoplasmic K^+ content and in the cyoplasmic volume. Omission of the compatible solute proline from MTEL medium rendered the inhibition more severe, while addition of the potent compatible solute glycine betaine to MTEL medium substantially reversed the inhibition. Supplementation of the MTEL medium containing 0.5 mM K^+ with the potassium analogue rubidium (19.5 mM) so that the total potassium plus rubidium concentration was 20 mM, completely reversed the inhibition. These results demonstrate that potassium accumulation plays an important role in the tolerance of *Lc. lactis* subsp. *cremoris* to osmotic stress.

4.1 Introduction.

The importance of potassium in the adaptation of bacteria to hyperosmotic stress (low a_w) has been recognised for many years (Epstein, 1986). Potassium is accumulated as a means of increasing the cytoplasmic osmolarity to counteract the increase in the osmolarity of the extracellular environment. Accumulation of potassium has been best studied in *Escherichia coli* and other Gram negative bacteria (Epstein, 1986; Csonka, 1989). In the case of Gram positive bacteria, osmotically induced potassium accumulation has been reported for some bacteria but not for others. The intracellular potassium content of *Bacillus subtilis* (Whatmore *et al.*, 1989; Whatmore and Reed 1990) *Enterococcus faecalis* (Kunin and Rudy, 1993) and *Listeria monocytogenes* (Patchett *et al.*, 1992) increased in response to osmotic stress. In contrast, potassium was not accumulated by *Staphylococcus aureus* grown at low a_w (Kunin and Rudy, 1991).

To our knowlege, the role of potassium in the response of *Lc. lactis* to low a_w has not been investigated. Many years ago, MacLeod and Snell (1948) reported that the potassium requirements of some lactic acid bacteria were increased by the presence of sodium or ammonium ions indicating some interaction between K^+, Na^+ and NH_4^+. In a recent report potassium accumulation was observed in *Lactobacillus plantarum* when KCl but not NaCl was added to the growth medium (Glaasker *et al.*, 1996a). In this chapter we examine the role of potassium in the osmoadaptation of *Lc. lactis*.

4.2 Results.

4.2.1 The potassium content of *Lc. lactis* grown in medium of decreased water activity.

The cytoplasmic potassium content of *Lc. lactis* subsp. *cremoris* NCDO 712 in complex (GM-17) or chemically defined (MTEL) medium in the absence of added solutes was approximately 1.6 μmol/mg protein. Addition of NaCl to reduce the a_w of GM-17 (Fig. 4.1) or MTEL media (Fig. 4.2) resulted in a decrease rather than an increase in potassium concentration reaching 1 μmol/mg protein at an a_w of 0.983. In contrast, if glucose or fructose were added to reduce the a_w of either medium, the potassium content of the cells increased (Figs. 4.1 and 4.2). The cytoplasmic potassium content of NCDO 712 cells in medium containing 0.88M monosaccharide (a_w = 0.983) was 3.12 μmol/mg protein for cells grown in MTEL and 3.36 μmol/mg protein for cells grown in GM-17.

4.2.2 Measurement of the potassium content of NCDO 712 during growth.

The values for the potassium content of NCDO 712 presented above (4.2.1) were taken at a single time point from exponentially growing cells. To determine whether or not the potassium content remained constant during growth, the potassium content of NCDO 712 cultures was measured at intervals during growth in GM-17 medium at low and high a_w.

The results of these experiments show that potassium accumulation occurred rapidly following inoculation and it remained reasonably constant but with wave-like fluctuations, especially when the a_w was reduced by glucose (Fig 4.3). The values obtained for the cellular potassium content confirmed the results from the earlier experiments. No significant accumulation of potassium occurred as a consequence of decreasing the a_w with NaCl, while potassium was accumulated by cells when the a_w was reduced by glucose. It was also noticeable that the lag phase of growth was shorter when the a_w was reduced by sugar rather than NaCl. The potassium content of cells

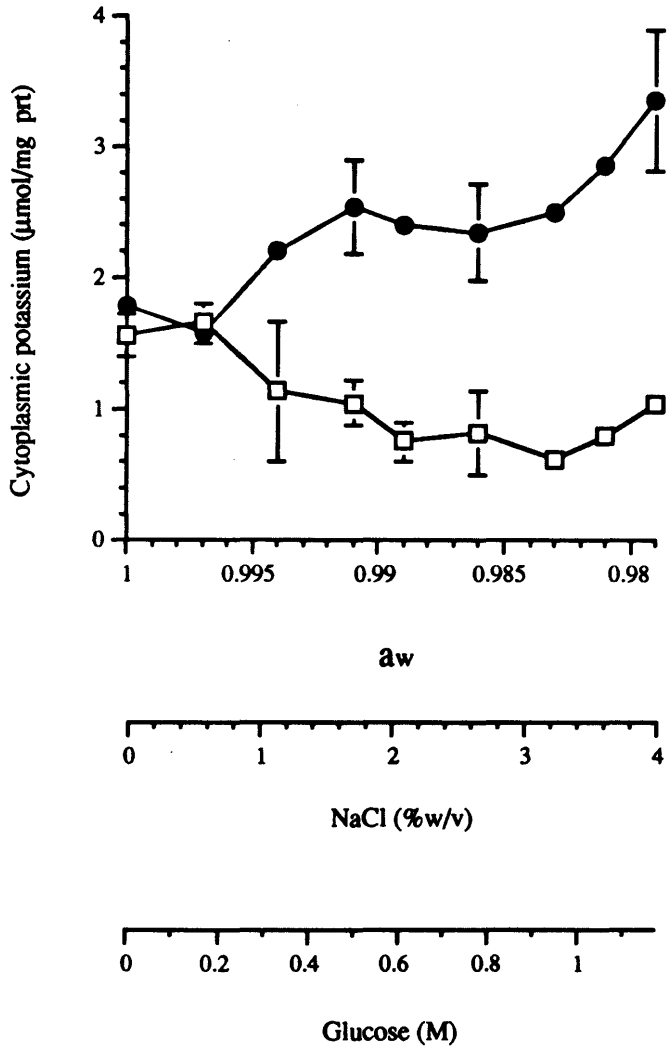


Fig. 4.1 The cytoplasmic content of potassium of *Lc. lactis* subsp. *cremoris* NCDO 712 cells growing in GM-17 medium with the aw decreased by addition of NaCl (□) or glucose (●).

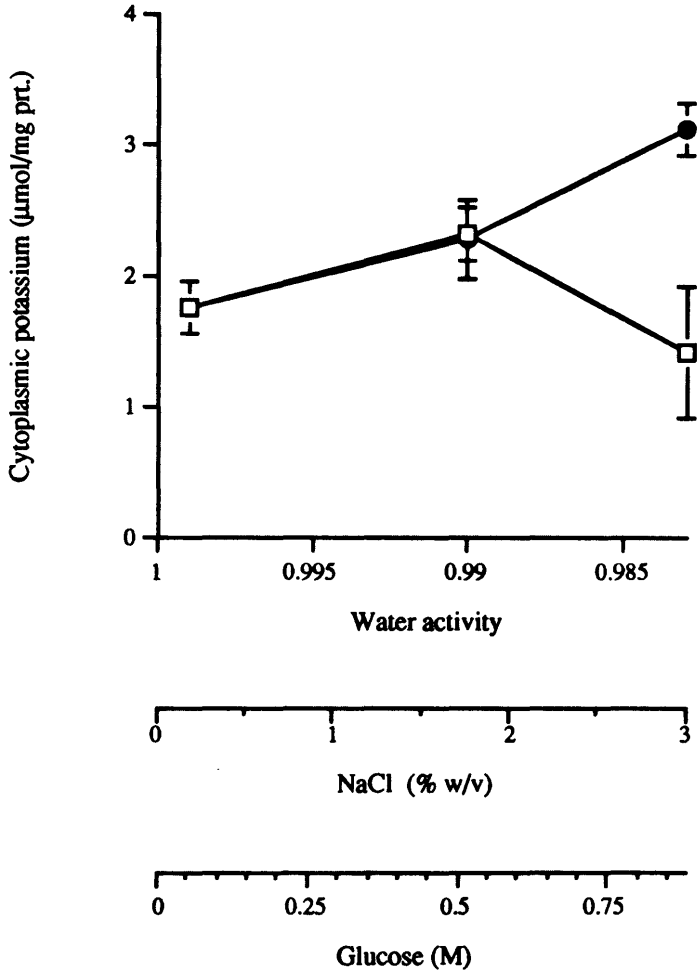


Fig. 4.2 The cytoplasmic content of potassium of *L. lactis* subsp. *cremoris* NCDO 712 cells growing in MTEL medium with the aw reduced by addition of NaCl (□) or glucose ●)

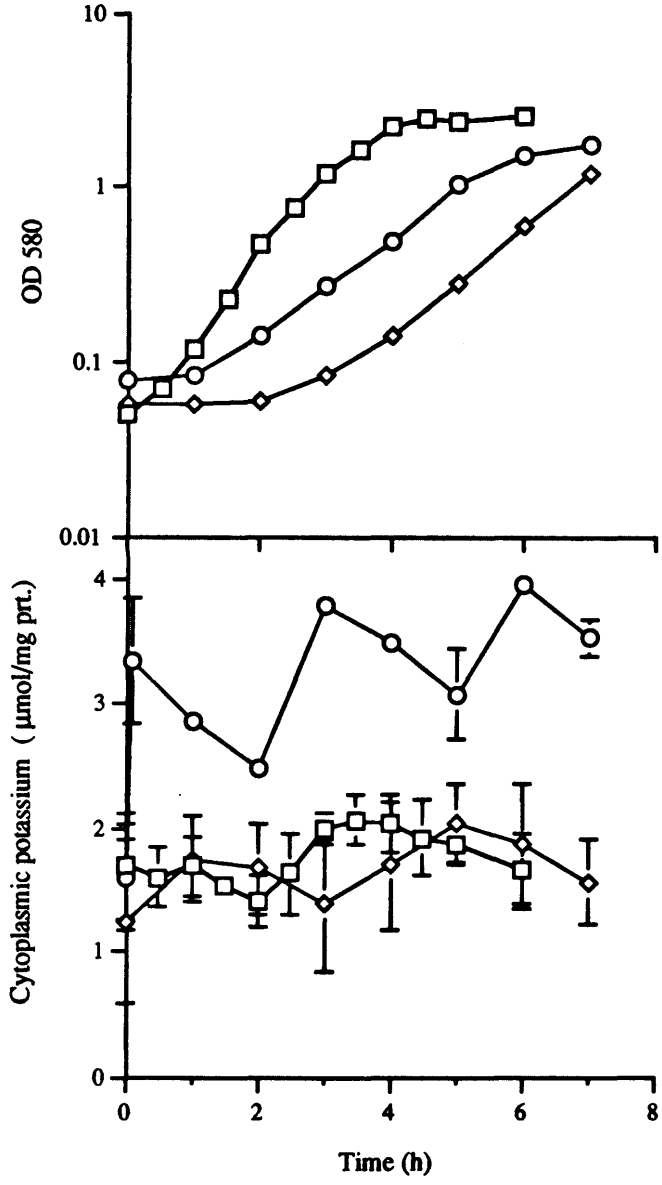


Fig. 4.3 Growth as OD580 and the potassium content of *Lc. lactis* subsp. *cremoris* NCDO 712 growing in GM-17 medium alone (□) and in GM-17 medium containing either 0.88M glucose (○) or 3% w/v sodium chloride (◇) to reduce the water activity to 0.983.

grown in medium with added sugar in these experiments was slightly higher than was observed in the earlier experiments. As the protein conc./ml of culture was available for each data point, it was possible to plot the potassium content against the protein content as the biomass of the culture increased; this gave a straight line graph, the slope of which was equal to the average potassium content of the culture per mg protein (Fig 4.4). This is probably the most accurate method of measuring the potassium content of a growing culture, provided that the potassium content remains reasonably constant during growth, so that there is a straight line relationship between the potassium and protein contents of the culture. Using this method the potassium content of *Lc. lactis* grown in the absence of osmotic stress was calculated as being 1.85 μmol/mg protein, while the potassium content of cells grown at an a_w of 0.983 in the presence of glucose or NaCl was 3.75 or 1.69 μmol/mg protein respectively.

4.2.3 The effect of potassium limitation on the growth of *Lc. lactis* subsp. *cremoris* in MTEL medium of low a_w.

The results of section 4.2.1 seemed to suggest that NaCl was in some way preventing uptake of potassium by the cells. If this was the case, then reducing the potassium content of the culture medium would be expected to result in severe inhibition by NaCl . The concentration of potassium which permitted growth at the maximal rate in the absence of NaCl was 0.5 mM (see Chapter V). This concentration of potassium was selected as the limiting concentration and growth was assayed at increasing NaCl concentrations in the chemically defined MTEL medium containing 0.5 mM potassium and the non-limiting concentration of 20 mM and the results are presented in Fig. 4.5.

In medium containing 0.5 mM potassium, the specific growth rate of NCDO 712 was substantially inhibited by NaCl and growth did not occur at NaCl concentrations >2% w/v (a_w 0.987). In medium containing 20 mM potassium, the inhibition of growth was much less severe and slow growth occurred at 3% w/v NaCl (a_w 0.983).

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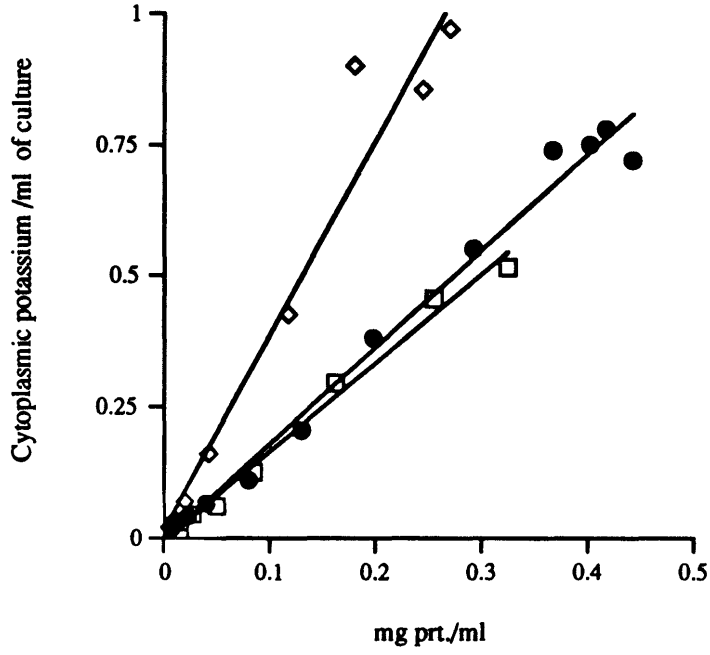


Fig. 4.4 The increase in the cytoplasmic content of potassium as a function of the increase in biomass (protein/ml) of *Lc. lactis* NCDO 712 cultures growing in GM-17 medium alone (●) or with the aw decreased to 0.983 by addition of 0.88M glucose (◇) or 3% w/v NaCl (□).

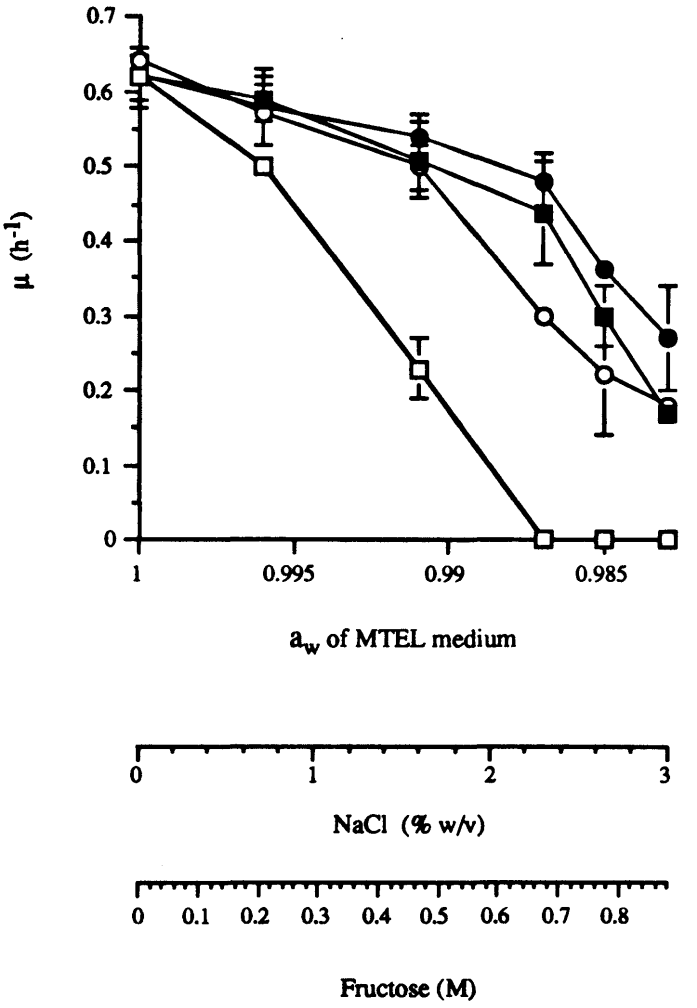


Fig. 4.5 The specific growth rate, μ of *Lc. lactis* subsp. *cremoris* NCDO 712 in MTEL medium containing either 0.5 mM (○, □) or 20 mM (●, ■) potassium. The aw was adjusted by addition of NaCl (■, □) or fructose (● ○ .

In MTEL medium in which the a_w was reduced by addition of fructose the reduction of the growth rate was mainly similar at both potassium concentrations.

The inhibition of growth by NaCl was due to the Na^+ rather than the Cl^- ions, as Na_2SO_4 had a similar inhibitory effect (Table 4.1). The inhibition of growth at low potassium concentrations was not specific to Na^+ ions however, as NH_4^+ ions were even more inhibitory (Table 4.1).

4.2.4 The effect of sodium and other monovalent cations on the potassium content of NCDO 712.

The data presented in 4.2.3, showed that Na^+ and NH_4^+ ions used to reduce the a_w, increased the potassium concentration required for maximal growth of NCDO 712. A possible explanation was that the cations caused a reduction in the cytoplasmic accumulation of potassium. The cytoplasmic potassium content of NCDO 712 in MTEL medium containing 20 mM KCl was 1.75 µmol/mg. protein and decreasing the potassium content of the medium to 0.5 mM did not significantly reduce the cytoplasmic potassium content (Table 4.2). In contrast, if NaCl , NH_4Cl or Na_2SO_4 was added to reduce the a_w, of MTEL medium, containing the lower concentration of potassium, from 0.999 to 0.990 the potassium content of the cells was substantially reduced (Table 4.2). It was notable that there was a strong correlation between the extent of the reduction in the specific growth rate and the reduction in the cellular potassium content. Cells growing in MTEL medium with the higher concentration of potassium had slightly higher cytoplasmic K^+ levels when the a_w was reduced. Likewise no reduction in cytoplasmic potassium was observed when the a_w of MTELwas reduced by fructose at low or high potassium levels.

4.2.5 Measurement of the cytoplasmic volume of cells grown in MTEL medium in the presence of NaCl .

The major function of compatible solutes in osmotically stressed bacteria, is to maintain turgor pressure and prevent plasmolysis. Following the observation that the

Table 4.1 Influence of the solute used to reduce the aw and the potassium concentration of MTEL medium on the specific growth rate (μ) of *Lc. lactis* subsp. *cremoris* NCDO 712 in MTEL medium.

Solute added	a_w	μ (h^{-1}) in MTEL medium containing	
		0.5 mM KCl	20.0 mM KCl
Control	0.999	0.66±0.06	0.66±0.06
0.25M NaCl	0.990	0.25±0.04	0.52±0.05
0.125 M Na_2SO_4	0.990	0.24±0.09	0.54±0.04
0.25M NH_4Cl	0.990	0.14±0.02	0.54±0.09
0.44 M Fructose	0.990	0.43 ±0.08	0.47±0.07

Table 4.2 Influence of the solute used to reduce the aw and the potassium concentration of the medium on the cytoplasmic content of K^+ of *Lc. lactis* NCDO 712 growing in MTEL medium.

Solute added	a_w	Cytoplasmic K^+ (µmol/mg. prt) of cells growing in MTEL medium containing	
		0.5 mM KCl	20.0 mM KCl
None	0.999	1.62±0.21	1.75±0.2
0.25M NaCl	0.990	1.12±0.20	2.31±0.20
0.125 M Na_2SO_4	0.990	1.07±0.23	2.10±0.02
0.25M NH_4Cl	0.990	0.64±0.02	1.94±0.02
0.44 M fructose	0.990	1.81 ±0..07	2.27±0.27

cytoplasmic potassium content of cells was substantially reduced when growing in MTEL medium containing 0.5 mM potassium and 1.5% w/v NaCl. It was decided to determine whether the low cytoplasmic potassium concentrations affected the cytoplasmic volume. The water-permeable/sorbitol-impermeable volume of NCDO 712 cells grown in MTEL medium containing 20 mM potassium and without added NaCl was 3.8 µl/mg protein. As shown in Table 4.3 reducing the potassium concentration to 0.5 mM in the absence of osmotic stress, or adding 1.5% w/v NaCl to medium containing 20 mM potassium, did not cause a reduction in the cytoplasmic volume. When the potassium content of the medium was reduced to 0.5 mM in the presence of 1.5% w/v NaCl the cytoplasmic volume decreased, by approximately 30%, to 2.78 µl/mg. protein. This decrease in cytoplasmic volume correlated strongly with the decrease in specific growth rate and cytoplasmic potassium content described earlier. If the a_w of the medium was reduced by adding fructose there was no significant difference between the cytoplasmic volume of cells grown at 0.5 mM and 20 mM potassium.

4.2.6 Glycine betaine, proline and rubidium as alternative osmoprotectants to potassium.

Glycine betaine and proline have previously been reported to act as osmoprotectants in *Lc. lactis* (Molenaar *et al.*, 1993). In Chapter VI of this thesis we confirmed that they are also osmoprotective in *Lc. lactis* subsp. *cremoris* NCDO 712. Assuming that the growth inhibition caused by the presence of NaCl at low potassium concentrations resulted from an osmotic effect, it is reasonable to suggest that a strong osmoprotectant such as glycine betaine could reverse the inhibition. Similarly, in the absence of glycine betaine, omission of proline from the growth medium might be expected to result in more severe inhibition. In Table 4.4 the specific growth rate of cells grown in MTEL medium from which proline was omitted, was compared with those observed in MTEL medium in the presence of proline and/or glycine betaine. Addition of glycine betaine partially reversed both the reduction in growth rate (Table 4.4) and cytoplasmic volume

Table 4.3 Influence of the solute used to reduce the aw, the potassium concentration of the growth medium and glycine betaine on the cytoplasmic volume of *Lc. lactis* NCDO 712 cells growing in MTEL medium

Solute added	a_w	Cytoplasmic volume (µl/mg. prt) of cells growing in MTEL medium containing.	
		0.5 mM KCl	20.0 mM KCl
None	0.999	3.74±0.51	3.88±0.34
0.25M NaCl	0.990	2.78±0.37	3.69±0.29
0.25M NaCl plus 2 mM glycine betaine	0.990	3.09±0.2	3.69±0.4
0.44M fructose	0.990	3.88±0.10	4.36±0.10
0.44 M fructose plus 2 mM glycine betaine	0.990	4.57±1.0	4.12±0.13

Table 4.4 The influence of the organic osmoprotectants proline and glycine betaine on the growth rate (μ) of *Lc. lactis* subsp. *cremoris* NCDO 712, in MTEL medium with a potassium concentration of 0.5 or 20 mM and the aw reduced to 0.990 by addition of 0.25M NaCl.

Solute added	Concentration of KCl (mM)	Specific growth rate, μ (h^{-1}) in MTEL medium :			
		Without proline	With proline (2 mM)	With glycinebetaine (2 mM)	With proline and glycinebetaine
None	0.5	0.61±0.01	0.63±0.06	0.63±0.06	N.D.
None	20	0.62±0.03	0.64±0.04	0.66±0.06	N.D.
0.25M NaCl	0.5	0.19± 0.04	0.25± 0.06	0.39±0.03	0.41±0.03
0.25 M NaCl	20	0.47±0.06	0.54±0.08	0.58±0.04	0.56±0.1

N.D.: not determined

(Table 4.3) caused by reducing the a_w with NaCl. Confirming that the inhibition observed, was at least partially due to osmotic stress, exacerbated by impaired potassium uptake. The presence of proline in MTEL medium led to a slight reversal of the NaCl inhibition of growth rate, but was not as stimulatory as glycine betaine. Adding proline and glycine betaine together did not cause any greater reversal of inhibition than did glycine betaine alone.

The monovalent cation rubidium acts as a potassium analogue and is transported by the potassium transport systems of *E. coli* and other bacteria. When rubidium was added to MTEL medium at a concentration of 19.5 mM in addition to 0.5 mM potassium (so that the total potassium plus rubidium concentration was 20 mM) the inhibitory effect of NaCl normally observed at the lower potassium level was not observed, suggesting that rubidium replaced potassium as an osmoprotectant (Fig. 4.6).

4.2.7 Measurement of the proton-motive force in NCDO 712.

The proton motive force (p.m.f., ΔP) is composed of two components, the transmembrane pH gradient (ΔpH) and the electrochemical potential ($\Delta\Psi$). Potassium transport has been shown to be essential for the generation of the p.m.f. in *Lc. lactis* (Kashket and Barker, 1977). In addition to the osmotic effects already demonstrated, it was conceiveable that the inhibition observed in the presence of NaCl at low potassium concentrations might have been partially due to perturbation of the cytoplasmic pH and p.m.f. due to impaired potassium transport. The ΔpH was measured by measuring the accumulation of ^{14}C benzoic acid, and the $\Delta\Psi$ was measured by measuring accumulation of the lipophilic cation trimethylphenylphosphonium (TPP^+). The ΔpH was slightly lowered by a decrease in the potassium concentration of MTEL at normal a_w and by decreasing the a_w with NaCl (Table 4.5). However, the effects do not seem to be sufficient to explain the growth inhibition by NaCl. The values for the p.m.f. of cells grown in MTEL with 1.5% w/v NaCl at both 0.5 and 20 mM potassium were similar (154±8 mV and 146±16 mV respectively) and were approximately the same as

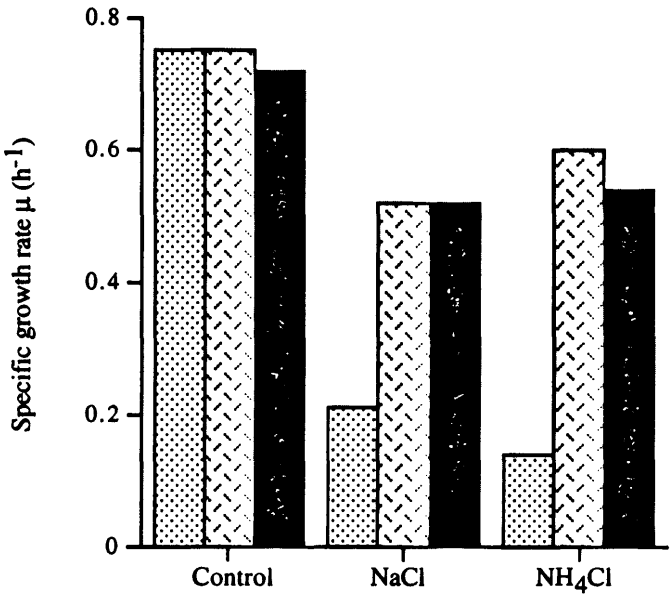


Fig. 4.6 The effect of partial substitution of rubidium for potassium in MTEL medium. on the specific growth rate μ of NCDO 712 in MTEL medium without added solute (control) 0.25M NaCl, or 0.25M NH_4Cl.. The MTEL contained 0.5 mM potassium. (■) or 0.5 mM potassium + 19.5 mM rubidium (▨) or 20 mM potassium (■).

Table 4.5 The influence of the potassium content of the medium and reducing the aw with NaCl on ΔpH, ΔΨ, and p.m.f. of *Lc. lactis* NCDO 712 growing in MTEL medium

NaCl (M)	KCl (mM)	Δ pH	-Z.ΔpH (mV)	Δψ (mV)	p.m.f. (mV)
0	0.5	1.03±0.09	62±6	85±8	147±12
0	20	1.17±0.04	70±3	52±12	122±13
0.25	0.5	0.93±0.11	56±7	99±1	154±8
0.25	20	1.05±0.13	63±8	83±9	146±16

Note: The samples were taken when the extracellular pH (pHo) of the cultures reached 5.8

that measured for cells grown in MTEL without NaCl containing 0.5 mM potassium (147±12 mV) The values obtained for the p.m.f. of cells grown in MTEL containing 20 mM potassium were lower than for the other cultures, due to the fact that the $\Delta\Psi$ value was extremely low. This appeared to be an accurate representation of the $\Delta\Psi$; part of the calculation of the $\Delta\Psi$ value involves correcting the TPP^+ content of the cells for TPP^+ bound non-specifically (i.e. not due to $\Delta\Psi$) and it was possible that this might have been a significant source of error. Analysis of the amount of TPP^+ bound by toluenised cells revealed that the amount of TPP^+ bound per mg. protein was similar for all cultures i.e. it was unlikely to be a source of error in the measurement. No correlation was therefore observed between ΔpH or p.m.f. and inhibition of *Lc. lactis* subsp. *cremoris* by NaCl.

4.3. Discussion.

In this chapter the accumulation of potassium by NCDO 712 following osmotic stress is described. It was also reported that potassium accumulation was dependent on the solute used to reduce the a_w of the medium. Solutes containing the monovalent cations Na^+ or NH_4^+ did not elicit potassium accumulation whereas the non-ionic solute fructose did. The reasons for the failure of NCDO 712 to accumulate potassium were then examined further.

Potassium accumulation by bacteria following osmotic stress has been widely reported (for reviews see Epstein, 1986; Csonka, 1989). Accumulation of potassium as a compatible solute has been best studied in *Escherichia coli*, and the transport systems responsible for its uptake have been well characterised. In Gram-positive bacteria there has been little study of the role of potassium in osmotolerance, and to our knowledge, there are no reports in the literature of osmotically induced potassium transport in *Lc. lactis*. Our results demonstrate clearly that potassium is accumulated by *Lc. lactis*, but that its accumulation is conditional on the solute which imposed the osmotic stress. This is a relatively novel concept, though there have been a few reports in the literature to indicate that potassium accumulation may be impaired in some circumstances. The

earliest report was that of MacLeod and Snell (1948) who reported that the presence of sodium and ammonium ions increased the potassium requirement of certain lactic acid bacteria. More recently, Harold and Baarda (1967) reported that sodium ions had a slight inhibitory effect on potassium transport by *Streptococcus faecium* (now *Enterococcus hirae*). The first report of this phenomenon, in the context of osmotic stress was by Glaasker *et al.* (1996a), who observed that potassium was accumulated by *Lactobacillus plantarum* if the a_w of the medium was reduced by potassium chloride, but not if NaCl was used. However, these workers did not investigate the matter further.

Potassium accumulation in *E. coli* is reported to be transient and accumulated potassium is subsequently replaced by other compatible solutes. In *Lc. lactis* subsp. *cremoris* NCDO 712, accumulation of potassium did not appear to be transient, even in complex medium (GM-17). This suggests that when possible, potassium accumulation plays a significant role in the adaptation of *Lc. lactis* NCDO 712 to osmotic stress.

We examined the influence of lowering the a_w with NaCl on growth at low potassium concentrations in some detail. NaCl was more inhibitory than fructose at low potassium concentration. This correlated well with the data presented on potassium accumulation at low a_w. The effect of NaCl was due to the cation as opposed to the chloride anion, as Na_2SO_4 and NH_4Cl were also inhibitory.

The potassium content of the growing cells also correlated well with the extent of growth inhibition observed. The potassium content of cells grown in MTEL medium at low potassium concentration in which the a_w was lowered by sodium or ammonium chloride was lower than that of cultures grown in the absence of osmotic stress, In contrast, if sufficient (20 mM) potassium was supplied the potassium content was greater in the presence of the solutes than in their absence. The potassium content of the cultures and the specific growth rate attained, appeared to be linked. Ammonium chloride was more inhibitory than was NaCl and this was also reflected in the potassium content of the respective cultures.

The role of potassium in the maintenance of turgor pressure in bacteria is well known. We tried to ascertain if the inhibition by NaCl at low potassium concentration related to a decrease in turgor pressure. The partial reversal of the inhibition by glycine betaine, and the slightly increased inhibition observed when proline was omitted from the medium suggest that reduced turgor pressure was at least partially responsible for the inhibition observed.

One of the consequences of reduced turgor pressure is a decrease in the cytoplasmic volume. The volume of cells grown in MTEL medium containing 0.5 mM potassium and 1.5% w/v NaCl was approximately 30% less than that observed for cells grown at the same NaCl concentration but with 20 mM potassium. In medium which included glycine betaine, the decrease in the cytoplasmic volume was not as large. These observations suggest that turgor pressure is reduced with a concomitant decrease in the cytoplasmic volume when the a_w is lowered with NaCl. The bacterial cell wall is somewhat elastic and subject to stretching forces as a result of internal turgor pressure (Koch, 1995). It is possible that the reduced cellular potassium content reduces the turgor pressure sufficiently to cause a reduction in the cell volume due to contraction of the cell wall. It is also possible however, that the reduction in volume was an artifact of the reduced growth rate and was independent of the turgor pressure.

Another important cell function which may be affected as a result of potassium limitation was the generation of the proton-motive force (p.m.f.). Generation of a transmembrane pH gradient in *E. coli* is dependent on potassium accumulation. Uptake of potassium ions by *Lc lactis* is thought to be essential to balance the the expulsion of protons from the cytoplasm, and tends to reduce the transmembrane electrochemical potential ($\Delta\Psi$) while promoting an increase in the ΔpH. (Kashket and Barker, 1977). Poolman *et al.* (1987) reported that potassium depleted *Lc lactis* cells were unable to regulate their cytoplasmic pH. Failure to accumulate potassium could theoretically impair the ability of the cells to maintain their cytoplasmic pH and also could lead to an increase in $\Delta\Psi$. The values which we obtained for the ΔpH and $\Delta\Psi$ in the absence of NaCl are similar to those of Kashket and Barker (1977). The $\Delta\Psi$ was lower in the

presence of 20 mM K^+ than with 0.5 mM K^+ which was partially compensated by a rise in the pH gradient so that only a relatively slight drop in the p.m.f. was observed. Our measurements reveal that the reduction in the cytoplasmic potassium content observed in the presence of NaCl at low extracellular potassium concentrations, did not greatly affect the cytoplasmic pH or $\Delta\Psi$. As would be expected, there was a slightly lower ΔpH and a slightly higher ΔY but the magnitude of the changes observed were unlikely to be the cause of a significant level of inhibition. The presence of NaCl did prevent the dissipation of $\Delta\Psi$ noted at 20 mM K^+ in the absence of NaCl , presumably due to a decrease in the rate of potassium transport.

These experiments also provide an insight into the importance of both potassium and glycine betaine in the osmoregulation of *Lc. lactis*. Provided that potassium is freely available, glycine betaine did not appear to be necessary for growth in the presence of 1.5% NaCl, and addition of glycine betaine did not result in any stimulation of growth. When potassium uptake was limited however, the addition of glycine betaine to the cultures was stimulatory. This suggests that turgor pressure may be the signal for activation of glycine betaine transport as there is evidence to suggest that loss of turgor had occurred in those circumstances. It is notable however, that addition of glycine betaine was not able to fully restore either the growth rate or the cell volume to their normal levels. This may imply that at least some potassium accumulation is necessary for proper osmoregulation.

Chapter V

Measurement of the potassium concentration required for growth and characterisation of potassium transport in *Lactococcus lactis* subsp. *cremoris* NCDO 712.

Abstract.

Growth of *Lc. lactis* subsp. *cremoris* NCDO 712 in chemically defined medium was dependent on the potassium concentration of the medium at potassium concentrations ≤ 0.5 mM. Growth was not initiated at potassium concentrations ≤ 30-40 μM and in medium containing higher potassium concentrations, growth ceased when the potassium concentration was reduced to 30-40 mM. Rubidium could substitute for potassium, but only on a temporary basis.

Assay of the rate of potassium transport by intact, exponential phase cells of NCDO 712 showed that the transport system displayed Michaelis-Menten kinetics. At pH 7.0 which was optimal for transport of potassium the apparent Km was 0.75 mM and the maximal velocity was 250 nmol/mg. prt/min. Eadie-Hofstee plots indicated that only one transport system was involved. No transport occurred in the absence of an energy source, either glucose or arginine and transport was strongly inhibited by dicyclohexylcarbodiimide (DCCD) and partially by nigericin. These results suggest that the system is an active transport system requiring either ATP, or proton-motive-force or both. Potassium transport was inhibited by the monovalent cations Na, Cs, Li and NH_4^+ at concentrations well below those which significantly reduce the a_w. Double-reciprocal plots indicate that the inhibition was competitive. Potassium transport was non-competitively inhibited when the a_w of the assay was reduced by addition of glucose. The specific activity of potassium transport was not increased when the potassium concentration of the growth medium was decreased from 20 to 0.2 mM but it was increased slightly (up to two-fold) when the a_w of the growth medium was reduced substantially with either glucose or NaCl. This suggests a slight induction (increased synthesis of transporter) when the cells are growing in conditions of osmotic stress.

5.1 Introduction.

Potassium is an essential nutrient for bacteria, and many bacteria possess both low-affinity and high affinity potassium transport systems, thereby ensuring their ability to adapt to changes in potassium availability. Some of these transport systems also transport rubidium; albeit at a lower rate, and in some bacteria rubidium can replace potassium as a nutrient.

The potassium transport systems of *Escherichia coli* have been well characterised; several different low affinity systems (Bakker, 1993) and one high affinity transport system (Siebers and Altendorf, 1993) have been identified. In Gram-positive bacteria, potassium transport by the lactic acid bacterium *Enterococcus hirae* is perhaps the best studied (Kakinuma, 1993). Potassium acts as a compatible solute and potassium transport is also intrinsic to pH homeostasis and maintenance of proton motive force (Kroll and Booth, 1983; Kashket and Barker, 1981).

The potassium content of many bacteria increases dramatically on exposure to osmotic stress. In *E. coli* the high affinity Kdp system is induced by either potassium limitation or osmotic stress (Gowrishankar, 1993; Malli and Epstein, 1998). Of the three low affinity systems, the Trk G and H systems are most involved in adaptation to higher osmolarity. Trk G and H are constitutively expressed (Rhoads *et al.*, 1976); however, the activities of both systems are greatly increased by osmotic upshock. The increased activity is a consequence of regulation of transport activity rather than synthesis of additional enzyme (Meury *et al.*, 1985). In *Bacillus acidocaldarius*, high (Bakker *et al.*, 1987), and low affinity (Michels and Bakker, 1987) potassium transport systems have been characterised and in *Methanobacterium thermoautotrophicum* a low affinity system, induced by potassium limitation has been described (Glasemacher *et al.*, 1996).

In the previous chapter it was demonstrated that *Lc lactis* NCDO 712 accumulated potassium in response to a decrease in a_w and that potassium accumulation was inhibited by Na^+ and other monovalent cations. In this chapter a low affinity potassium transport system in *Lc. lactis* NCDO 712 is described. Inhibition by Na^+

and NH_4^+ ions was determined, together with the effect of growth under osmotic stress on the kinetics of potassium transport.

5.2. Results.

5.2.1 The effect of potassium limitation on the growth of *Lc lactis* subsp. *cremoris* NCDO 712.

In MTEL medium buffered with MOPS-Tris at pH 6.8, a potassium concentration of 0.5 mM was sufficient to support growth of *Lc. lactis* NCDO 712 at the maximal rate. Buffering with MOPS rather than disodium *B*-glycerophosphate allowed the sodium content of the medium to be kept to a minimum. If the potassium concentration of the growth medium was less than 0.5 mM the specific growth rate and biomass yield decreased (Fig. 5.1, Table 5.1). The influence of potassium limitation on both the specific growth rate and growth yield was most pronounced at potassium concentrations < 0.1 mM, below which there was a good linear relationship between potassium concentration and specific growth rate. It was not possible to eliminate all traces of potassium from MTEL medium; on average the potassium content of MTEL without added potassium was 29 μM. This concentration was not sufficient to support growth; the lowest concentration added was 20 μM which gave a total potassium content of 49 μM and a small amount of growth occurred at this concentration (Table 5.1). There was a linear relationship between the potassium concentration and growth at potassium concentrations ≤ 0.1 mM; it is therefore reasonable to assume that at potassium concentrations ≤ 0.1 mM, cessation of growth was due to potassium limitation. The residual potassium concentrations i.e. the potassium remaining after cessation of growth in MTEL medium, are shown in Table 5.1. It is clear from these results that potassium concentrations ≤ 30 μM approximately are incapable of supporting the growth of NCDO 712. The yield of biomass was relatively constant at 60-70 g dry wt/g potassium (Table 5.1). The relationship between the specific growth rate and the potassium content of the MTEL medium displayed approximate Michaelis-menten kinetics.

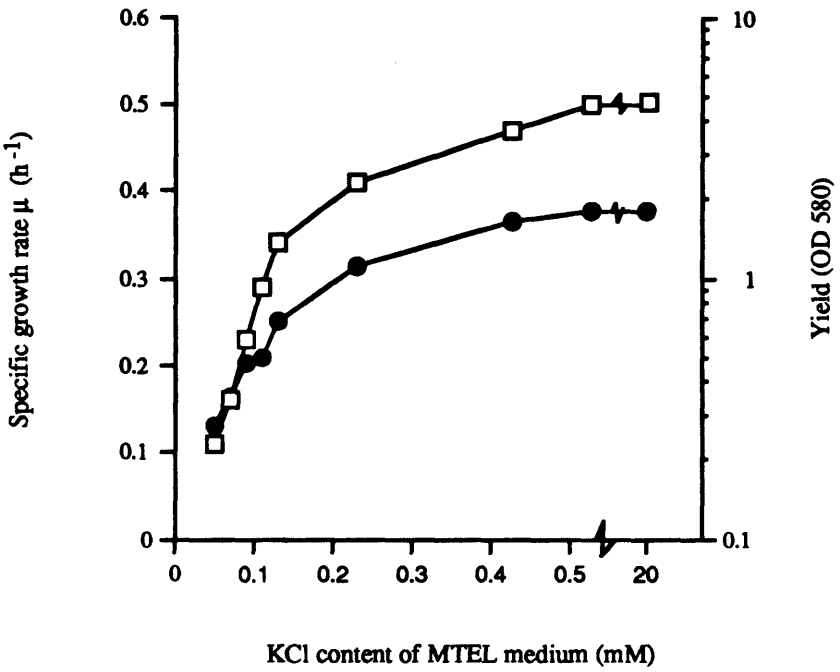


Figure 5.1 The specific growth rate μ (□) and final growth yield (●) of *L. lactis* subsp. *cremoris* NCDO 712 growing in MTEL medium at varied potassium concentrations. The data are representative of triplicate experiments.

Table 5.1 The specific growth rate, μ (h^{-1}), growth yield and residual potassium concentrations (following growth) of *Lc. lactis* subsp *cremoris* NCDO 712 in MTEL medium buffered with MOPS-Tris and containing different levels of KCl.

KCl (μM)	μ (h^{-1})	Final biomass (OD 580)	Residual K^+ (μM)	Yield coefficient (g dry weight/g K^+)
49	0.11±0.03	0.19±0.04	33±3	78
69	0.16±0.04	0.28±0.1	36±4	66
89	0.23±0.03	0.39±0.1	42±8	65
109	0.29±0.02	0.5±0.16	44±10	60
129	0.34±0.03	0.57±0.05	45±5	63

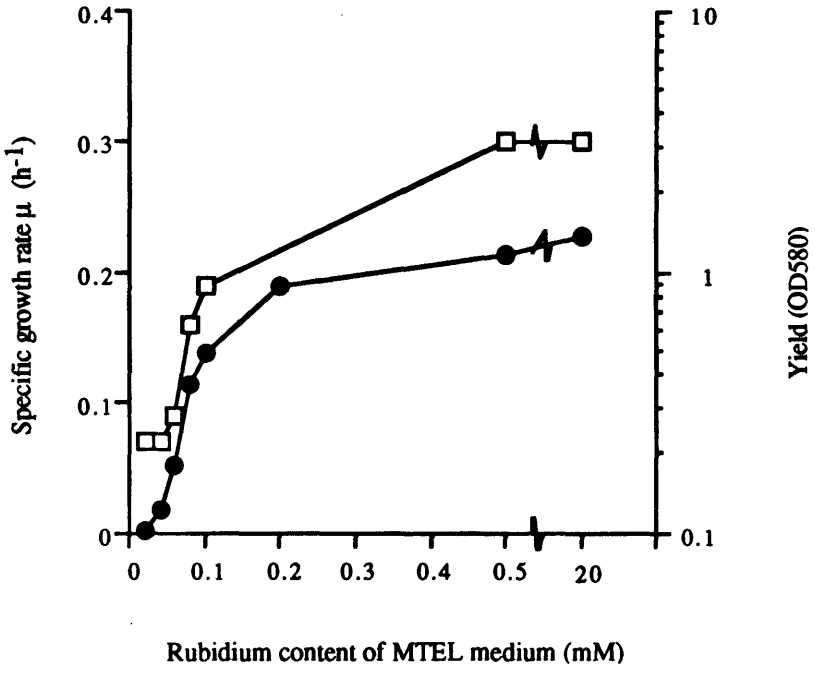


Figure 5.2 The specific growth rate, μ (□) and final growth yield (●) of *Lc. lactis* subsp. *cremoris* NCDO 712 growing in MTEL medium at varied rubidium concentrations. The data are representative of triplicate experiments

The apparent Km value (Ks) was 100 μM which is the potassium concentration required to maintain the specific growth rate at half the maximal rate.

When the potassium of MTEL was substituted by rubidium there was a similar relationship between the concentration of rubidium in the growth medium, and the specific growth rate and growth yield (Fig. 5.2). The Ks value for rubidium appeared to be similar It is clear that rubidium did not support growth at the same rate as an equivalent potassium concentration but the growth yield was similar to that observed with potassium. These results were obtained using cells which had been grown in medium containing potassium, washed, and inoculated into medium with rubidium replacing potassium. When it was attempted to subculture cells a second time in medium containing rubidium, growth was poor.

5.2.2 Characterisation of potassium transport by NCDO 712.

Potassium transport was assayed in exponential phase cells grown in GM-17 medium, harvested, washed and depleted of potassium by loading with Na^+ as described in the materials and methods section (Chapter II). The specific activity of the system was pH dependent, with an optimum pH of approximately 7.0 (Fig. 5.3), and all kinetic measurements were performed at pH 7.0. The transport of potassium by NCDO 712 displayed Michaelis-Menten kinetics with a Km value of 0.74 mM and a Vmax of 256 nmol/mg. prt./ minute (Fig. 5.4 a and b). Measurement of potassium transport over a wide range of substrate concentrations and plotting the data as Eadie-Hofstee plots, indicated that under the assay conditions used, there was only one transport system active (Fig. 5.4c). Transport required the presence of an energy source either glucose or arginine though the latter was not as effective as the former. (Fig. 5.5). The ionopore nigericin, which dissipates the transmembrane pH gradient,substantially reduced the accumulation of potassium (Fig.5.5). Addition of the H^+-ATPase inhibitor dicyclohexylcarbodiimide (DCCD) greatly reduced the rate of potassium transport (Fig. 5.5).

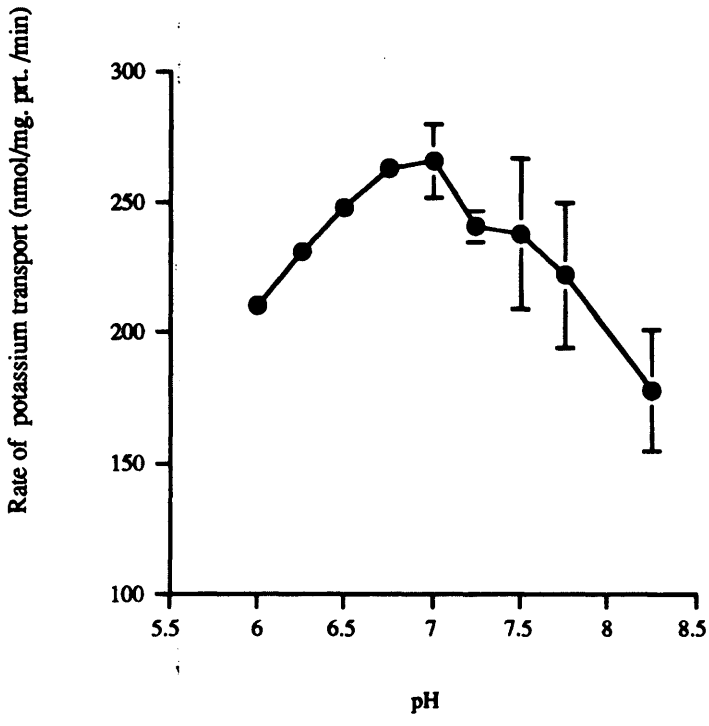


Fig. 5.3 The effect of the pH of the assay buffer on potassium transport by *Lc lactis* subsp. *cremoris* NCDO 712. The cells were harvested from exponentially growing cultures at an OD580 of 0.5-0.7 and depleted of potassium by loading with Na^+ as described in materials and methods. The potassium depleted cells were assayed in MTB-$MgSO_4$ buffer at the appropriate pH.

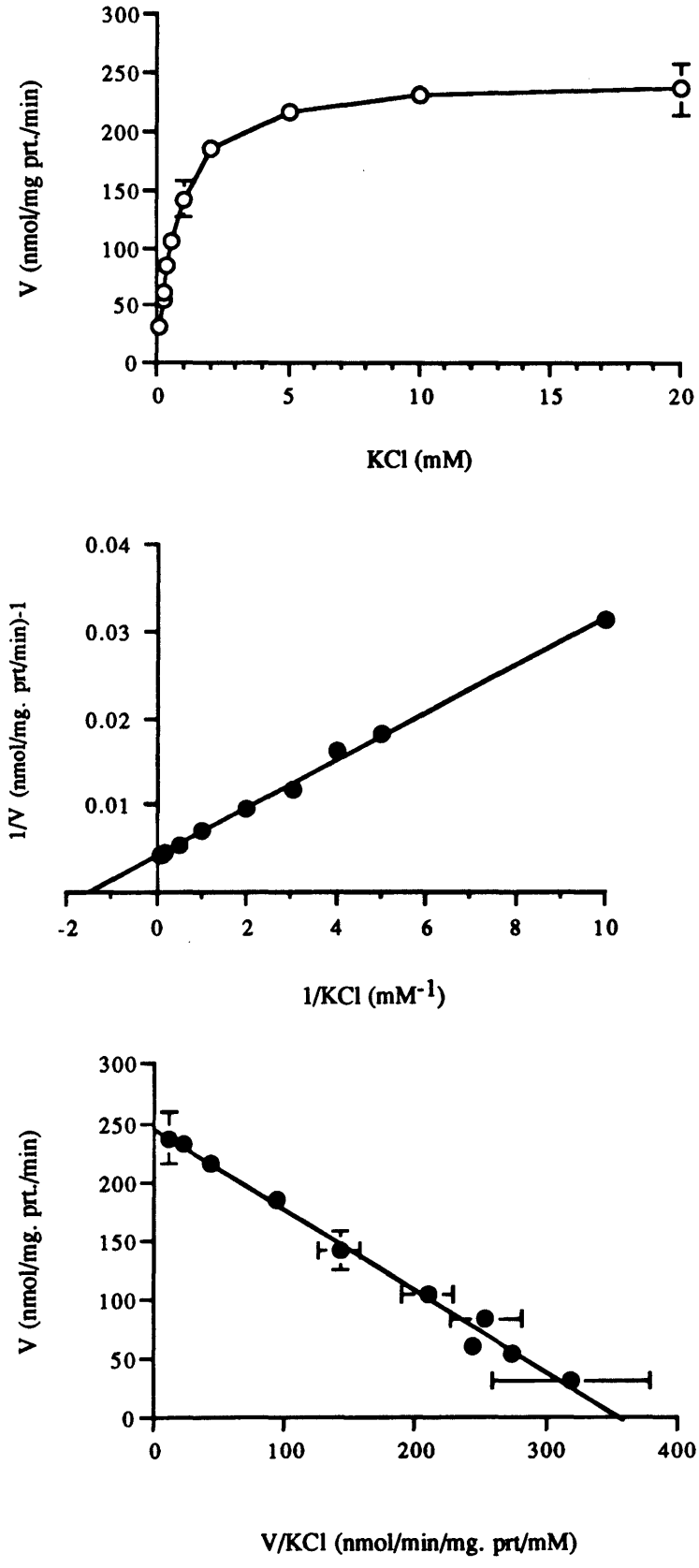


Fig. 5.4 The kinetics of potassium transport by *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in GM-17 medium, depleted of potassium and assayed in MTB-$MgSO_4$ buffer. (A) Rate of transport vs substrate concentration (B) Lineweaver-Burk plot, (C) Eadie-Hofstee plot.

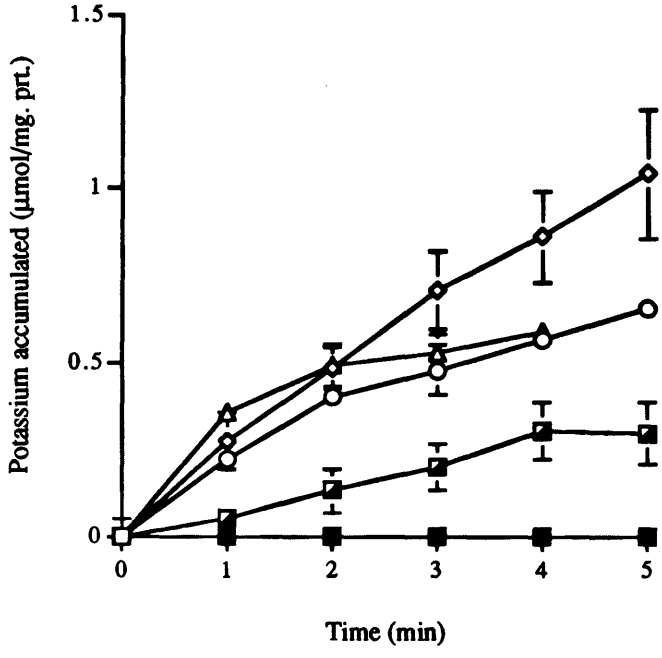


Fig. 5.5 The requirement for an energy source and the effect of nigericin and DCCD on potassium transport by *Lc. lactis* subsp. *cremoris* NCDO 712. The cells were grown in GM-17 medium, depleted of potassium and assayed in MTB-$MgSO_4$ in the absence of an energy source (■), with 27 mM glucose (◆), or 27 mM arginine (○). The effect of adding 1µM nigericin (▲) or 0.3 mM DCCD (◪) to the assay systems containing glucose is also shown.

5.2.3 Inhibition of potassium transport by other monovalent cations

The results presented in the previous chapter suggest that the presence of monovalent cations such as sodium inhibits potassium transport by NCDO 712. This was confirmed by measuring the rate of potassium transport by NCDO 712 in the presence and absence of different cations. In the absence of potential inhibitors, reducing the potassium content of the assay to 0.5 mM from 20 mM resulted in a 50% decrease in the rate of potassium transport (Fig. 5.6a). In contrast, if sodium chloride was present in the assay, reducing the potassium concentration resulted in a substantial further reduction in the rate of potassium transport at both potassium concentrations (Fig. 5.6a). The cells were depleted of potassium prior to these assays by loading with sodium ions. To verify that the inhibition was not due to inhibition of sodium efflux, choline loaded cells were also assayed (Fig. 5.6b) and the same patterns of inhibition were observed. In chapter IV the inhibition of growth by potassium limitation was not observed when the a_w was reduced by fructose or glucose. The influence of glucose on transport of potassium is shown in Fig. 5.7, and it is clear that the presence of these sugars in the assay only slightly inhibited the rate of potasssium transport.

In order to rapidly assess the inhibitory effect of other monovalent cations a simple experiment was devised to allow the effects of different cations to be compared. A potassium concentration (2 mM) was selected with which the rate of potassium transport was reduced substantially (≥30%) in the presence of 100 mM NaCl in the assay. The rate of potassium transport in the presence of 100 mM cesium chloride, lithium chloride, ammonium chloride and 50 mM sodium sulphate was then determined. LiCl was the most inhibitory of the compounds tested; the effect of Cs^+ was similar to that of Na^+ while NH_4^+ ions were more inhibitory than Na^+ but less so than Li^+. The use of Na_2SO_4 confirmed that it was the cation rather than the anion which was responsible for the inhibition (Table 5.2).

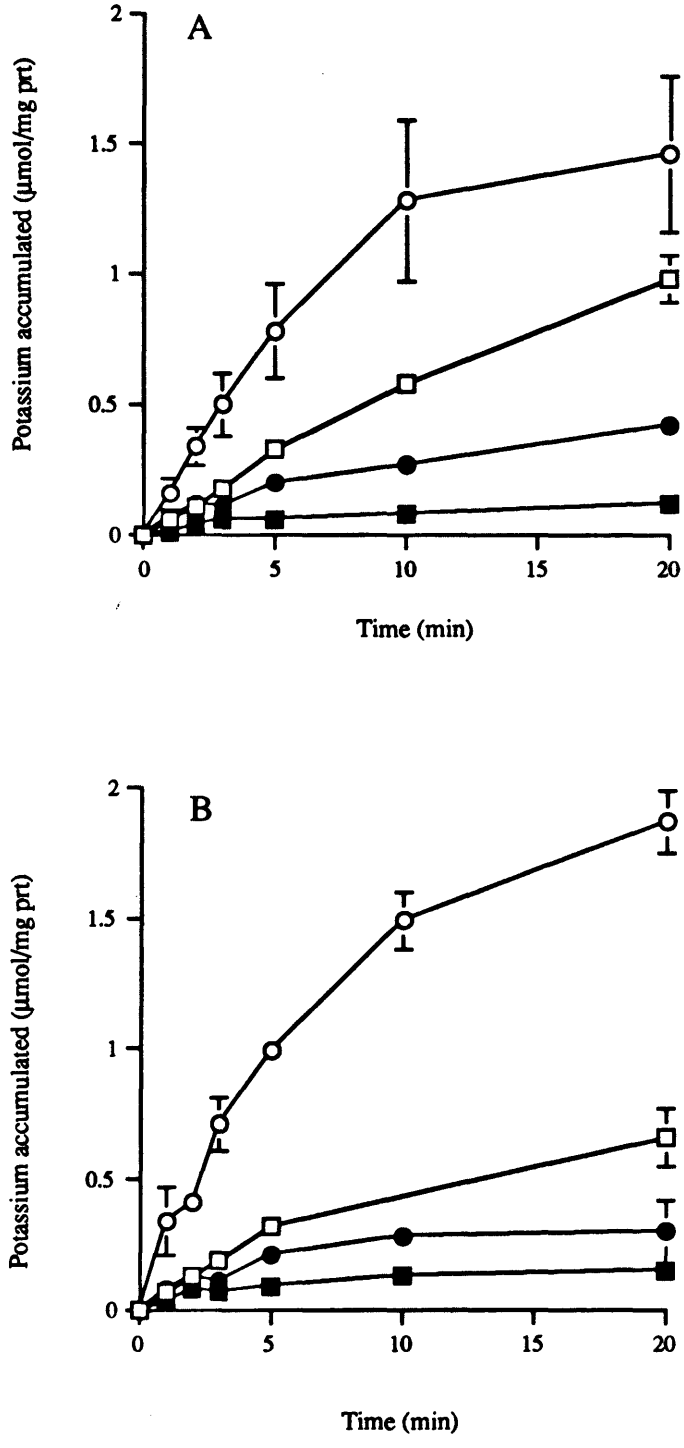


Fig. 5.6 Potassium transport by *Lc. lactis* subsp. *cremoris* NCDO 712 cells depleted of potassium by loading with either sodium (A) or choline (B) and assayed in MTB-$MgSO_4$ buffer (pH 7.0) with either 0.5 (■, □) or 20.0 mM (●, ○) potassium in the absence (open symbols) or presence (closed symbols) of 0.25M NaCl (a_w, 0.990).

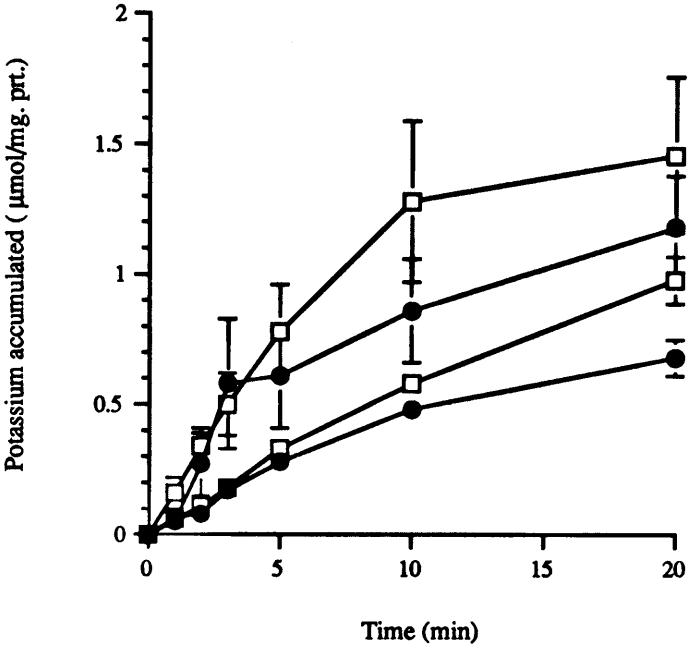


Fig. 5.7 Potassium transport by *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in GM-17 medium and assayed in MTB-MgSO 4 with either 0.5 mM (□, ■) or 20 mM (●, O) potassium in the absence (open symbols) or presence (closed symbols) of 0.44M glucose (aw, 0.990).

The inhibition of potassium transport by Na^+ and NH_4^+ was due to competitive inhibition; the Vmax value in the presence of either cation was unchanged whereas the Km value increased in proportion to the inhibitor concentration (Fig. 5.8) NH_4^+ was a much more potent inhibitor than was Na^+. The Ki value for each inhibitor (defined as the inhibitor concentration required to halve the slope of a Lineweaver-Burk plot) was determined by plotting the apparent Km values against the inhibitor concentrations, the slope of the resulting straight-line relationship being equal to Km/Ki (Fig. 5.9). The Ki value for NH_4^+ (38 mM) was much less than that for Na^+ (200 mM).

Lc. lactis NCDO 712 cells depleted of potassium by loading with choline did not accumulate sodium ions when sodium was used instead of potassium in transport assays at concentrations up to 100 mM.

5.2.4 The effect of decreased a_w on the activity of the potassium transporter.

In many bacteria the activity of compatible solute transporters is enhanced if the osmolarity of the assay buffer is increased. Our measurements of the activity of the potassium transport system in NCDO 712 cells indicate that decreasing the a_w of the assay buffer results in a reduction in potassium transport. In buffer containing 0.88M glucose, the rate of potassium transport by intact cells which ahad been grown in GM-17 medium containing 0.88M glucose was approx 50% of that observed in the absence of the solute (Fig. 5.10). The inhibition observed was non-competitive. i.e the Vmax for potassium transport was reduced, without any effect on the Km (Fig. 5.10).

5.2.5 Potassium transport was induced by pregrowth in medium with reduced a_w but not by potassium limitation.

We have previously observed that potassium was accumulated by *Lc. lactis* subsp. *cremoris* in response to a decrease in a_w (4.2.2) but that decreased a_w tends to

Table 5.2 Inhibition of potassium transport of NCDO 712 cells by monovalent cations.

Solute added	Conc. (mM)	Rate of transport nmol/mg. prt./min
None	-	186±6
NaCl	100	132±10
Na_2SO_4	50	137±17
CsCl	100	101±30
NH_4Cl	100	119±3
LiCl	100	97±1

The KCl concentration in the assay was 2 mM. The cells were depleted of potassium by sodium loading as described in the materials and methods section.

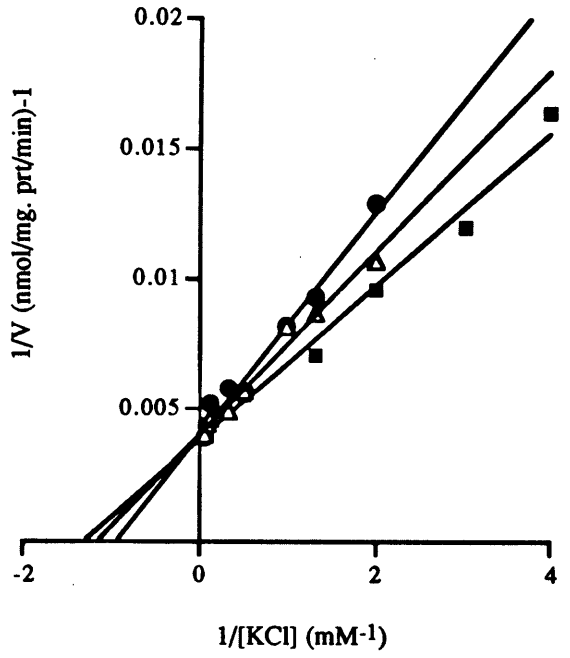


Fig. 5.8a Lineweaver-Burk plot showing inhibition of potassium transport in *Lc. lactis* subsp. *cremoris* NCDO 712 by NaCl. The NaCl concentration of the assay was either 0 (■) 50 mM (▲) or 100 mM (●).

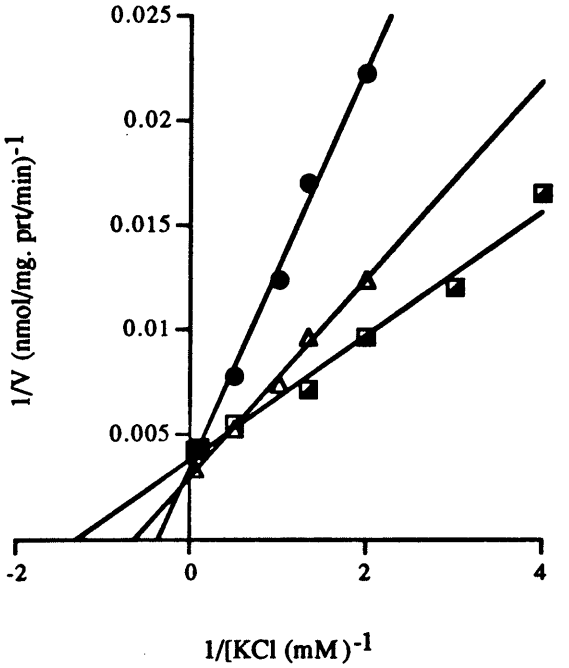


Fig. 5.8b Lineweaver-Burk plot showing inhibition of potassium transport in *Lc. lactis* subsp. *cremoris* NCDO 712 by NH_4Cl ions. The NH_4Cl concentration of the assay was either 0 (■) 50 mM (▲) or 100 mM (●).

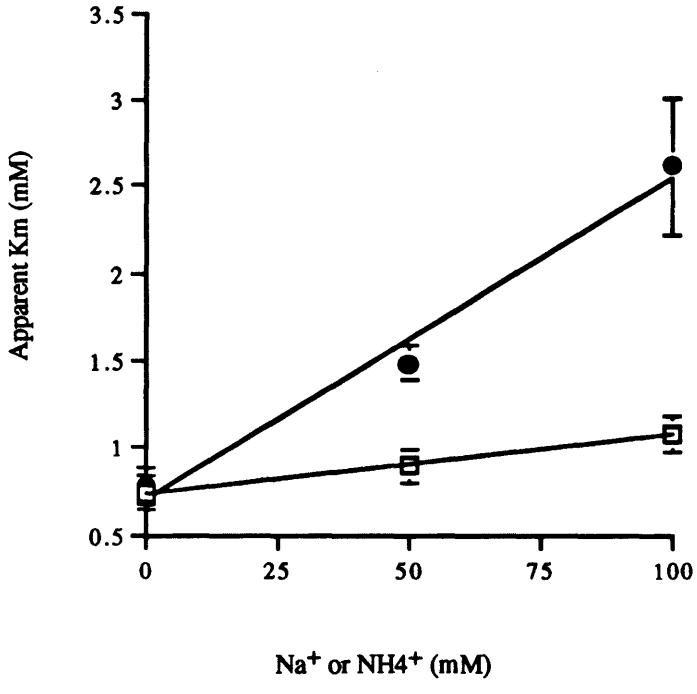


Fig. 5.9 Plot of the apparent Km for potassium transport in *Lc. lactis* NCDO 712 against the inhibitor concentration for Na^+ (□) and NH_4^+ (●). The slope of the lines is Km/Ki.

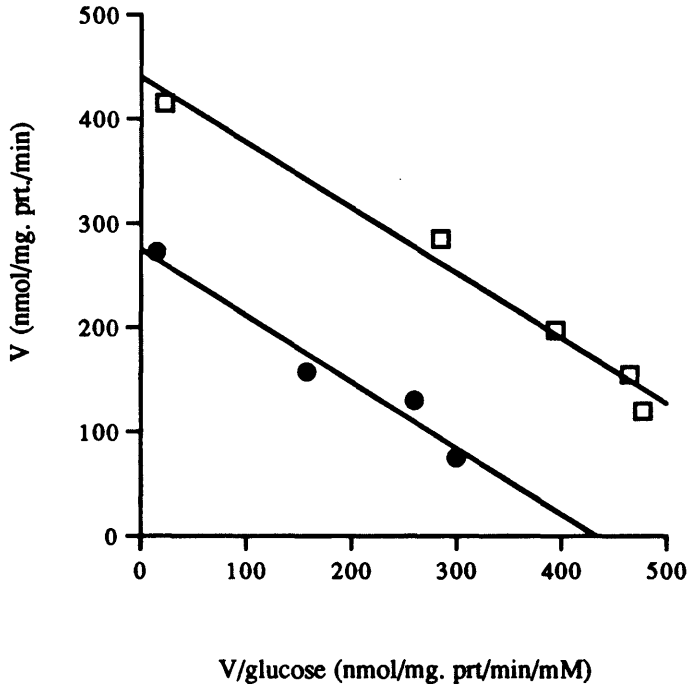


Fig. 5.10 Inhibition of potassium transport by increasing the solute concentration of the assay in *Lc. lactis* subsp. *cremoris* NCDO 712. The cells were grown in GM-17 broth containing 0.88M glucose, depleted of potassium and assayed in MTB-$MgSO_4$ buffer pH 7.0 without (□) or with 0.88M glucose (●).

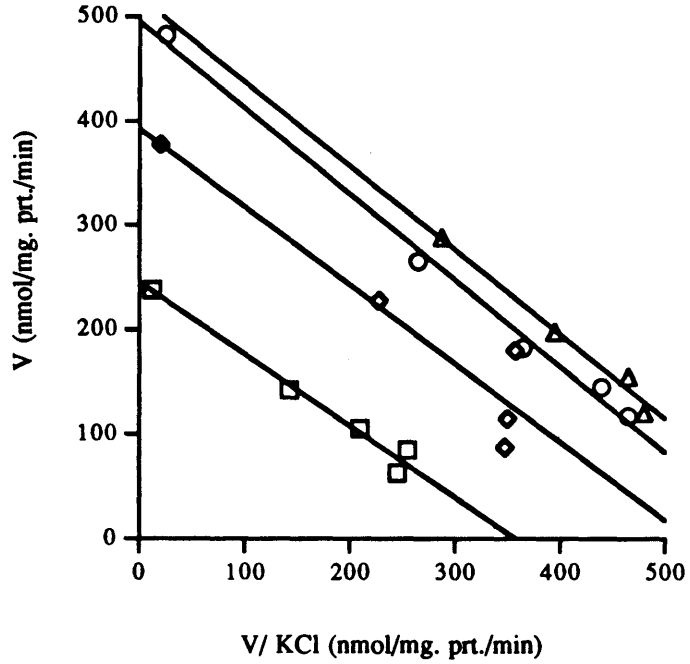


Fig. 5.11a Kinetics of potassium transport by *Lc. lactis* subsp. *cremoris* NCDO 712. The cells were grown in GM-17 medium without added solute (□), or with glucose at 0.3 M (◇), 0.6M (○), or 0.88M (△). The cells were depleted of potassium and assayed in MTB-$MgSO_4$ buffer at pH 7.0 without added solute

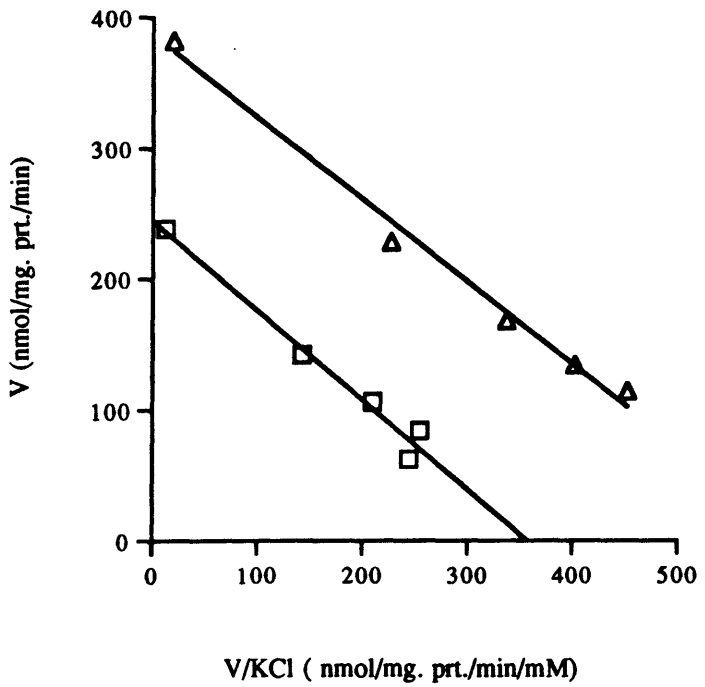


Fig. 5.11b Kinetics of potassium transport by *Lc lactis* subsp. *cremoris* NCDO 712 grown in GM-17 medium (□) or GM-17 medium containing 3% w/v NaCl (△) The cells were depleted of potassium and assayed in MTB-$MgSO_4$ buffer pH 7.0 without added solute.

Table 5.3 The effect of reducing the potassium content of MTEL medium on the apparent Km and Vmax for potassium transport by *Lc. lactis* subsp. *cremoris* NCDO 712.

KCl concentration of MTEL (mM)	Apparent Km (mM)	Maximal rate of transport (Vmax) (nM/mg prt./min)
0.2	0.72±0.3	279±21
0.3	0.29±0.03	238±20
0.5	0.26±0.06	233±15
1.0	0.42±0.09	272±14
20	0.5±0.15	272±78

reduce the rate of transport (Fig. 5.2a). This suggests that the increased cytoplasmic potassium levels observed may result from an increase in the amount of potassium transporter in the cells growing at low a_w which would overcome the loss in activity due to osmotic inhibition by high concentrations of solute. As mentioned already, the maximum rate of potassium transport by cells grown in GM-17 medium was 250 nmol/mg prt/ min. Decreasing the a_w of the growth medium by addition of glucose or NaCl caused an increase in the specific activity of the potassium transport system (Fig. 5.11 a, b). Growth in medium with low a_w did not alter the apparent Km for potassium transport (Fig. 5.11 a, b) indicating that the induced transporter had the same affinity for potassium as the uninduced transporter.

In some bacteria potassium transport systems are induced in response to potassium limitation. Since low a_w inhibited the rate of potassium transport by NCDO 712 cells it was conceivable that the increased specific activity for potassium transport was a response to potassium limitation rather than to osmotic stress. Reducing the potassium concentration of MTEL medium to 0.2 mM did not produce any change in the specific activity, or the apparent Km of the potassium transport system (Table 5.3). The rate of potassium transport predicted under these conditions (estimated from the kinetic data obtained earlier) was less than 30% of the predicted rate expected in cells grown and assayed in medium containing 0.88 M monosaccharide.

5.3 Discussion.

At potassium concentrations < 100 μM approximately, the specific growth rate and growth yield of NCDO 712 were dependent on the potassium concentration of the growth medium. No growth occurred at potassium concentrations ≤ 30 μM, and the final potassium concentration following cessation of growth was approx 30 μM also. This suggests that *Lc. lactis* subsp. *cremoris* NCDO 712 does not possess a high affinity potassium transport system similar to the Kdp system of *Escherichia coli* (Rhoads *et al.* 1976) and several other bacteria (Bakker *et al.*, 1987; Abee *et al.*, 1992). The Kdp system in *E. coli* has a Km value for potassium of about 2 μM (Bakker,

1993) and allows growth to occur under conditions of low potassium availability as it can scavenge potassium down to concentrations of around 50 nM. The yield of *Lc. lactis* NCDO 712 biomass per gram of potassium utilised was 60-70g dry wt/g potassium which is in agreement with values determined for other bacteria (Pirt, 1975).

Potassium transport by NCDO 712 had a pH optimum of 7.0 and obeyed Michaelis-Menten kinetics. Eadie-Hofstee plots of transport assay data at potassium concentrations from 0.1 to 20 mM were straight lines which indicated that only one potassium transport system was present. Eadie Hofstee plots are an accepted method of detecting transport systems with differing affinities (for an example see Pourkomailan and Booth, 1992). Transport of potassium was substantially reduced by the presence of DCCD which inhibits the H^+-ATPase and addition of the ionopore nigericin also inhibited potassium transport, particularly later in the assay. This suggests that proton motive force is necessary for potassium transport. DCCD inhibits the H^+ ATPase preventing export of protons and nigericin short-circuits the proton-motive force by allowing re-entry of exported protons. ATP production by metabolism of glucose or arginine is also necessary for activity of the potassium transport system. The data suggest that the transport system is similar to the Ktr I system of *Ent. hirae* (Bakker and Harold, 1980) or the TrkG and TrkH systems of *E. coli.* (Dosch *et al.*, 1991). It has been suggested that potassium transport in *Ent. hirae* occurs via a secondary potassium/proton symport which requires ATP and efflux of two protons via the H+ ATPase (Bakker and Harold, 1980; Kakinuma, 1993). Unlike the Ktr I and Trk systems, potassium transport in *Lc lactis* was induced by high solute concentrations. The Trk systems are constitutively expressed and controlled at the activity level by extracellular osmolarity (Bakker, 1993). KtrI is neither induced nor activated by high solute concentration (Kakinuma, 1993). Inhibition by high solute concentration was observed for potassium transport by NCDO 712.

In Chapter IV we reported that the growth rate and potassium content of NCDO 712 were substantially reduced in cells grown in the presence of NaCl or NH_4Cl at low potassium concentrations. The data in Fig. 5.5 support the proposition that the

inhibition of growth by NaCl was caused by inhibition of potassium transport. The inhibitory effect was not confined to Na^+ ions as Cs^+, Li^+ and NH_4^+ ions also had an inhibitory effect on potassium transport (Table 5.2). Inhibition of potassium uptake was by competitive inhibition and $NH4^+$ ions produced more substantial inhibition than did Na^+ ions (Figs.5.7 and 5.8). There was therefore a good correlation between the degree of inhibition of potassium transport and inhibition of growth rate by Na^+ and NH_4^+ ions. Apart from the observations of Macleod and Snell (1948) there have been few reports in the literature of interference with the potassium requirements of lactococci by other monovalent cations. In general, potassium transport systems are regarded as having a high degree of discrimination between potassium and other monovalent cations (Doyle *et al.* 1998). The degree of discrimination is greater against the smaller cations Li^+ (0.6 Å) and Na^+ (0.95 Å) than the larger alkali cations rubidium (1.48 Å) and cesium (1.69 Å). The close similarity in size of rubidium and potassium (1.33 Å) is thought to be the reason that rubidium can act as a potassium analogue (Doyle *et al.* 1998). In the case of NCDO 712, discrimination by the potassium transport system against other monovalent cations seems to be poor and it is the smaller cations which tend to be more inhibitory. The system in NCDO 712 may be similar to that of *Ent. hirae* in which a mutant with a potassium transport system more strongly inhibited by Na^+ than the wild type transport has been reported. The mutation is presumably due to an alteration in the structure of the potassium transporter giving it a greater affinity for Na^+ than its parent enzyme (Harold and Baarda, 1967).

In addition to competitive inhibition by monovalent cations, potassium transport in NCDO 712 was also subject to non-competitive inhibition by high solute concentration. 0.88M glucose reduced the rate of potassium transport by approx 50%. This inhibition is possibly due to reduced turgor pressure affecting the conformation of the membrane bound transporter protein. The inhibitory effect of high solute concentration on the rate of potassium transport was unexpected; in general, transport systems with a role in osmotolerance are activated rather than inhibited by osmotic stress.

Induction of the potassium transport system was observed when glucose or NaCl was added to reduce the a_w of the growth medium, though the extent of the induction was at most two-fold. The possibility existed that the induction of potassium transport activity that was observed was not due to osmotic stress *per se*, but rather to the lower rate of potassium transport resulting from non-competitive inhibition by high solute concentration. However, reducing the potassium concentration in MTEL medium from 20 mM to 0.2 mM did not induce a greater amount of potassium transporter, even though the rate of potassium transport (calculated from the Michaelis-Menten equation) decreased from 240 nmol/mg prt./min to 50 nmol/mg prt/min. It therefore seems likely that the increased rate of potassium transport observed in cells grown in reduced a_w conditions was due to osmotic induction. The fact that low a_w caused by either glucose or NaCl which are structurally different induced increased levels of the potassium transporter supports this view.

It is clear that the potassium transport system of *Lc. lactis* subsp. *lactis* NCDO 712 responds to osmotic stress. What is not clear is how the activity of the system is controlled so as to increase the cell potassium content when required. The activity of the *E. coli* Trk systems is stimulated at low turgor pressure (Bakker, 1993), whereas the activity of the *Lc. lactis* system is reduced. A further factor to be taken into consideration is the role of potassium in maintaining the pH gradient and $\Delta \Psi$. Potassium accumulation is essential for the maintenance of a transmembrane pH gradient in *Lc. lactis*, as potassium uptake is necessary to balance the expulsion of protons from the cytoplasm (Kashket and Barker, 1977). Presumably, potassium accumulation in response to proton efflux occurs electrogenically and in response to changes in the membrane potential, $\Delta \Psi$. In contrast, osmotically mediated potassium uptake needs to be regulated by turgor pressure. Otherwise the increased uptake resulting from the higher specific activity of the transport system will result in efflux of excess potassium once turgor is poised at the required level. Such futile cycling of potassium is wasteful of energy; it is therefore likely that the potassium transport system is also regulated in some way by turgor pressure.

In conclusion, the data presented in this chapter provides an explanation for the observations made in the previous chapter, on potassium accumulation by *L. lactis* subsp. *cremoris* NCDO 712 and its inhibition by other monovalent cations.

Chapter VI

Utilisation of glycine betaine and other compatible solutes by *Lactococcus lactis*: correlation between low a_w sensitivity and absence of glycine betaine transport activity.

Abstract

This study investigates the utilisation of compatible solutes by *Lc. lactis* subsp. *cremoris* NCDO 712. Both glycine betaine and proline relieved the inhibition of growth rate and yield of NCDO 712 caused by growth at low water activity (a_w). Proline was much less stimulatory than was glycine betaine. The dipeptide leucyl-proline relieved the inhibition of growth rate to a similar degree as proline. A similar degree of growth inhibition was observed when the a_w was reduced by either NaCl or glucose and glycine betaine was equally effective in counteracting the inhibition by either. Carnitine and ectoine, which are known to act as compatible solutes in other bacteria did not relieve the inhibition of growth of NCDO 712 at low a_w. The difference between the *Lc. lactis* strains capable of growth at 4% w/v NaCl and those which do not grow at this NaCl concentration was investigated. The sensitive *cremoris* strains have an upper limit for growth of approximately 2% w/v NaCl. Inhibition of growth of these strains by NaCl was not relieved by addition of glycine betaine while a slight degree of relief was observed for one strain if the a_w was reduced by glucose. The *lactis* strains were capable of growth at 4% w/v NaCl and growth inhibition by either NaCl or glucose was substantially relieved by glycine betaine. All the strains capable of growth at 4% w/v NaCl accumulated significant amounts of glycine betaine when growing at low a_w. Very little accumulation of glycine betaine occurred during growth of the NaCl sensitive strains. The NaCl tolerant strains had substantial levels of glycine betaine transport activity *in vitro*, whereas the NaCl sensitive strains had little or no such activity. A low a_w sensitive mutant of *Lc. lactis* subsp. *cremoris* MG1363 was isolated following IS*S1* insertional mutagenesis. This mutant was inhibited at the a_w of 0.988 produced by addition of 2% w/v NaCl or the equivalent glucose concentration (0.58M). The mutant did not accumulate glycine betaine when growing at low a_w, and did not transport glycine betaine when assayed *in vitro*.

6.1 Introduction.

Inhibition of microbial growth under conditions of hyperosmotic stress occurs chiefly as a result of loss of cellular water. Under optimum growth conditions the solute concentration in the cytoplasm is greater than that of the environment and there is a tendency for water to flow from the exterior into the cytoplasm. The resulting hydrostatic pressure, known as turgor pressure, exerts an outward force on the cell wall (Csonka, 1989). Turgor pressure is thought to provide the force necessary for cell expansion (Koch *et al.*, 1981; Koch, 1995). It follows, that any decrease in the ratio between the solute content of the cytoplasm and that of the extracellular environment will result in an efflux of water from the cell. In bacteria, and indeed in many eukaryotic organisms, the most widespread means of counteracting such losses of turgor pressure is by accumulation of 'compatible' solutes to reduce the a_w of the cytoplasm (Csonka, 1989).

Glycine betaine (N,N,N,-trimethylglycine) is probably the most widely used organic compatible solute, not alone in bacteria but also in eukaryotic cells (Csonka, 1989). Glycine betaine accumulation occurs either as a result of transport or by synthesis from choline; only a few bacteria are capable of *de novo* glycine betaine synthesis. Bacteria subjected to osmotic stress in medium which does not contain glycine betaine grow at substantially reduced rates (Csonka, 1989). Similarly, mutant strains in which the transport systems for glycine betaine are inactivated grow poorly under conditions of osmotic stress (Haardt *et al.*, 1995; Kappes *et al.*, 1996) and are not stimulated by glycine betaine.

Lactococcus lactis is an industrially important microorganism, many strains of which are used as starter cultures in the manufacture of numerous cheese varieties and also in other dairy products. In recent years the response of *L. lactis* to environmental stress such as low pH (Hartke *et al.*, 1994; O'Sullivan and Condon, 1997), high (Kilstrup *et al.*, 1997; Whitaker and Batt, 1991) and low temperatures (Panoff *et al.*, 1995), and starvation (Hartke *et al.*, 1994) have been studied.

However, there have been only a few studies (Molenaar *et al.*, 1993; O'Sullivan and Condon, 1997; Troller and Stinson, 1981) which examined osmotic stress in *L. lactis* and to our knowlege, there has been only one report to date concerned with the accumulation of compatible solutes by this bacterium. The accumulation of glycine betaine by *L. lactis* ML3 has been reported to occur via a constitutively expressed high affinity transport system and also via an inducible transport system which also transports proline (Molenaar *et al.*, 1993). There have been several reports that other lactic acid bacteria also use glycine betaine as an osmoprotectant (Glaasker *et al.*, 1996a; 1996b; 1997; Hutkins *et al.*, 1987; Kunin and Rudy, 1991; Sakaguchi, 1960).

The species *Lactococcus lactis* is subdivided into two subspecies, *lactis* and *cremoris* on the basis of a number of phenotypic traits, among which is the ability to grow in the presence of NaCl. An upper limit for growth of 2% w/v NaCl has been used to distinguish members of the subspecies *cremoris* from members of the subspecies *lactis* for which growth at 4% w/v salt is a general characteristic. In recent years, DNA sequence analysis has been used to reclassify some *lactis* strains as *cremoris* strains and *vice versa* (Godon *et al.*, 1992) This reclassification has led to a situation in which the reclassified subspecies *lactis* and *cremoris* now contain strains with different levels of tolerance to NaCl.

The osmotolerance of *Lactobacillus* strains appears to be dependent on their ability to accumulate glycine betaine (Hutkins *et al.*, 1987). We have investigated the possibility that the failure of *L. lactis* subsp. *cremoris* strains to grow at NaCl concentrations in excess of 2% w/v is related to an inability to utilise glycine betaine as an osmoprotectant. In addition, an osmotic stress sensitive mutant of *Lc. lactis* subsp. *cremoris* MG 1363 was isolated by insertional mutagenesis, and characterised in relation to its ability to transport glycine betaine and to grow at low a_w.

6.2 RESULTS.

6.2.1 Inhibition of growth of *Lc. lactis* subsp. *cremoris* NCDO 712 in complex medium at low a_w.

Lactococcus lactis subsp. *cremoris* NCDO 712 is one of those strains formerly classified as being a member of the subspecies *lactis*, but which has now been reclassified as belonging to the subspecies *cremoris*. An initial series of experiments in complex medium had a twofold purpose. Firstly, to quantify the effect of low a_w on the growth rate of this strain and secondly to determine if the nature of the solute used to reduce the a_w influenced the sensitivity of NCDO 712 to osmotic stress. The specific growth rate in GM-17 decreased gradually from a value of 1.0 h-1 at an a_w of 0.999 to about 0.25 h-1 at an a_w of 0.977 (Fig. 6.1). , the specific growth rate in GM-17 broth at any specific a_w was the same when the a_w was reduced with NaCl or glucose.

6.2.2 The effect of compatible solutes on the growth of NCDO 712 at low a_w.

It was not possible to assess the effect of adding compatible solutes to GM-17 medium, which is rich in sources of these compounds. Instead, a chemically defined medium based on that of Thomas *et al.* (1979) was used (MTEL medium). Glycine betaine and proline were used initially as it has previously been shown that these are accumulated by *Lc. lactis* ML3 in response to osmotic stress. Growth of NCDO 712 was strongly inhibited by addition of NaCl to MTEL medium and little growth occurred at 4% w/v NaCl. If 2 mM glycine betaine was added, there was a substantial relief of inhibition of both growth rate and of growth yield (Fig 6.2). By comparison, proline was much less significant; there was only a slight relief of inhibition of growth rate (Fig. 6.3 a). The Km for proline transport by the lactococci is quite high (Molenaar *et al.*, 1993) and as only 2 mM proline was used it was possible that the low level of stimulation by proline (relative to that observed with glycine betaine) was due to limited proline uptake. However, increasing the proline concentration to 20 mM only slightly

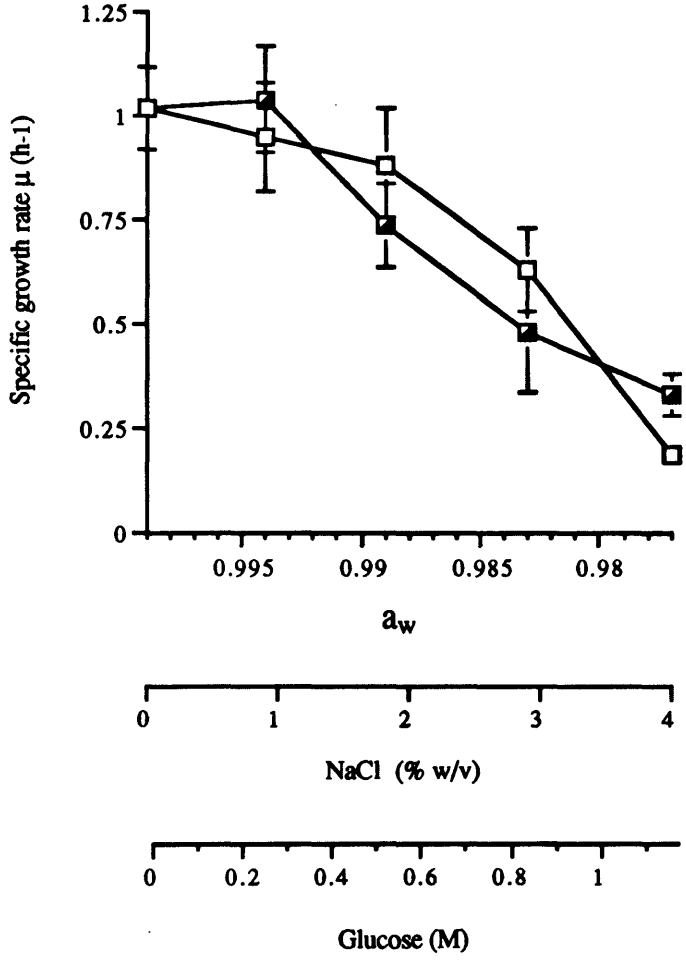


Fig. 6.1 The specific growth rate, μ (h^{-1}), of *Lc. lactis* subsp. *cremoris* NCDO 712 in GM-17 medium with the aw adjusted by addition of NaCl □) or glucose ◪).

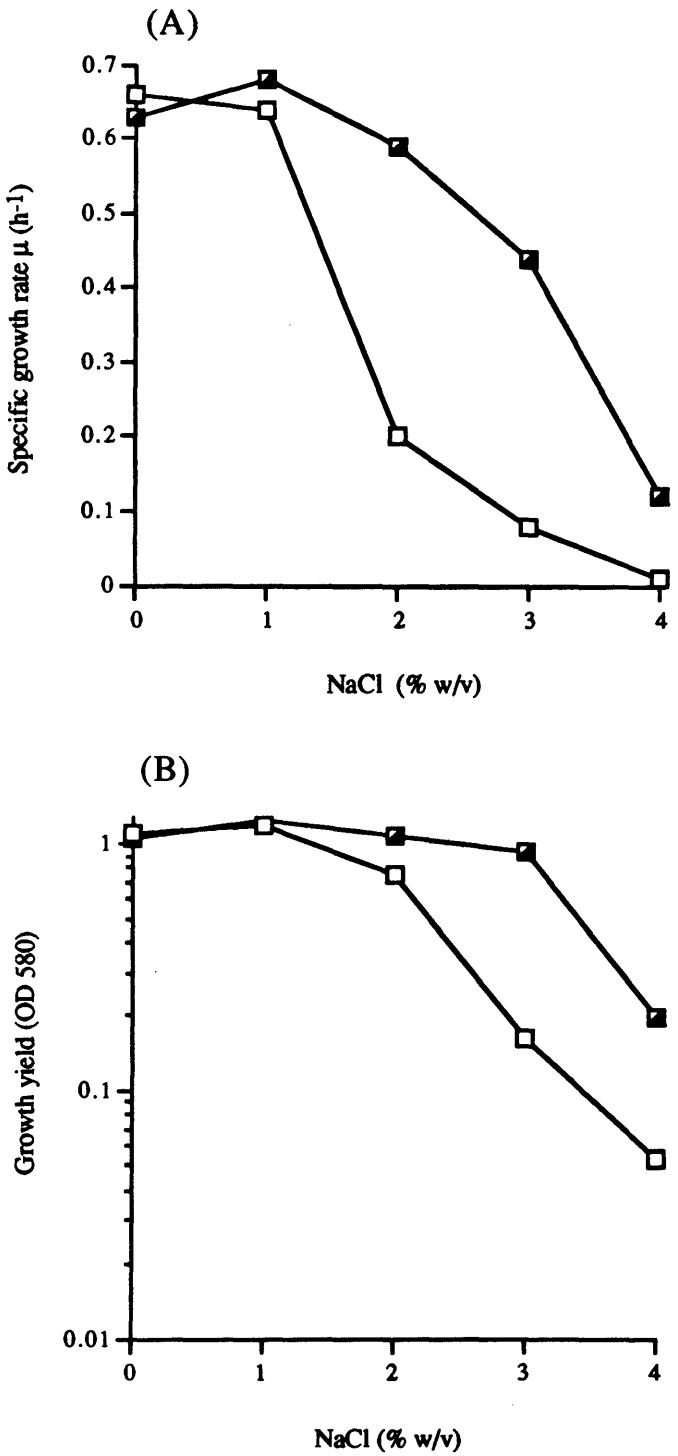


Fig. 6.2 Specific growth rate, μ (h^{-1}), (A) and growth yield (B) of *Lc. lactis* subsp. *cremoris* NCDO 712 in MTEL medium with the aw reduced by adding NaCl, in the presence (■) and absence (□) of 2 mM glycine betaine.

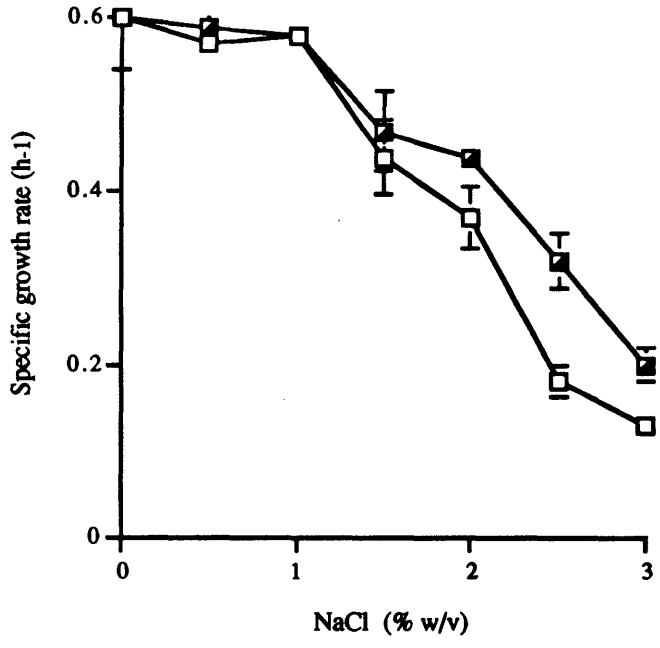


Fig. 6.3a The specific growth rate, μ (h^{-1}), of *Lc. lactis* subsp. *cremoris* NCDO 712 in MTEL medium with the aw reduced by adding NaCl in the presence (■) and absence (□) of 2 mM proline

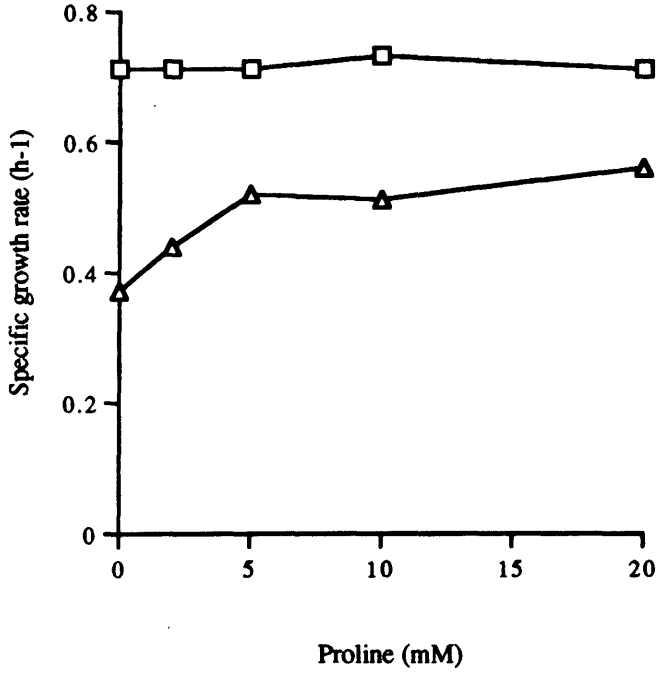


Fig. 6.3b The effect of proline concentration on the specific growth rate, μ (h^{-1}), of *Lc. lactis* subsp. *cremoris* NCDO 712 in MTEL medium containing 0% (□) or 2% w/v (▲) NaCl.

increased the relief of inhibition observed (Fig. 6.3b), indicating that limitation of proline uptake was unlikely to be the reason for the lack of stimulation by proline. Addition of the dipeptide leucyl-proline to MTEL medium relieved the inhibition of growth of NCDO 712 at low a_w, but only to the same extent as proline (Fig. 6.4).

6.2.3 The effect of other potential osmoprotectants on the inhibition of growth of NCDO 712 at low a_w.

Although proline and glycine betaine are the only osmoprotectants which have been reported to be utilised by *Lc. lactis*, there are a number of other compounds which act as osmoprotectants in other bacteria. We examined the ability of two of these compounds, carnitine and ectoine, to stimulate NCDO 712 at low a_w. The addition of carnitine to MTEL medium had no effect on the specific growth rate at low a_w (Fig. 6.5). Ectoine had no effect on either the specific growth rate or the final biomass yield of NCDO 712 at low a_w. The compound was quite expensive to buy, so the effect of ectoine addition was only determined at 3% w/v NaCl and the equivalent concentration of glucose (0.88M) (Table 6.1).

6.4.4 The effect of glycine betaine on the growth of other *Lactococcus lactis* strains

When it was attempted to extend the study of glycine betaine accumulation to other strains, a problem was encountered in that a number of the strains were unable to grow on MTEL. However, these strains grew satisfactorily in another chemically defined medium (CDM) as described by Molenaar *et al.* (1993). It would have been advantageous to omit proline from this medium so that glycine betaine, if added, would be the only osmoprotectant present. Unfortunately, all the *cremoris* strains were proline auxotrophs and some of the *lactis* strains grew at sub-optimal rates in the absence of proline. Proline was therefore present in the CDM for all experiments. To allow comparison with previous results, the relevant experiments previously done in MTEL using NCDO 712 were repeated using the new medium.

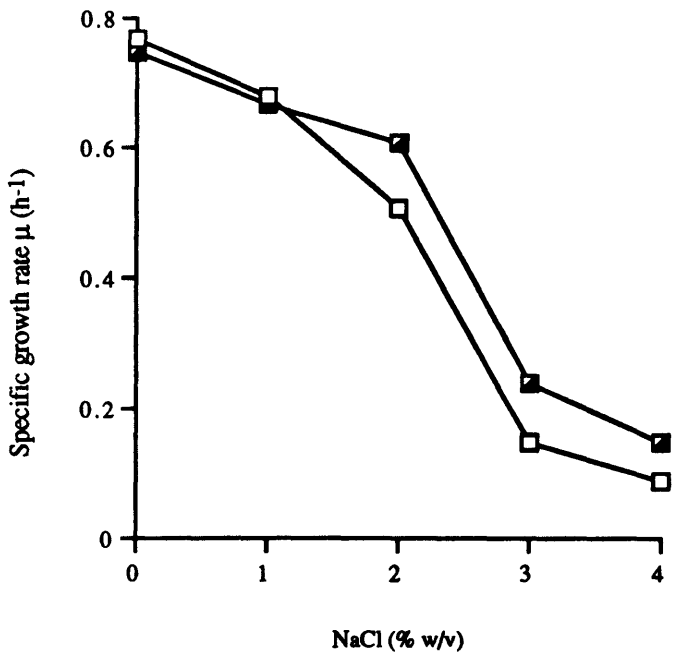


Fig. 6.4 The specific growth rate μ (h^{-1}) of *Lc. lactis* subsp. *cremoris* NCDO 712 in MTEL medium with the aw reduced by addition of NaCl, in the presence (■) and absence (□) of 2 mM leucyl-proline.

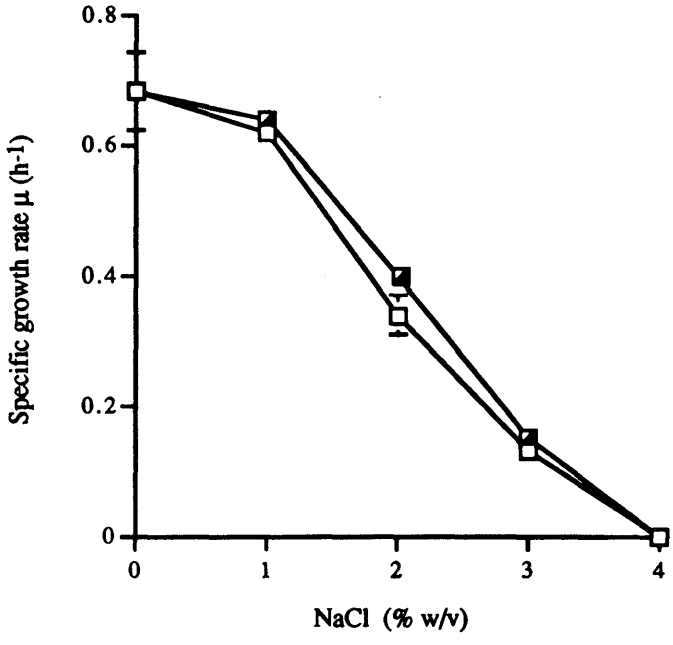


Fig. 6.5 Specific growth rate, μ (h^{-1}), of *Lc. lactis* subsp. *cremoris* NCDO 712 in MTEL medium with the aw reduced by adding NaCl, in the presence ( ) and absence ( ) of 2 mM D/L-carnitine.

Table 6.1 The specific growth rate, μ (h^{-1}) and growth yield of *Lc. lactis* subsp. *cremoris* NCDO 712 in MTEL medium with the aw reduced by addition of NaCl or glucose in the presence and absence of 1 mM ectoine.

Culture additions	μ (h^{-1})	Growth Yield (OD 580)
None	0.66±.02	1.34±0.03
ectoine	0.66±0.01	1.27±0.1
3% NaCl	0.20±0.01	0.25±0.05
3% NaCl + ectoine	0.22±0.01	0.31±0.07
0.88M Glucose	0.25±0.01	0.58±0.01
0.88M Glucose + ectoine	0.25±0.01	0.59±0.03

The inhibition of the growth rate of the three *lactis* strains and the *cremoris* strain NCDO 712 at NaCl concentrations >1% w/v was substantially relieved by glycine betaine (Fig. 6.6). The relief of inhibition was most apparent at 4% w/v NaCl, where three of the four strains (NCDO 712, BA2, and C10) did not grow unless glycine betaine was present. One strain (BA1) was capable of growth at 4% w/v NaCl in the absence of glycine betaine, but at a greatly reduced rate. If the a_w of the medium was reduced by addition of glucose, the effect of betaine on the growth rate was similar to that observed when NaCl was used (Fig. 6.6). Glycine betaine did not have as strong an influence on growth yield as on growth rate (Fig. 6.7) The yields of strain BA1 were similar in the presence and absence of glycine betaine. The growth yield of strains 712, C10 and BA2 were reduced at low a_w when NaCl was used and this reduction was prevented by glycine betaine. NCDO 712 was the only strain whose yield was reduced at low a_w by glucose and this was prevented by glycine betaine.

Addition of glycine betaine did not significantly relieve the inhibition of the growth rate of the other *cremoris* strains HP, BK5 and US3 in CDM medium containing NaCl (Fig. 6.8). In the case of HP and BK5 inhibition of growth by glucose was not relieved either; however, inhibition of the growth rate of the US3 strain by glucose was relieved by glycine betaine. Growth of strain US3 occurred at glucose concentrations $\leq$ 0.88M (equivalent a_w to 3% w/v NaCl) provided that betaine was present. Some stimulation by glycine betaine, of the growth yield in CDM when the a_w was reduced by glucose was also observed, for the *cremoris* strains BK5 and US3 (Fig. 6.9).

6.2.5 Glycine betaine transport by *L. lactis* strains.

NCDO 712 was used as a model strain to try to identify the main characteristics of glycine transport in *L. lactis*. When NCDO 712 was grown in CDM without added salt or sugar the harvested cells transported glycine betaine via a high affinity system which essentially obeyed Michaelis-Menten kinetics (Fig. 6.10). The apparent Km was 1.4 μM, the Vmax was 8.0±1 nmol. mg. prt.$^{-1}$. min^{-1}, and the pH optimum was 6.0

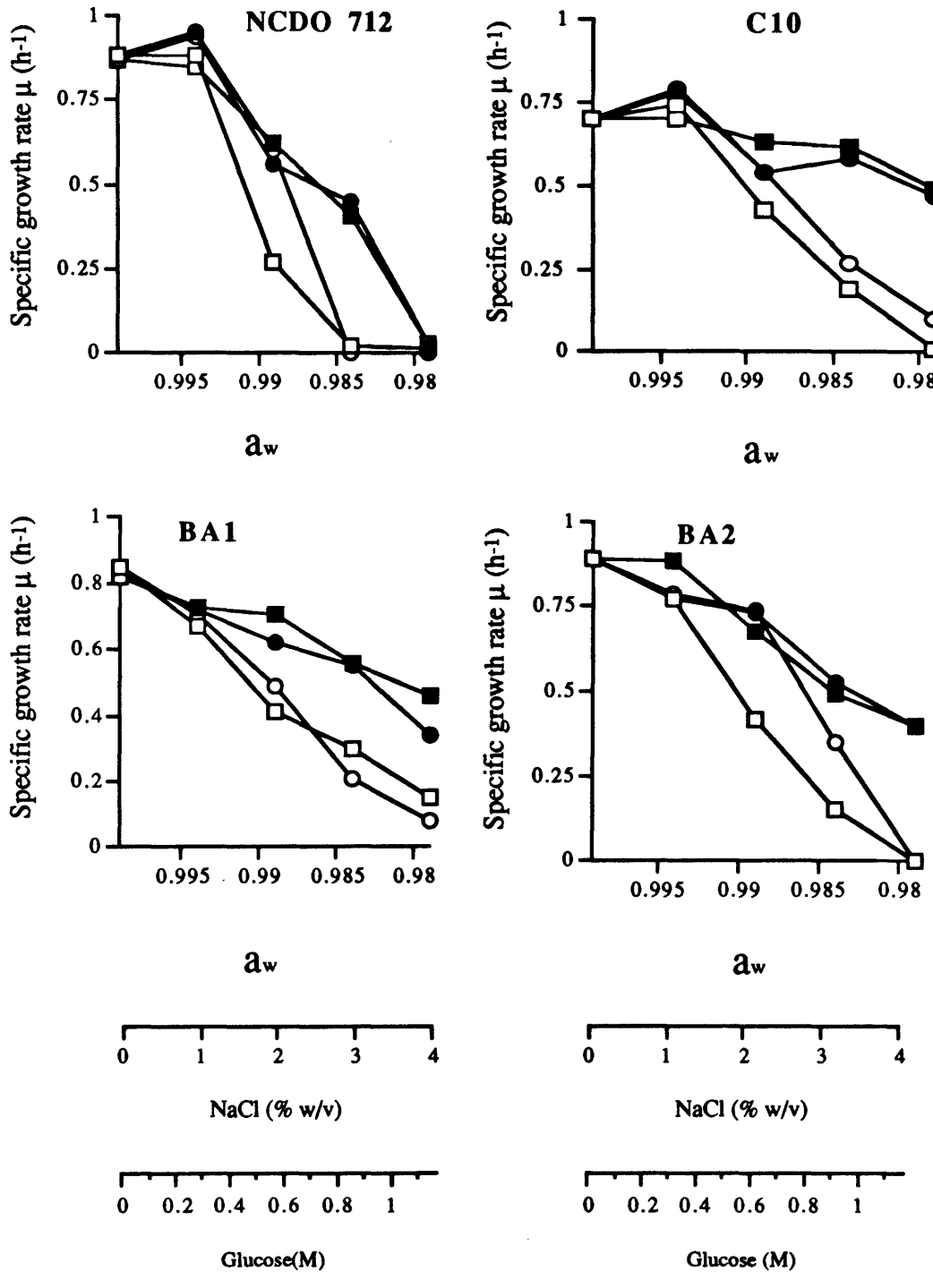


Fig. 6.6 The specific growth rates, μ (h^{-1}), of *Lc. lactis* subsp. cremoris NCDO 712 and *Lc. lactis* subsp. *lactis* strains C10, BA1 and BA2 in CDM medium with the aw reduced by glucose (circles) or NaCl (squares) in the presence (closed symbols) or absence (open symbols) of 2 mM glycine betaine. The data are representative of triplicate experiments.

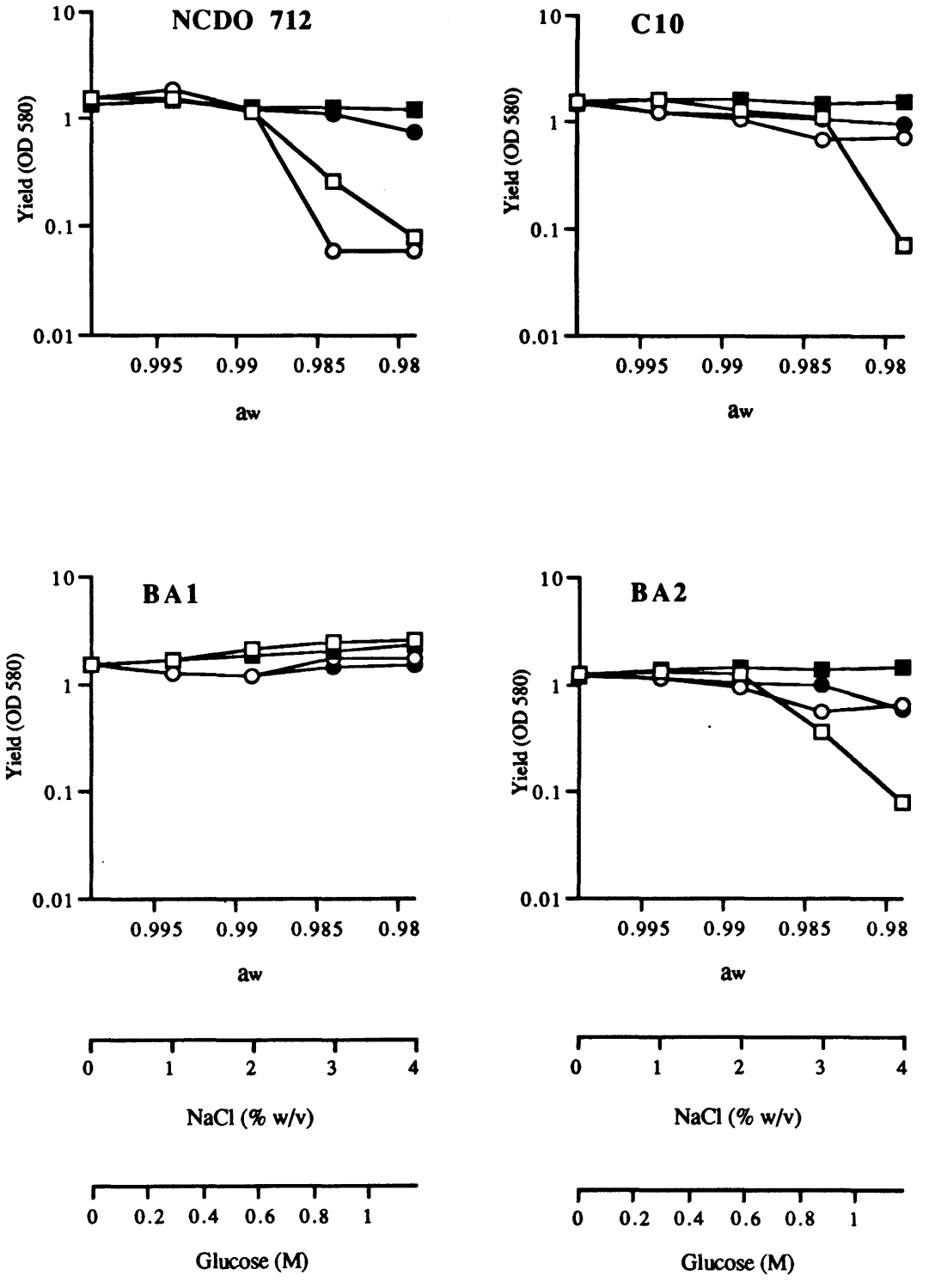


Fig.6.7 The growth yields (OD580) of *Lc. lactis* subsp. *cremoris* NCDO 712 and the *Lc. lactis* subsp. *lactis* strains C10, BA1 and BA2 in CDM with the aw reduced by addition of NaCl (squares) or glucose (circles), in the presence (filled symbols) or absence (open symbols) of 2 mM glycine betaine.

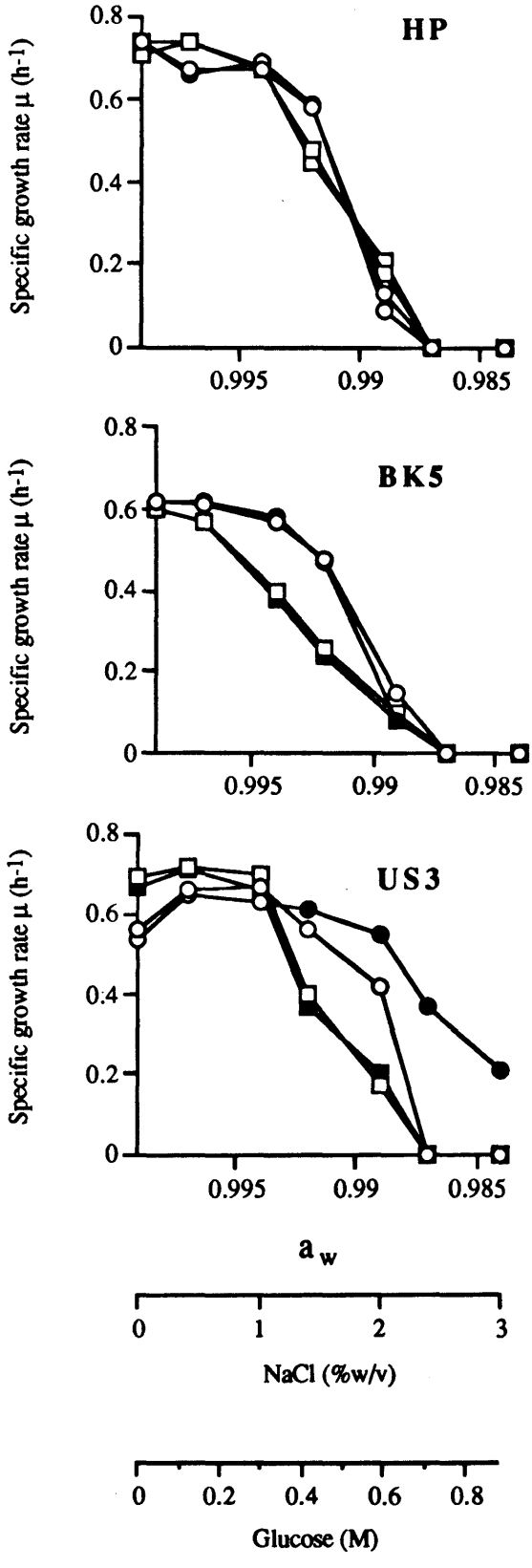


Fig. 6.8 The specific growth rates, μ (h^{-1}), of *Lc. lactis* subsp. *cremoris* strains HP, BK5 and US3 in CDM medium with the aw reduced by addition of glucose (circles) or NaCl (squares), in the presence (filled symbols) or absence (open symbols) of 2 mM glycine betaine. The data are representative of triplicate experiments.

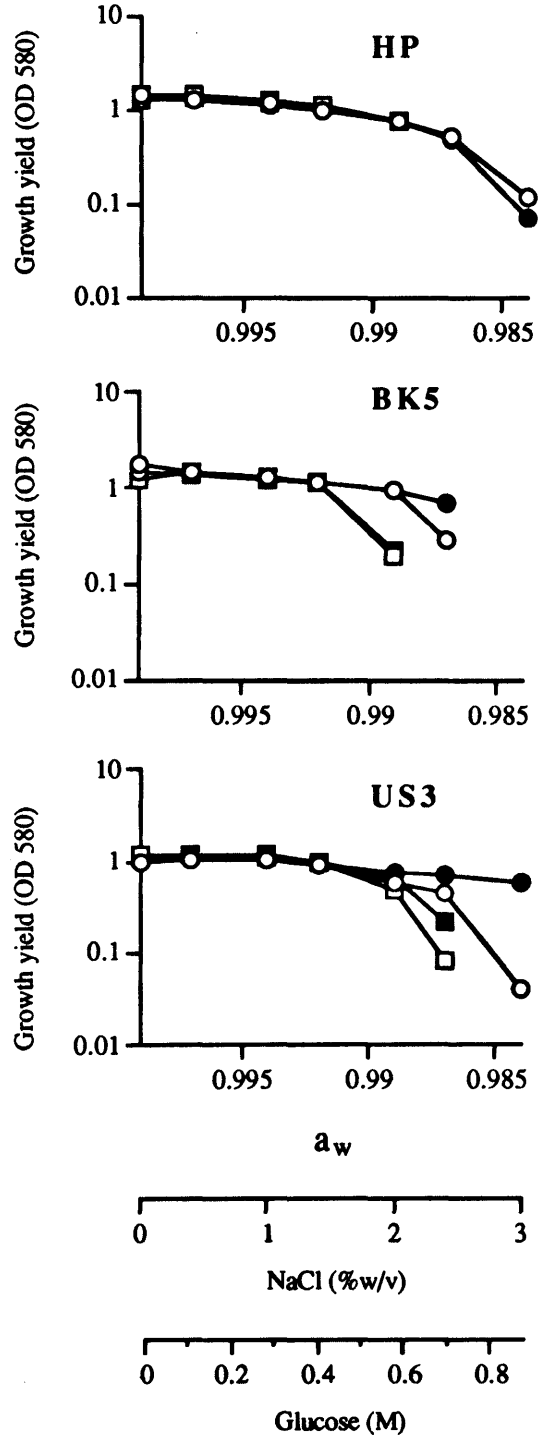


Fig. 6.9 The growth yield of *Lc. lactis* subsp. *cremoris* strains HP, BK and US3 in CDM with the aw reduced by addition of NaCl (squares) or glucose (circles) in the presence (filled symbols) or absence (open symbols) of 2 mM glycine betaine.

(Fig. 6.10). No transport was observed in the absence of an energy source in the assay buffer (Fig. 6.11). When NCDO 712 cells were harvested from CDM to which NaCl or glucose was added to decrease the a_w their rate of glycine betaine transport was increased, (Table 6.2 a and b). The apparent Km was similar to those of cells grown in CDM alone and again no transport occurred in the absence of an energy source in the assay buffer (Fig. 6.11). However, the transport of glycine betaine by cells grown in CDM with a decreased a_w, differed from that by cells grown in CDM alone. The rate of glycine betaine transport by cells grown at a low a_w was stimulated by decreasing the a_w of the assay buffer, by addition of salt (Table 6.2a) or glucose (Table 6.2b) whereas no such stimulation was detected when cells grown in CDM alone were assayed with or without solute. The accumulation of glycine betaine transport by cells grown in CDM or CDM with 1% w/v NaCl was slightly reduced by the the presence of 1 μM nigericin in the assay; 1 μM valinomycin did not affect glycine betaine transport to the same extent as did nigericin (Table 6.3).

The general characteristics of glycine betaine transport were similar to that of NCDO 712 in those *L. lactis* subsp. *lactis* strains C10, BA1 and BA2 whose growth rates in CDM at low a_w were stimulated by glycine betaine (Fig. 6.12). The transport rates of these cells grown in CDM with NaCl were stimulated by the same concentration of salt in the assay buffer (Fig. 6.12). Glycine betaine transport was not detected in the *L. lactis* subsp. *cremoris* strains HP or BK5 (Fig. 6.12), neither of which could grow in CDM at a_w values below 0.987 (2.5% NaCl or 0.75M glucose) and which did not respond to glycine betaine in the growth medium. A low rate of transport was detected in the other *cremoris* strain US3; this strain did respond to glycine betaine when growing in CDM containing sugar but not salt (Fig. 6.8). The low rate of transport of US3 cells was not increased by growth in CDM with 1% NaCl; activation of transport was observed when NaCl was added to the assay buffer but the rate of transport was less than that in NCDO 712 or the *L. lactis* subsp. *lactis* strains (Fig. 6.12).

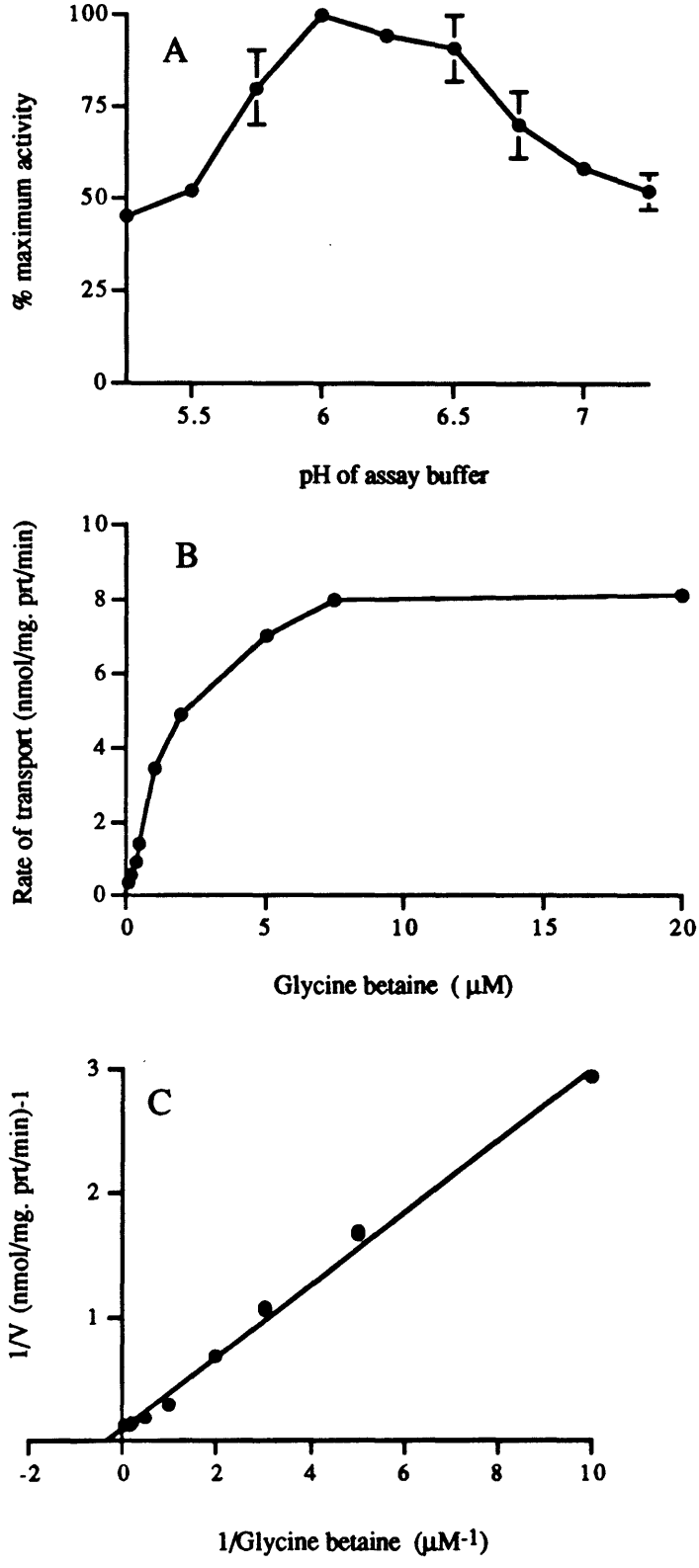


Fig. 6.10 Transport of glycine betaine by exponential phase *Lc. lactis* subsp. *cremoris* NCDO 712 cells in phosphate buffer, (A) at different pH values with 10 µM ^{14}Cglycine betaine and (B) at pH 6.0 at different glycine betaine concentrations. In (C) the data of (B) are plotted in the Lineweaver-Burk form.

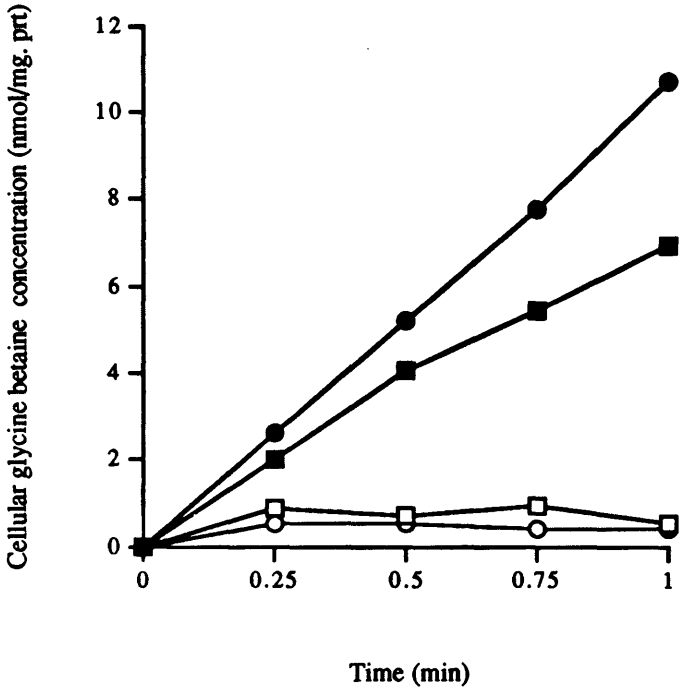


Fig. 6.11 Glycine betaine transport by exponential phase *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in CDM (squares) or CDM with 1% NaCl (circles). The cells were assayed with (filled symbols) or without (open symbols) 25 mM glucose. The data are representative of triplicate experiments.

Table 6.2a Glycine betaine transport by exponential phase *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in CDM with different NaCl concentrations and asssayed with and without NaCl in the buffer.

CDM Additions	Rate of transport (nmol/mg prt/min) in phosphate buffer with:		
	0% NaCl	1% NaCl [a]	2% NaCl [b]
None	9.6±0.2	10.55±0.8	9.74±0.5
1% NaCl [a]	16.5±1.5	36±2	33±1
2% NaCl [b]	32±7	N.D.	81±14

a and b refer to similar a_w values

N.D.: not determined

Table 6.2b Glycine betaine transport by exponential phase *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in CDM with different glucose concentrations and assayed with and without glucose in the buffer.

CDM Additions	Rate of transport (nmol/ mg prt/min) in phosphate buffer with:		
	O	0.29M glucose [a]	0.58M glucose [b]
CDM	9.6±0.2	N.D.	N.D.
0.29M Glucose [a]	18.2±4	34±7	N.D.
0.58M Glucose [b]	27±9	N.D.	125±10

a and b refer to similar a_w values

N.D.: not determined

Table 6.3 Influence of the ionopores nigericin and valinomycin on glycine betaine transport by *Lc. lactis* subsp. *cremoris* NCDO 712 cells grown in CDM with and without 1% NaCl. The NaCl concentration of the assay buffer in each case was identical to that of the CDM in which the cells were grown.

NaCl of CDM (%w/v)	Rate of glycine betaine transport (nmol/mg. prt./min) in assay buffer with:			
	No additions	1 µM nigericin	1 µM valinomycin	nigericin plus valinomycin
0%	9.0±0.7	1.0±0.1	5.6±5.	0.9±0.2
1%	37.3 ±3	11.5±3	38.8±10	5.8±0.9

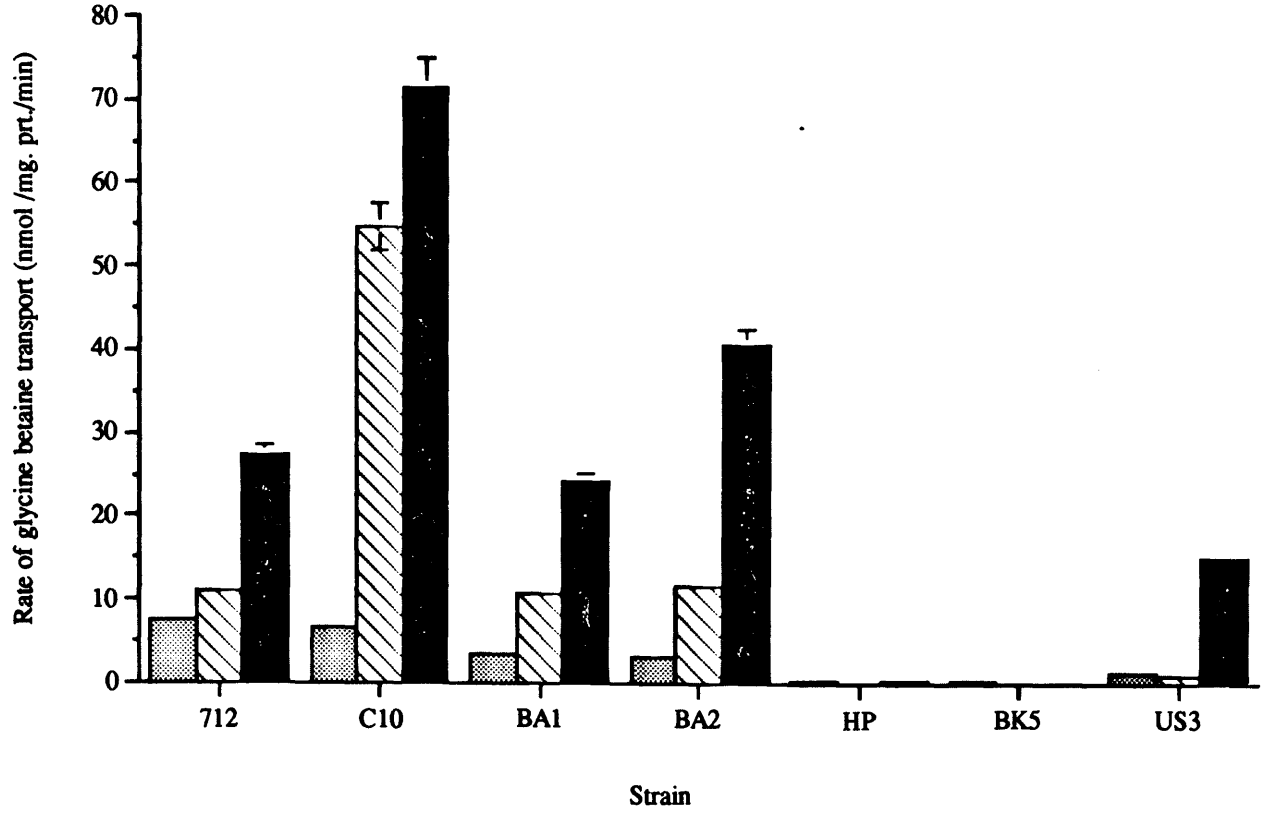


Fig. 6.12 The rate of glycine betaine transport by *Lc. lactis* subsp. *lactis* strains C10, BA1 and BA2 and *Lc. lactis* subsp. *cremoris* strains NCDO 712, HP, BK5 and US3. The cells were grown in CDM (▩) or CDM with 1% NaCl (▨ and assayed in phosphate buffer or grown in CDM with 1% NaCl and assayed in phosphate buffer containing 1% NaCl (■).

6.2.6 Accumulation of glycine betaine during growth at low a_w.

NCDO 712 cells accumulated glycine betaine when growing in CDM with the a_w reduced by either NaCl or glucose (6.13). A low level of glycine betaine was present even in cells growing in CDM without any added solute; addition of NaCl or glucose caused the cellular content of glycine betaine to increase. At NaCl concentrations between 1 and 3% w/v and glucose concentrations between 0.29M and 0.88M, there was a linear relationship between the extracellular solute concentration and the glycine betaine content of the cells. Cells growing in CDM containing glucose accumulated higher quantities of glycine betaine than did cells growing in CDM containing NaCl at the equivalent a_w.

To determine whether the remaining strains accumulated betaine during growth, glycine betaine accumulation was measured in cells growing in CDM and also in CDM containing 1.5% w/v NaCl or the equivalent glucose concentration (0.44M). These solute concentrations were selected so as to obtain a reasonably rapid rate of growth by the *cremoris* strains while being sufficiently high to elicit a reasonable level of glycine betaine accumulation in those strains which were positive. A basal level of 20-40 nmol.mg prt^{-1} was detected in all strains grown in CDM alone. With either NaCl or glucose as the solute added to the CDM, all of the *lactis* strains tested accumulated betaine to higher levels than did NCDO 712, with each strain accumulating > 200 nmol/mg protein (Fig. 6.14). Accumulation of glycine betaine in response to NaCl or glucose by the *cremoris* strains was much lower than that for the *lactis* strains. HP was unique in that no increase in its cellular content of glycine betaine was observed at reduced a_w whether by addition of NaCl or glucose. The glycine betaine content of the other *cremoris* strains increased moderately at low a_w, US3 and BK5 appeared to increase their betaine content in response to NaCl but not glucose.

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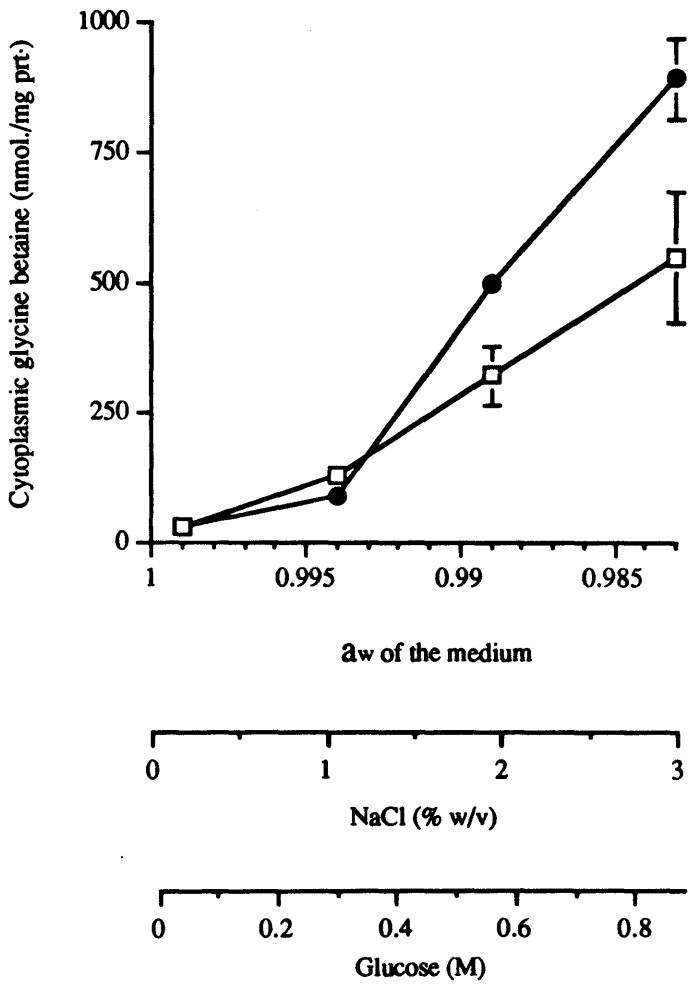


Fig. 6.13 Accumulation of ^{14}C glycine betaine by *Lc. lactis* subsp. *cremoris* NCDO 712 cells growing in CDM medium with the aw adjusted by addition of glucose (●) or NaCl (□).

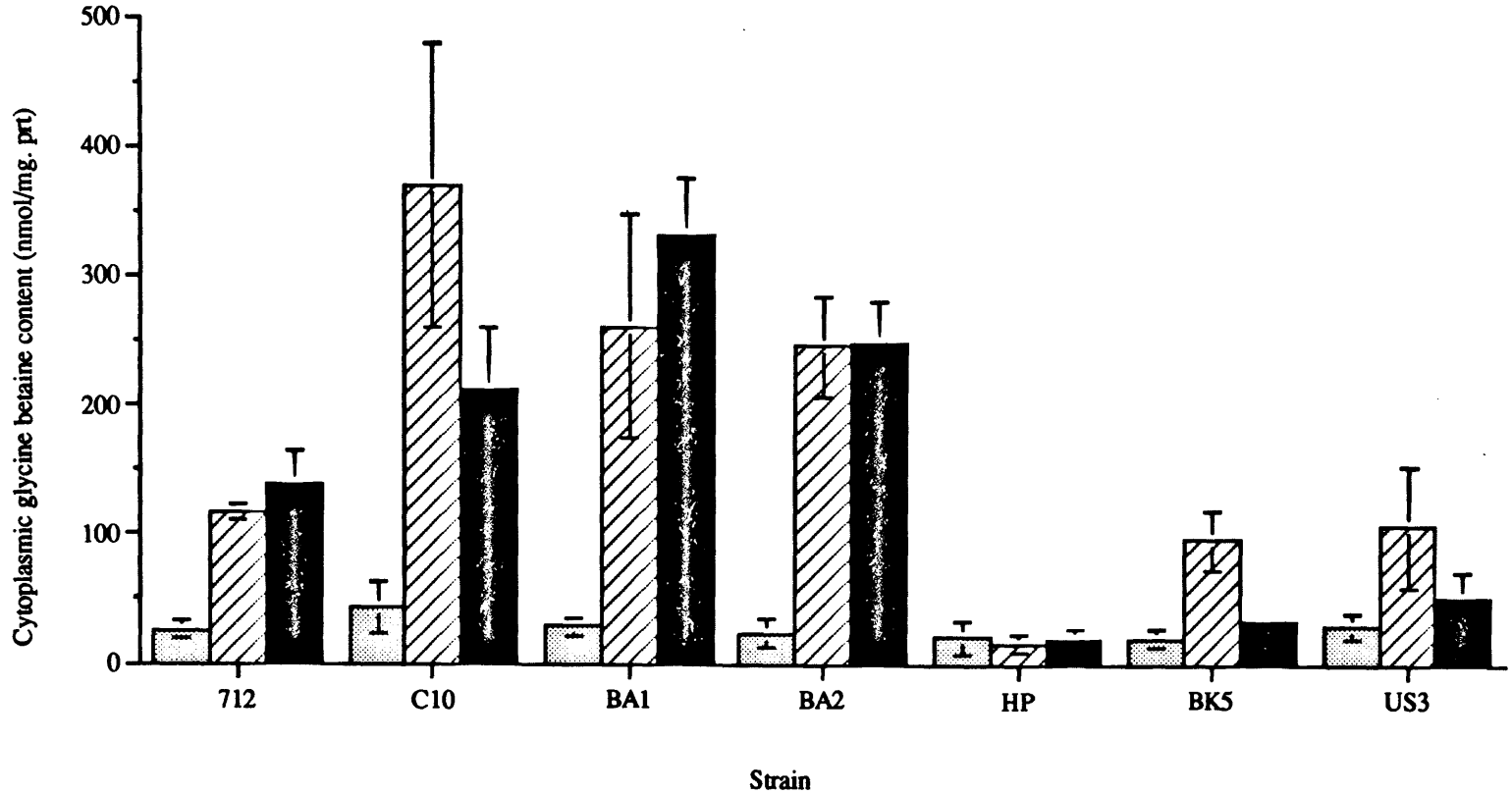


Fig. 6.14 Accumulation of ^{14}C labelled glycine betaine by *Lc. lactis* subsp. *cremoris* strains NCDO 712, HP, BK5, and US3 and *Lc. lactis* subsp. *lactis* strains C10, BA1 and BA2 growing in CDM (▩), CDM with 1.5% w/v sodium chloride (▨) or CDM with 0.44M glucose (■). The NaCl and glucose concentrations each reduced the aw of the CDM medium to 0.990

6.2.7 Isolation of a *L lactis* subsp. *cremoris* MG1363 mutant unable to accumulate betaine.

NCDO 712 was unable to grow at 4% w/v NaCl unless betaine was present, and there was a strong correlation between the ability to transport betaine and the ability to grow at low a_w. These observations suggested that it should be possible to obtain mutants of NCDO 712 deficient in glycine betaine transport by replica plating mutagenised cells on medium with either low or high a_w in the presence of glycine betaine and selecting colonies unable to grow at the low a_w. An insertional mutagenesis system (Maguin *et al.*, 1996) was used to obtain a bank of mutants of *L. lactis* subsp. *cremoris* MG1363 a plasmid-free derivative of NCDO 712 (Gasson, 1983). A concentration of NaCl of 3.5% w/v was selected because NCDO 712 was incapable of growth on CDM plates containing this NaCl concentration unless glycine betaine was present. Following the replica plating of 1,000 colonies on GM-17 and GM-17 with 3.5% NaCl, 13 colonies incapable of growth at 3.5% w/v NaCl were identified. One of these (OSM2) was selected for verification that it was inhibited by both NaCl and glucose, i.e that the inhibition was due to osmotic stress rather than a specific inhibitory effect of NaCl (Fig. 6.15). A ^{32}P-labelled fragment of DNA from the inserted plasmid hybridised to digested OSM2x chromosomal DNA, but not to MG1363 chromosomal DNA (Fig. 6.16). The mutant (OSM 2) was then treated to excise the integrated plasmid so as to create a stable IS*S1* mutant (OSM 2x) which could be grown without fear of excision at 30°C. This isolate was tested by bacteriophage typing to verify that it was the same phage type as the MG1363 parent strain.

Several attempts were made to clone the sequences flanking the IS*S1* insertion in OSM2 using the method described by Maguin *et al.* (1996). Unfortunately, all attempts proved unsuccessful. As an alternative to this method, it was attempted to clone the flanking sequences by inverse PCR. Chromosomal DNA from OSM2x was digested with either *Eco*RI or *Hin*dIII, neither of which had recognition sites within IS*S1*. Following religation at low DNA concentrations so as to favour intramolecular ligation the DNA was used as a template in an inverse PCR reaction. The primers used

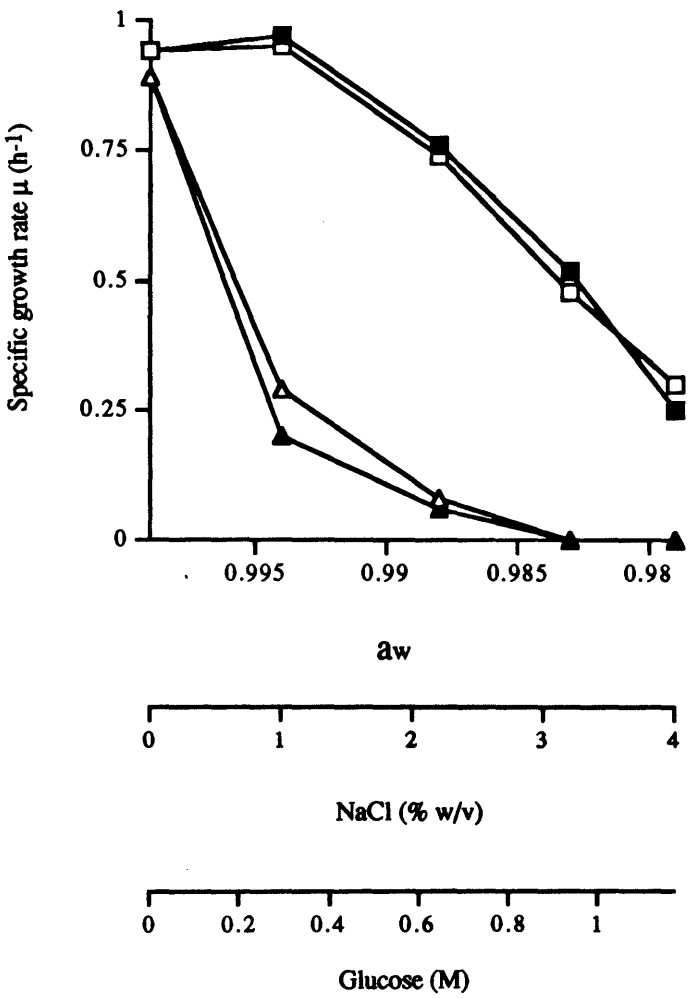


Fig. 6.15 Specific growth rate in GM-17 of *Lc. lactis* subsp. *cremoris* MG 1363 (squares) and the osmotic stress sensitive mutant OSM 2 (triangles) in GM-17 medium with the aw adjusted by addition of NaCl (filled symbols) or glucose (open symbols).

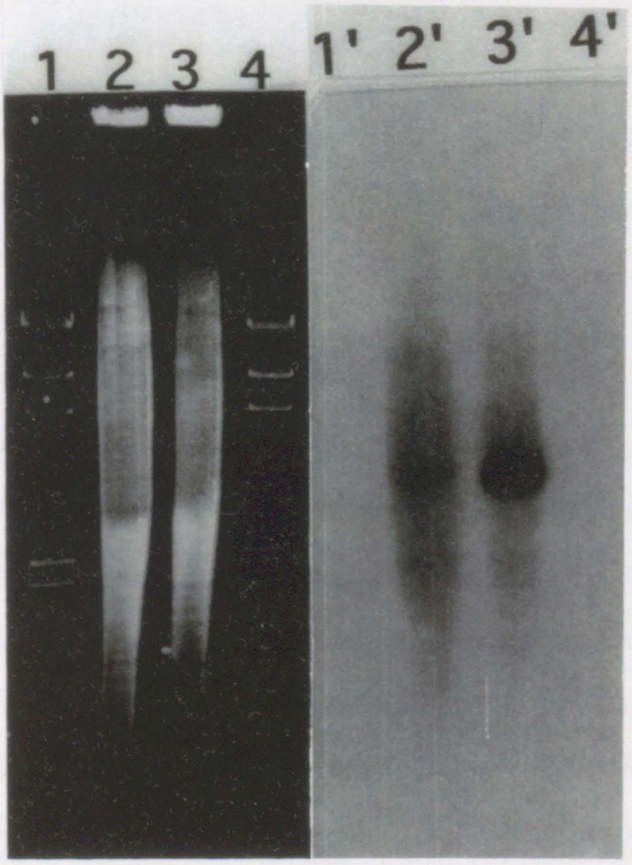


Fig. 6.16 Hybridisation of a ^{32}P-labelled fragment from pGhost^{+}9 to *Eco*RI digested chromosomal DNA from *Lc. lactis* MG 1363 and OSM2. Lanes 2 and 3 contain digested chromosomal DNA from MG1363 and OSM2x respectively. Hybridisation of the ^{32}P labelled fragment is shown in the corresponding lanes 2' and 3'. Lane 1 and 4 contain Hind III digested bacteriophage λ DNA as a size marker.

were 21 bp in length and designed to amplify outwards from the inverted repeat sequences at the ends of IS*51*. Several attempts were made using this method, including variation of the primer annealing temperature but no PCR product was obtained.

6.2.8 Characterisation of the OSM2x strain.

In GM-17 medium the presence of 3.5% w/v NaCl was bacteriocidal to OSM 2x (Fig. 6.17a). It was noticed that after prolonged incubation (>24 hours) in medium 3.5% w/v NaCl growth of OSM 2x occurred. Plating of 10^8 CFU/ml on GM-17 agar plates containing 3.5% w/v NaCl indicated that reversion occurred at a frequency of 1.42×10^{-7}. The ability of the mutant OSM 2x to survive a challenge with 5% or 10% w/v NaCl did not differ from that of the parent strain (Fig. 6.17b).

OSM 2x grew somewhat slower than did its parent strain in CDM and was inhibited by low concentrations of NaCl (Fig. 6.18). Addition of glycine betaine to the growth medium did not result in any stimulation of growth at low a_w (Fig. 6.18). Glycine betaine transport was absent in this strain (Fig. 6.19) whereas MG1363 had approximately the same rate of transport as NCDO 712. Growth in medium containing added NaCl did not lead to an increase in the cytoplasmic glycine betaine content of the mutant (Fig. 6.20).

6.2.9 Complementation of OSM2x with a glycine betaine transporter gene from *Listeria monocytogenes*.

A gene encoding a glycine betaine transporter from *L. monocytogenes* was used in an attempt to restore the salt tolerant phenotype in OSM 2x. The 2.5 kb fragment encoding the gene was originally cloned into pUC18 (pCPL 1) (R. Sleator Dept. of Microbiology UCC, personal communication). pCPL 1 was digested with *Sca* 1 to linearise the fragment. Following phenol extraction and ethanol precipitation, the resulting linear DNA was digested with *Xma* 1 and *Xba* 1 which restrict sites flanking the insert in the multiple cloning site of pCPL 1 and the 2.5 kb fragment containing the

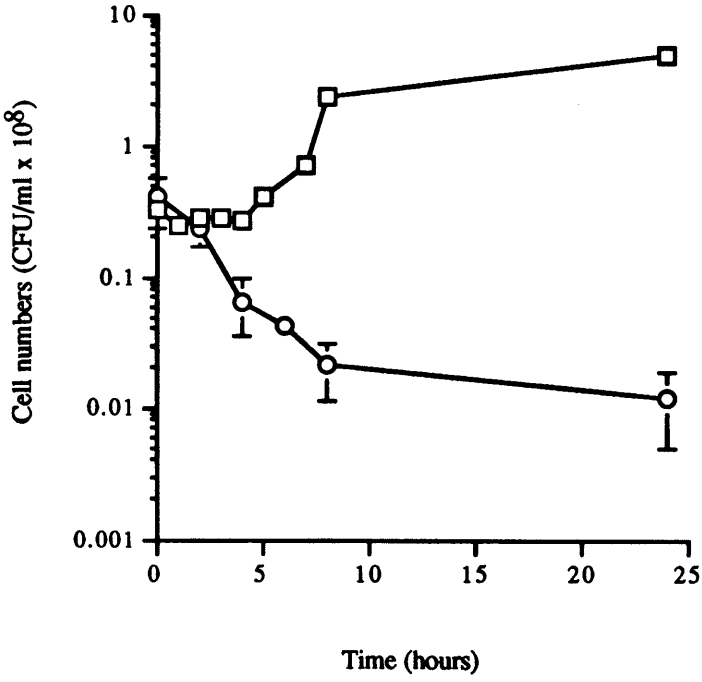


Fig. 6.17a Viable cell counts following inoculation of *Lc. lactis* subsp. *cremoris* MG 1363 (□) and OSM 2x (○) into GM-17 broth containing 3.5% w/v NaCl.

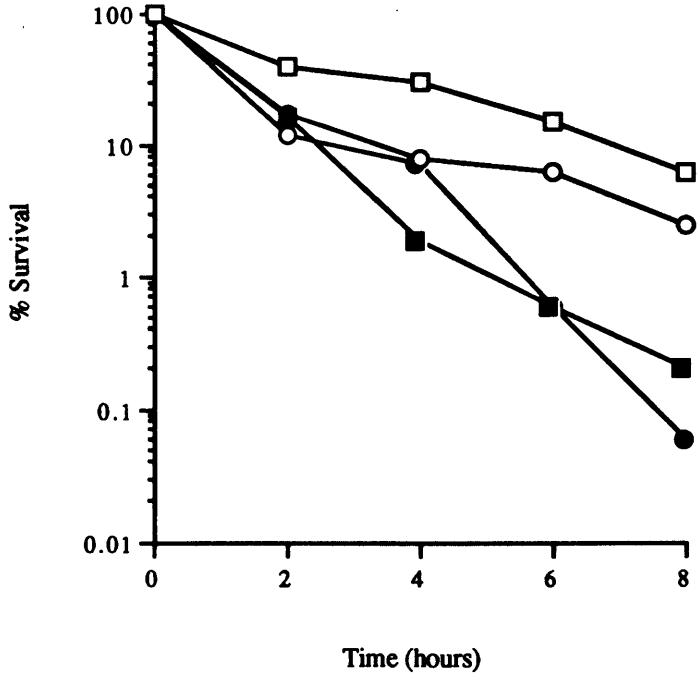


Fig. 6.17b Survival of exponential-phase *Lc. lactis* subsp. *cremoris* MG1363 (circles) and OSM 2x (squares) during challenge with 5% (open symbols) and 10% (filled symbols) w/v NaCl in GM-17 broth. The data are representative of triplicate experiments.

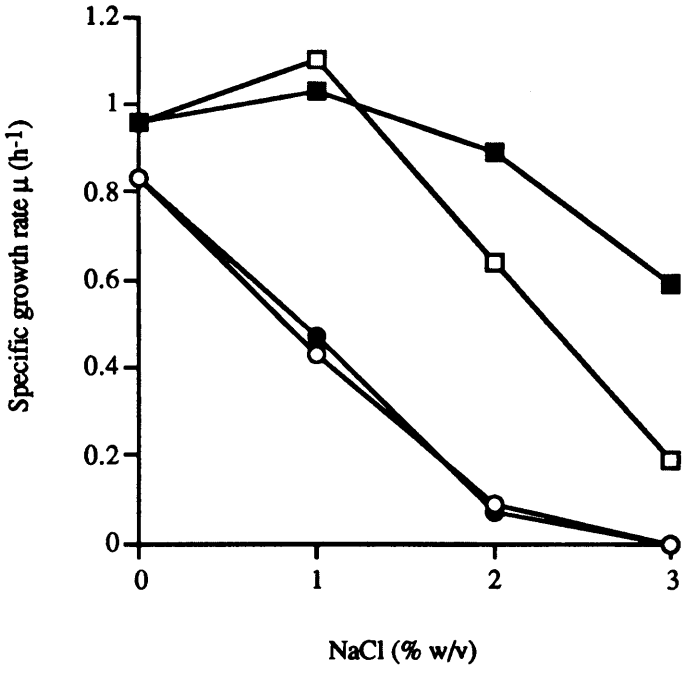


Fig. 6.18 The specific growth rate of *Lc. lactis* subsp. *cremoris* MG1363 (squares) and OSM2x (circles) in CDM containing NaCl in the presence (filled symbols) and absence (open symbols) of 2 mM glycine betaine. The data are representative of triplicate experiments.

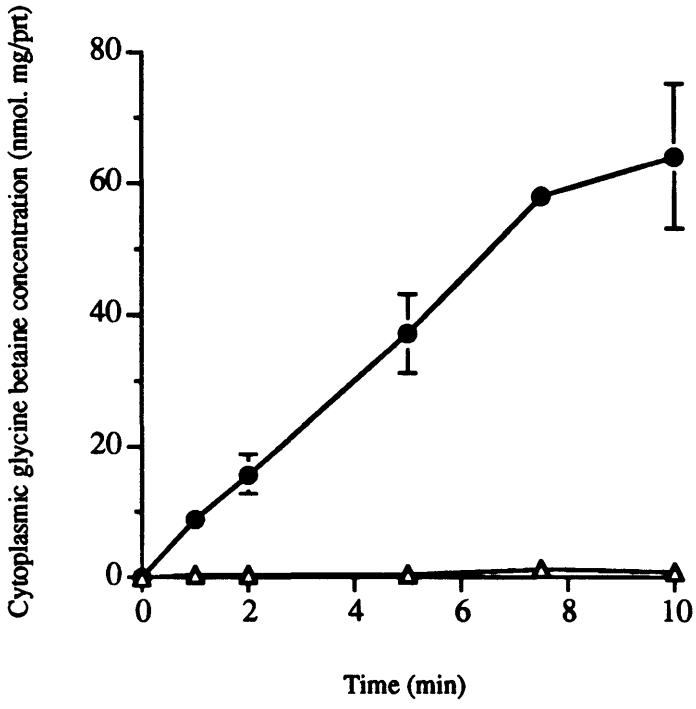


Fig. 6.19 Standard glycine betaine transport assay by *Lc. lactis* subsp. *cremoris* MG1363 (●) and the mutant OSM2x (▲) cells grown in CDM medium.

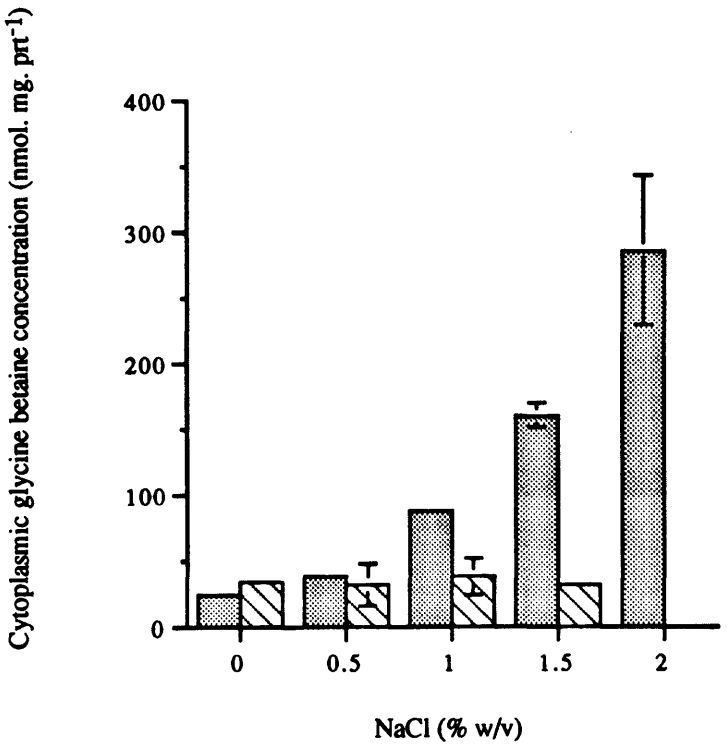


Fig. 6.20 Accumulation of glycine betaine by *Lc. lactis* subsp. *cremoris* MG1363 (▩) and the mutant OSM2x (▧) growing in CDM with its a_w reduced by NaCl.

Table 6.4 The specific growth rate, μ (h^{-1}) of *Lc. lactis* subsp. *cremoris* MG1363 and OSM2x containing the plasmids pCI 372 or pCPL 3[a] in GM-17 medium in the presence and absence of 3% w/v NaCl

Growth Medium	Specific growth rate μ (h^{-1}) of :			
	1363 (pCI372)	1363 (pCPL 3)	OSM 2x (pCI372)	OSM 2x (pCPL 3)
GM-17	0.97 ± 0.05	0.97 ± 0.1	0.99 ± 0.07	0.94 ± 0.08
GM-17 + 3% NaCl	0.45 ± 0.07	0.38 ± 0.05	No growth	No growth

[a] Contains the glycine betaine transport gene from *Listeria monocytogenes* cloned into the vector plasmid pCI 372

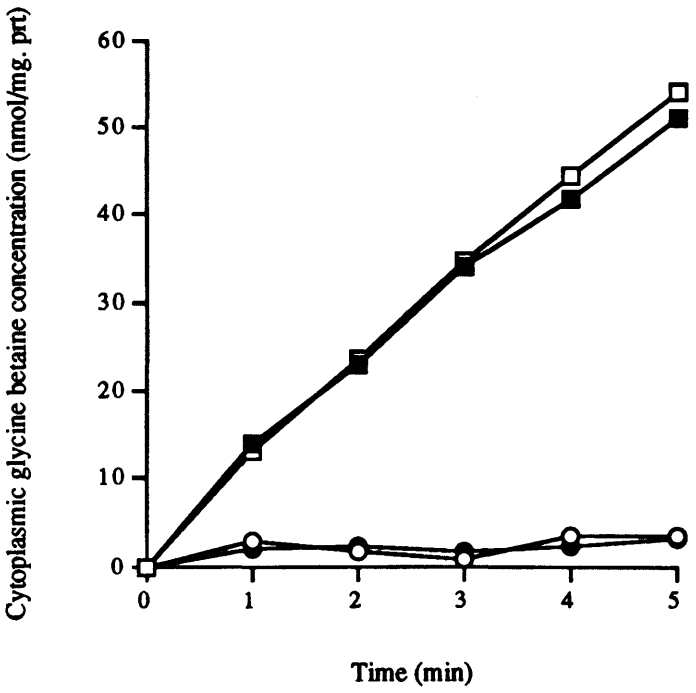


Fig. 6.21 Glycine betaine transport by *Lc. lactis* subsp. *cremoris* MG1363 (squares) and OSM 2x (circles) containing pCI372 (open symbols) or pCPL 3 (filled symbols). pCPL 3 consists of the glycine betaine transport gene from *Listeria monocytogenes* cloned into the vector plasmid pCI 372.

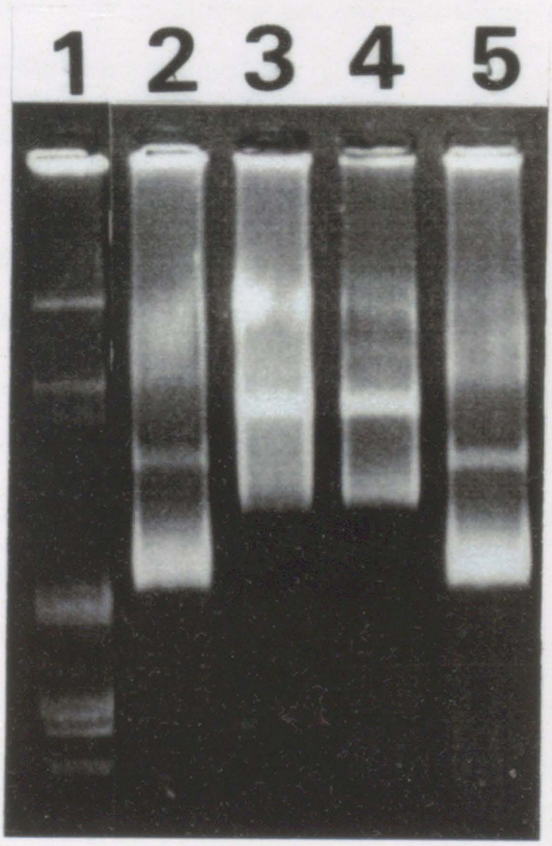


Fig. 6.22 Plasmid profiles of MG 1363 and OSM2x following transformation with the plasmids pCI372 and pCPL3. Lane 1 contains size marker plasmids from *Escherichia coli* V517 (55.9, 7.5, 5.8, 5.3, 4.1, 3.1 and 2.8 kb).

Lane 2: OSM2x with pCI372

Lane 3: OSM2x with pCPL3

Lane 4: MG1363 with pCPL3

Lane 5: MG1363 with pCI372

gene was extracted from an agarose gel and ligated into the shuttle vector pCI 372 (Hayes *et al.*, 1990) to create pCPL 3. The ligation mix was transformed into *Escherichia coli* DH5a cells by electroporation and the plasmid DNA isolated from transformed cells. Ligation of the 2.5kb fragment containing the glycine betaine transport gene into the vector pMG36ct was also attempted, however no transformants were obtained following electroporation of the ligation mix into *E. coli.*

The strains MG1363 and OSM 2x were readily transformable with pCPL 3,by electroporation however no increase in tolerance to low a_w was observed in OSM 2x containing pCPL 3 (Table 6.4). The presence of pCPL 3 did not restore the ability to transport glycine betaine either (Fig. 6.21). The presence of pCPL 10 in OSM 2x and MG1363 was confirmed by gel electrophoresis of isolated plasmid DNA (Fig. 6.22). The *Lc. lactis* subsp. *cremoris* strain HP did not display any glycine betaine transport activity, and this strain was also transformed with pCPl 3. As was the case with OSM 2x, the presence of the plasmid did not confer the ability to transport glycine betaine (data not shown).

6.3 Discussion.

The data presented in this chapter suggest that glycine betaine is the most important of the compatible solutes for lactococci. Proline, reported by Molenaar *et al* (1993) to be accumulated during osmotic stress was not nearly as effective as glycine betaine. There are two possible reasons for this. Firstly, glycine betaine may be a more effective compatible solute than proline and its accumulation may be more beneficial to the cell. There is a certain amount of evidence to support this proposition as glycine betaine is preferred over proline by most bacteria, including *Lc. lactis* (Molenaar *et al.*, 1993). The second possibility is that some proline accumulation occurs as a result of synthesis and that the apparent effect of added proline is thereby reduced. The slight stimulatory effect of added proline would then serve to augment that from synthesised proline levels. Stimulation of growth by added leucyl-proline was also observed. What is not known is whether intracellular peptidase activity results in the generation of free

proline or if the peptide itself acts as a compatible solute. *Lc lactis* strains are known to have prolidase enzymes which hydrolyse X-pro dipeptides thus generating free proline (Kunji *et al.*, 1996). A similar osmoprotective effect of proline-containing peptides has been reported for *Listeria monocytogenes* (Amezaga *et al.*, 1995) in which stimulation by the dipeptide proline-hydroxyproline was observed. It appeared that accumulation of the dipeptide as well as the free amino acid residues ocurred in *L. monocytogenes* (Amezaga *et al.*, 1995). The slight stimulation observed with leucyl-proline raises the question of whether the peptide transport system necessary for its uptake is osmotically regulated. *Lc. lactis* has at least two transport systems which transport di/tri peptides one of which (DtpT) is proton motive force driven and transports hydrophilic substrates (Smid *et al.*, 1989); the other (DtpP) is driven by ATP hydrolysis and transports hydrophobic substrates (Foucaud *et al.*, 1995). Leucyl-proline has hydrophobic tendencies and therefore is likely to be transported by DtpP. At present there is no information available on the osmotic regulation of peptide transport systems.

Carnitine is similar to glycine betaine in that it has a tri-methylated amino group and is utilised as a compatible solute by many Gram-positive bacteria, including *Lactobacillus plantarum* (Kets *et al.*, 1994) and *Listeria monocytogenes* (Beumer *et al.*, 1994). The data presented in Fig. 6.5 demonstrate that carnitine does not appear to stimulate the growth of *Lc. lactis* at low a_w. In *Lb. plantarum*, glycine betaine and carnitine are transported by the same system (Glaasker *et al.*, 1998b), while in *L. monocytogenes* separate systems are present and the glycine betaine system does not appear to transport carnitine (Verheul *et al.*, 1997). It is therefore, reasonable to assume that despite the structural similarity of both compounds, that some glycine betaine systems are incapable of transporting carnitine and this appears to be the case for *Lc. lactis*.

The low a_w sensitive mutant, OSM2x, constructed by transposing the insertion sequence IS*S1* into the genome of *L. lactis* subsp. *cremoris* MG1363, a plasmid free derivative of NCDO 712, has lost the ability to take up the substantial quantities of glycine betaine needed to grow in low a_w environments. Low concentrations of glycine

betaine were accumulated by the mutant growing in CDM at high a_w, but these basal levels were not increased when the a_w was decreased, in contrast to the response of the parent cultures (Fig. 6.20). When assayed for glycine betaine transport using the conventional assay no glycine betaine transport was detected in the mutant (Fig. 6.19). Since the mutation in OSM 2x is probably caused by a single insertion into the parent genome (Fig. 6.16), it suggests that the parent culture has only one system for glycine betaine transport which is capable of the response needed when the a_w of the environment is substantially reduced.

The results presented in Chapter III of this thesis show that for NCDO 712, NaCl concentrations only slightly in excess of the upper limit for growth, are bacteriocidal. This was also true in the case of the mutant as 3.5% w/v NaCl was lethal to OSM 2x even though the parent strain MG 1363 was capable of growth above this NaCl concentration. The mutation did not appear to result in increased sensitivity to NaCl concentrations higher than 3.5% w/v as both the parent strain and the mutant were equally sensitive to 5 and 10% w/v NaCl. These data suggest that the mechanism of cell death which is preventable by glycine betaine at concentrations of 3.5-4% w/v NaCl may be different to that at higher NaCl concentrations such as 10-20% NaCl or that glycine betaine is ineffective at these higher concentrations.

Transforming the mutant with a plasmid encoded glycine betaine transport gene from *Listeria monocytogenes* did not complement the mutation in OSM 2x. The most likely reason for this is that the promoter of the cloned gene is not recognised in *Lc. lactis*. Sequence data for the gene indicates that it is under the control of the alternative sigma factor σ^B (R.Sleator, Dept of Microbiology U.C.C., personal communication). It is unlikely that this promoter is recognised by *Lc. lactis*. Attempts to clone the gene into the vector pMG36ct were unsuccessful; this vector contains a strong *Lc. lactis* promoter upstream of the cloning site which should ensure strong expression in *Lc. lactis*. It is possible that strong constitutive expression may be lethal, leading to failure to obtain transformant colonies containing pMG 36ct ligated to the glycine betaine gene.

Molenaar *et al.* (1993) suggested that there were two systems for glycine betaine uptake in *L. lactis* strain ML3, a close relative of NCDO 712 (Davies *et al.*, 1981) from which MG 1363 is derived. They suggested that the first was a high affinity transport system which was not induced by increasing the osmolarity of the growth medium. The second system was a low affinity proline uptake system which also transported glycine betaine. The second system was induced by growing the cells in a chemically defined medium at high osmolarity. Strain OSM 2x did not transport glycine betaine at high or low a_w suggesting a complete loss of glycine betaine transport as a result of a single insertional mutation event. This result does not support the conclusion of Molenaar *et al.* (1993) that two glycine betaine transport systems exist in lactococci.

There are several possibilities raised by this apparent difference in results. It is possible that glycine betaine transport in ML3 differs from that in NCDO 712 even though both strains are regarded as being very similar (Davies *et al.*, 1981). A second possibility is that two glycine betaine transport systems exist in NCDO 712 (and MG1363) but that both are eliminated in the OSM 2x mutant. A third possibility is that there is only one system for glycine betaine transport in NCDO 712 and this is eliminated in the OSM 2x mutant. Our data fit the latter model as it is likely that the OSM 2x mutation is due to a single insertion into the chromosome. However, there is one important observation which does not fit this model for glycine betaine transport. When NCDO 712 cells were grown at high a_w, glycine betaine transport was not stimulated by the presence of NaCl in the assay, whereas the transport system in cells grown at low a_w were stimulated. The Km values for glycine betaine transport for cells grown at high or low a_w were similar which is evidence for one system or two systems with similar affinities. It is possible that the difference in glycine betaine transport kinetics in cells grown at high and low a_w is due to a fundamental change in the cells growing at low a_w which causes an activation of the glycine betaine transport protein at low a_w. Such an activation of glycine betaine transport could account for a similar observation in *Lb. plantarum* by Glaasker *et al.*(1996a). The only data to support two transport systems is the absence of osmotic activation for cells grown at high a_w.

Glaasker (1998) proposed that the failure of some workers (including Molenaar *et al.*) to observe activation may be an artifact of the experimental conditions. For example, osmotic activation of glycine betaine or carnitine transport in *L. monocytogenes* was only observed when cells contained pre accumulated compatible solutes (Verheul *et al.*, 1997). On balance, glycine betaine transport in *Lc. lactis* probably occurs via a single system, the activity of which is osmotically regulated if the cells are grown at low a_w.

The transport of glycine betaine in Gram-positive and Gram-negative bacteria has been well studied. Multiple transport systems have been reported in many cases. The best studied example being the ProU and ProP systems of *E. coli* and *Salmonella typhimurium* (Cairney *et al.*, 1985 a, b). The ProU system has a high affinity for betaine while the ProP system has a lower affinity for glycine betaine and also transports proline. Similar high- and low-affinity transport systems for glycine betaine were observed in *Staphylococcus aureus* with the low affinity system also transporting proline (Pourkomailan and Booth, 1992). In *Bacillus subtilis*, three transport systems for glycine betaine have been reported (Kappes *et al.*, 1996). The presence of multiple systems of different affinities allows the cell to accumulate betaine efficiently over a wide range of extracellular betaine concentrations (Koo and Booth, 1994). In *Corynebacterium glutamicum* (Peter *et al.*, 1995) and *Lb. plantarum* (Glaasker, 1998) it appears that only one system for glycine betaine is present. Single glycine betaine transport systems tend to have high substrate affinity, be expressed constitutively or semi-constitutively and are strongly regulated by solute concentration at the activity level. The characteristics of the glycine betaine transport system in NCDO 712 are similar to those reported for *Lb. plantarum* and *C. glutamicum* in that a significant degree of control is exerted at the level of transporter activity.

The *Lc. lactis* strains examined formed two distinct groups with respect to their ability to grow in the presence of added NaCl or sugar. All of the *cremoris* strains with the exception of NCDO 712 were much more sensitive to low a_w than were the *lactis* strains. As was the case with the OSM 2x mutant, failure to grow at low a_w appeared be related to the ability of the cells to accumulate the compatible solute glycine betaine.

Two of the strains, did not transport glycine betaine in the assay systems used here. Growth of these strains at low a_w did not result in increased glycine betaine transport rates. The third *cremoris* strain examined, US3, transported glycine betaine at a much lower rate than did the osmotolerant strains. The low levels of betaine transport were reflected in the glycine betaine content of exponential phase growing cells. Some betaine accumulation was measureable for strains US3 and BK5, but only when the solute used to reduce the a_w was NaCl, unlike the *lactis* strains which accumulated betaine when the a_w was reduced by NaCl or sugar. It appears that glycine betaine accumulation by the salt sensitive strains differs from that observed in the salt tolerant strains. When the amount of glycine betaine accumulated by each strain is compared, it is obvious that NCDO 712 accumulates much less betaine than the other salt tolerant strains which is reflected in the fact that NCDO 712 was stimulated by glycine betaine to a lesser extent than the other strains. This correlation between the accumulation of glycine betaine and the ability to grow at low a_w is similar to that reported for *Lactobacillus* spp. A strain of *Lactobacillus acidophilus* which was abnormally osmotolerant, was able to transport betaine at higher rates than less osmotolerant strains due to the presence of a constitutively expressed transport system activated at low a_w (Hutkins *et al.*, 1987).

The possible reasons for such a substantial difference in glycine betaine transport between strains which are so closely related are intriguing. Strains of *Lc. lactis* subsp. *cremoris* which have some of the traits formerly associated with *Lc. lactis* subsp. *lactis* (growth at pH 9.2, 4% w/v NaCl and at 40°C and arginine hydrolysis) are not unusual. In one study only 26 out of 59 genotypically *cremoris* strains had the classical *cremoris* phenotype. The remaining 33 strains had some or all of the classical *lactis* traits including 25 which grew at 4% w/v NaCl (Urbach *et al.*, 1997). Analysis of the genome of *Lc. lactis* strains has revealed that the overall high degree of DNA homology between the two strains is a composite of highly conserved regions with 80-90 % homology and variable regions of <40% homology. It has been suggested, based on studies of codon usage and base composition, that the variable regions arose as a

result of horizontal transfer of genes from distantly related species (Delorme *et al.*, 1994). Maybe gene transfer in this manner has led to differences in phenotype between *lactis* and *cremoris* strains (Urbach *et al.*, 1997).

Chapter VII
Concluding Remarks.

In this study the mechanisms by which *Lc. lactis* subsp. *cremoris* NCDO 712 adapts to osmotic stress were investigated. Chapter I of the thesis provides a detailed review of the literature concerning osmotic stress in bacteria and mechanisms by which bacteria adapt to fluctuations in the a_w of their environment. The role of compatible solutes in bacterial osmotolerance was reviewed in some depth, as compatible solute accumulation is almost universal during osmotic stress (Csonka, 1989).

In Chapter III of this thesis, the survival of *Lc. lactis* at NaCl concentrations in excess of those that permit growth were investigated. It has previously been shown that the resistance of *Lc. lactis* subsp *cremoris*. NCDO 712 to lethal acid challenge is dependent on the growth phase of the culture (O'Sullivan, 1996). A similar trend was observed, in this study, for the tolerance of NCDO 712 to 20% NaCl. There are a number of possible explanations for the variation in tolerance. It is possible that the cell density plays a role in triggering the development of resistance in mid-exponential phase. Cell density dependent regulation, also known as quorum sensing, has been shown to have a role in induction of the *lux* operon in *Vibrio fischeri*. In addition, bacteriocin production by lactobacilli and catalase production by *Rhizobium leguminosarum* appear to be related to the cell density of the respective cultures. The basic model for quorum sensing involves the production of an autoinducer molecule by the cells; when the cell density of a culture increases there is an accelerated increase in the inducer concentration, which accelerates the induction of the response.

Another explanation for growth phase dependent variation in stress tolerance is that it results from growth phase dependent changes in gene expression. The alternative sigma factor s^S in *E. coli* is present only in late exponential phase/early stationary phase cells. As s^S functions in the transcription of genes involved in the response to a number of environmental stresses (Hengge-Aronis, 1996), it is easy to see how it could participate in growth phase dependent stress responses.

Lc. lactis subsp. *cremoris* NCDO 712 was capable of growth at up to 4% w/v NaCl; increasing the NaCl concentration to 5% w/v was bacteriocidal. There is therefore a very narrow margin between those NaCl concentrations which allow growth

and those which are bacteriocidal. Little is known about the mechanism by which NaCl damages bacterial cells. However, efflux of water following osmotic stress probably reduces the a_w to a level where some damage to cell proteins would be expected to occur. Loss of turgor pressure, if it occurred, might also inhibit the activity of membrane bound enzymes. It has been reported that carbohydrate transport in *E. coli* is inhibited by osmotic stress (Roth *et al.*, 1995). In addition, osmotic stress inhibited glycolysis in *Clostridium pasteurianum* (Walter *et al.*, 1987). In both examples, the generation of ATP is likely to be reduced, so that in effect, the cells are starved. Prolonged starvation is in itself lethal to *Lc. lactis* (Hartke *et al.*, 1994). If transport of carbohydrates or glycolysis were inhibited in *Lc. lactis* then starvation stress would be imposed, together with the other expected effects at low a_w such as low turgor pressure.

If NCDO 712 cells were exposed to 3% w/v NaCl for 1 hour prior to challenge with lethal concentrations of NaCl they were more resistant to the challenge than cells which were not pre-exposed to 3% w/v NaCl. The enhanced tolerance to NaCl challenge required protein synthesis and appeared to be only effective at NaCl concentrations $\leq$ 10% w/v. The induced tolerance to NaCl appears to be analogous to other inducible stress responses such as heat shock and the acid tolerance response (ATR). The ATR was first reported for *Escherichia coli* (Goodson and Rowbury, 1989) and subsequently for a number of other Gram-positive and Gram-negative bacteria. Induction of an ATR in *Lc. lactis* NCDO 712 which also induced resistance to heat, H_2O_2, ethanol, and 20% w/v NaCl has been reported (O'Sullivan and Condon, 1997). In this study, the effect of induction of NaCl tolerance on resistance to other stresses was not investigated, but it is known that exposure to 4% w/v NaCl for 1 hour does not induce tolerance to pH 4.0 in NCDO 712 (O'Sullivan and Condon, 1997). It is likely that the induced protection is due to induction of chaperone proteins. A number of these proteins are synthesised by *Lc. lactis* during exposure to 2.5% w/v NaCl (Kilstrup *et al.*, 1997). A major function of chaperone proteins is to degrade proteins which have become damaged or malformed, with the classic example being the action

of heat-shock proteins. DnaK, which is a well known heat-shock protein is known to have a role in restoring turgor pressure in *E. coli*, during osmotic stress (Meury and Kohiyama, 1991).

The mechanisms which bacteria use to survive exposure to high concentrations of NaCl are not well understood. Survival of NCDO 712 during NaCl challenge was enhanced by the presence of magnesium ions, as was the survival during challenge at 42°C and low pH. It is possible that magnesium has a protective effect against a number of different stresses in *Lc. lactis* as it also increases survival during prolonged starvation (Thomas and Batt, 1967). Magnesium therefore, either directly stabilises some cell component(s) damaged by exposure to environmental stress or is essential for the action of a stress tolerance mechanism(s). There is evidence to support both possibilities as magnesium can act directly to stabilise ribosomes (Hui Bon Hoa *et al.*, 1980) and ribosomal RNA (Tolker-Nielsen and Molin 1996). Intact ribosomes may be needed for initiation of growth when the lethal stress is removed; therefore cells challenged in the absence of magnesium may be unable to resume growth due to lack of functional ribosomes. Magnesium is also essential for the ATPase activity of the chaperone DnaK (Skowyra and Wickner, 1995). The involvement of magnesium in DnaK activity may explain its role in the tolerance of *Lc. lactis* to a number of stresses as DnaK is induced in response to heat (Kilstrup *et al.*, 1997), low pH (Hartke *et al.*, 1996) and NaCl (Kilstrup *et al.*, 1997).

The role of potassium in the adaptation of *Lc. lactis* subsp. *cremoris* NCDO 712 to osmotic stress was examined in Chapters IV and V. When the concentration of potassium was not limiting, potassium was accumulated to high cytoplasmic levels when the a_w of the medium was decreased by addition of glucose or fructose, but not when NaCl was used. If the potassium concentration of MTEL medium was limited, the reduction of the a_w with NaCl led to a decrease in the specific growth rate, cytoplasmic potassium content and cytoplasmic volume changes which were not observed when potassium was not limiting. Addition of NH_4Cl instead of NaCl led to similar but slightly more severe effects on growth rate and potassium content. Similar

results were reported by MaCleod and Snell (1948) who observed that the potassium requirement of lactic acid bacteria increased if other monovalent cations were present in the medium. The degree of growth inhibition by NH_4Cl and NaCl correlated well with the inhibitory effect of each compound on potassium transport.

Growth in the presence of NaCl in MTEL medium containing 0.5 mM potassium clearly demonstrated the role of potassium in maintaining turgor. The volume of the cells growing in MTEL containing 0.25M NaCl decreased by 30% when compared to cells growing in the absence of salt stress. This reduction in volume may explain the reduction in specific growth rate observed under these conditions.

Potassium transport occurred via a single system which had a low affinity for potassium. Potassium transport was competitively inhibited by Na^+ and other cations and non-competitively inhibited by low a_w. Na^+ ions were not accumulated in place of potassium, which was confirmed by the absence of Na^+ transport by choline loaded cells. The data suggest that Na^+ ions bind to the potassium transporter, yet are not transported. Normally, potassium transport systems are highly discriminatory in their substrate binding (Doyle *et al.*, 1998). The situation in *Lc. lactis* is therefore unusual in that the potassium transporter discriminates poorly between potassium and other monovalent cations.

The apparent Km for potassium transport (0.75 mM) is approximately 7-fold greater than the potassium concentration (100 μM) which supports growth at 50% of the maximal rate. The cells are therefore capable of a much higher rate of potassium transport than is needed for growth at high a_w. The fact that the cell can transport potassium at rates higher than are needed for growth suggests that the extra potassium transport capability could be used for osmoregulation. The 2-fold increase in the specific activity of the system in cells growing at low a_w may contribute to the raised cellular potassium content of the cells. However, the increased specific activity is almost exactly counterbalanced by the inhibitory effect of low a_w on the rate of transport. It is therefore likely that there is another means of control of potassium transport. The inhibition of the rate of transport by low a_w seems to rule out activation

by reduced turgor; however, it must be remembered that the potassium transport assays were performed using cells artificially depleted of potassium. The effect of low a_w on the activity of the system *in vivo* may be different. It is also possible that the intracellular potassium concentration is controlled by the activity of efflux systems. Potassium efflux in *E. coli* occurs rapidly if cells growing at low a_w are shifted to high a_w (Epstein and Schultz, 1965; Meury *et al.*, 1985; Bakker, 1993; Schleyer *et al.*, 1993). Rapid efflux of cytoplasmic solutes in this manner is probably due to the activity of stretch activated channels and is probably an emergency response to counteract the risk of the cells bursting during hypo-osmotic stress. There is a requirement for tight control of potassium movement across the cytoplasmic membrane; uncontrolled accumulation followed by efflux amounts to futile cycling and is extremely wasteful of energy. In *E. coli*, the rate of uptake of potassium when turgor is high is only 1 to 2% of the value when turgor is low (Meury *et al.*, 1985; Bakker, 1993). It is likely that control of potassium levels in *Lc. lactis* is mediated in a similar manner.

Growth of *Lc. lactis* did not occur at potassium concentrations below 30 μM. Therefore *Lc. lactis* does not appear to possess a high affinity potassium transport system similar to the Kdp system of *E. coli* or *Bacillus acidocaldarius* (Bakker et al., 1987) which allows the cells to scavenge potassium efficiently at nanomole concentrations. The relationship between the growth rate of *Lc. lactis* NCDO 712 and the available potassium in MTEL medium approximately obeyed Michaelis-Menten kinetics and the apparent Km value was 100 μM.

In Chapter VI the role of glycine betaine and other organic compatible solutes was investigated. Of the compatible solutes tested, only glycine betaine produced substantial stimulation of growth at low a_w. Proline was also stimulatory but to a lesser degree. Carnitine and ectoine were not stimulatory even though both have been reported as being stimulatory in other Gram-positive bacteria. Leucyl-proline stimulated growth to about the same extent as did proline. We cannot say whether the stimulation results from accumulation of intact leucyl-proline as reported for other dipeptides in *Listeria*

monocytogenes (Amezaga *et al.*, 1995) or if leucyl-proline is hydrolysed intracellularly to provide free proline which is then accumulated.

Glycine betaine was the only significant organic compatible solute identified. The ability of glycine betaine to reverse the inhibition of growth rate and reduction of volume caused by NaCl at low potasssium concentrations is a good demonstration of its ability to restore turgor pressure. There was an excellent correlation between the ability to accumulate glycine betaine and growth at low a_w. The *cremoris* strains incapable of growth at 4% w/v NaCl or the equivalent glucose concentration, accumulated less glycine betaine than did the strains capable of growth at 4% w/v NaCl. As would be expected, the ability to accumulate glycine betaine seemed to be dependent on the presence of a significant degree of glycine betaine transport activity and the NaCl sensitive strains displayed little or no glycine betaine uptake in *in vitro* assays. Variation in the ability to accumulate glycine betaine within a genus is not unknown, and has been observed in *Lactobacillus* (Hutkins *et al.*, 1987) and in *Propionibacteriium* (Deborde,1998). *

The mutant strain OSM2x which was extremely sensitive to low a_w did not accumulate glycine betaine, nor did it display any measurable glycine betaine transport activity. The abolition of glycine betaine transport in OSM 2x with what appeared to be a single IS*S1* insertion suggests that there is only one transport system for glycine betaine. This does not agree with the results of Molenaar *et al.* (1993) who reported two glycine betaine transport systems in a strain (ML3) closely related to NCDO 712. Some of our data also suggested a second transport system, in that no activation of transport by the a_w of the assay buffer is observed for cells grown at normal a_w while activation was observed for cells grown at low a_w. However, growth at low a_w causes several physiological changes; these could include the synthesis of an 'activator' which enhances the rate of transport at low a_w but not at normal a_w. In such a scenario a single glycine betaine transporter could have different rates of transport depending on the a_w of the medium in which the cells were grown and on the a_w of the assay buffer.This topic requires further study.

Literature Cited

Abee, T., J. Knol, K.J. Hellingwerf, E.P. Bakker, A. Siebers and W.N. Konings. 1992. A kdp-like, high affinity, K^+-translocating ATPase is expressed during growth of *Rhodobacter sphaeroides* in low potassium media. *Arch. Microbiol.* **158:**374-380.

Alemayehu, D. The log-phase acid tolerance of *Lactococcus lactis* MG1363 during growth at constant pH 7.0 1997. M.Sc thesis. National University of Ireland, Dublin.

Amezaga, M.-R., I. Davidson, D. McLaggan, A. Verheul, T. Abee, and I.R. Booth. 1995. The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. *Microbiology.* **141:**41-49.

Anderson, C.B. and L.D. Witter. 1982. Glutamine and proline accumulation by *Staphylococcus aureus* with reduction in water activity. *J. Bacteriol.* **143:**1501-1503.

Anderson, D.G. and L.L. McKay. 1983. A simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.* **46:**549-552.

Anderson, R.R., R. Menzel, and J.M. Wood. 1980. Biochemistry and regulation of a second L-proline transport system in *Salmonella typhimurium. J. Bacteriol.* **141:**1071-1076.

Andresen, P.A., I. Kaasen, O.B. Styrvold, G. Boulnois and A.R. Strom. 1988. Molecular cloning, Physical mapping and expression of the bet genes governing the osmoregulatory choline-glycine betaine pathway of *Escherichia coli. J. Gen. Microbiol.* **134:**1737-1746.

Arnold, K.W. and C.W. Kasper. 1995. Starvation- and stationary-phase induced acid tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **61:**2037-2039.

Asha, H. and J. Gowrishankar. 1993. Regulation of *kdp* operon expression in *Escherichia coli*: evidence against turgor as signal for transcriptional control. *J. Bacteriol.* **175:**4528-4537.

Badger, J.L. and V.L. Miller. 1995. Role of *rpoS* in survival of *Yersinia enterocolitica* to a variety of environmental stresses. *J. Bacteriol.* **177:**5370-5373.

Bae, J.H. and K.J. Miller. 1992. Identification of two proline transport systems in *Staphylococcus aureus* and their possible roles in osmoregulation. *Appl. Environ Microbiol.* **58:**471-475.

Bakker, E.P. 1993. Cell K^+ and K^+ transport systems in prokaryotes. In E.P. Bakker, (ed) Alkali cation transport systems in prokaryotes. CRC Press Inc. Boca Raton Fl USA.

Bakker, E.P. and F.M. Harold. 1980. Energy coupling to potassium transport in *Streptococcus faecalis. J. Biol. Chem.* **255:**433-440.

Bakker, E.P., A. Borchard, M. Michels, K. Altendorf, and A. Siebers. 1987. High-affinity potassium uptake system in *Bacillus acidocaldarius* showing immunological cross-reactivity with the *kdp* system from *Escherichia coli. J. Bacteriol.* **169:**4342-4348.

Ball, C.A., R. Osuna, K.C. Ferguson and R.C. Johnson. 1992. Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli. J. Bacteriol.* **174:**8043-8056.

Becker, L.A., M.S. Çetin, R.W. Hutkins and A.K. Benson. 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. *J. Bacteriol.* **180:**4547-4554.

Benjamin, M. and A.R. Datta. 1995. Acid tolerance of enterohemorrhagic *Escherichia coli. Appl. Environ. Microbiol.* 61:1669-1672.

Berkower, I. J. Leis and J. Hurwitz. 1973. Isolation and characterisation of an endonuclease from *Escherichia coli* specific for ribonucleic acid in ribonucleic acid deoxyribonucleic acid hybrid structures. *J. Biol. Chem.* **248:**5914-5921.

Bernard, T., M. Jebbar, Y. Rassouli, S. Himdi-Kabbab, J. Hamelin and C. Blanco. 1993. Ectoine accumulation and osmotic regulation in *Brevibacterium linens. J. Gen. Microbiol.* **138:**1629-1638.

Beumer, R.R., M.C. Te Giffel, L.J. Cox, F.M. Rombouts and T. Abee. 1994. Effect of exogenous proline, betaine, and carnitine on growth of *Listeria monocytogenes* in a minimal medium. *Appl. Environ. Microbiol.* **60:**1359-1363.

Boch, J., B. Kempf and E. Bremer. 1994. Osmoregulation in *Bacillus subtilis*: Synthesis of the osmoprotectant glycine betaine from exogenously provided choline. *J. Bacteriol.* **176:**5364-5371.

Boch, J., B. Kempf, R. Schmid and E. Bremer. 1996. Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: characterisation of the *gbs*AB genes. *J. Bacteriol.* **178:**5121-5129.

Boch, J., G. Nau-Wagner, S. Kneip and E. Bremer. 1997. Glycine betaine aldehyde dehydrogenase from *Bacillus subtilis*: characterisation of an enzyme required for the synthesis of the osmoprotectant glycine betaine. *Arch. Microbiol.* **168:**282-289.

Bohringer, J., D. Fischer, G. Mosler, and R. Hengge-Aronis. 1995. UDP-glucose is a potential intracellular signal molecule in the control of expression of s^S and s^S-dependent genes in *Escherichia coli. J. Bacteriol.* **177:**413-422.

Booth, I.R., R.M. Douglas, G.P. Ferguson, A.J. Lamb, A.W. Munro and G.Y. Ritchie. 1993. K^+ Efflux systems. In Alkali cation transport systems in prokaryotes. E.P. Bakker (Ed). CRC Press Inc. Boca Raton FL USA.

Bossemeyer, D.A., A. Borchardt, D.C. Dosch, G.C. Helmer, W. Epstein, I.R. Booth and E.P. Bakker. 1989a. K^+ transport protein TrkA of *Escherichia coli* is a peripheral membrane protein that requires other *trk* gene products for attachment to the cytoplasmic membrane. *J. Biol. Chem.* **264**:16403-16410.s

Bossemeyer, D., A. Schlösser and E.P Bakker. 1989b. Specific cesium transport via the *Escherichia coli* Kup (TrkD) K^+ uptake system. *J. Bacteriol.* **171**:2219-2221.

Botsford, J.L. 1990. Analysis of protein expression in response to osmotic stress in *Escherichia coli. FEMS Microbiol. Lett.* **72:**355-360.

Brown, A.D. and J.R. Simpson. 1972. Water relations of sugar-tolerant yeasts; the role of intracellular polyols. *J. Gen. Microbiol.* **72:**589-591.

Cairney, J., I.R. Booth and C.F. Higgins. 1985a. *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. *J. Bacteriol.* **164:**1218-1223.

Cairney, J., I.R. Booth and C.F. Higgins. 1985b. Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. *J. Bacteriol.* **164:**1218-1223.

Cheville, A.M., K.W. Arnold, C. Buchrieser, C.M. Cheng and C.W.Kaspar. 1996. *rpoS* regulation of acid, heat, and salt tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **62:**1822-1824.

Clark, D. and J. Parker. 1984. Proteins induced by high osmotic pressure in *Escherichia coli. FEMS Microbiol. Lett.* **25:**81-83.

Cogan, T.M. 1995. Flavour production by dairy starter cultures. *J. Appl. Bacteriol. Symp. Suppl.* **79:**49S-64S.

Csonka, L.N, and A.D Hanson. 1991. Prokaryotic osmoregulation: genetics and physiology. *Annu. Rev. Microbiol.* **45:**569-606.

Csonka, L.N. 1981. Proline over-production results in enhanced osmotolerance in *Salmonella typhimurium. Mol. Gen. Genet.* **182:**82-86.

Csonka, L.N. 1982. A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. *J. Bacteriol.* **151:**1433-1443.

Csonka, L.N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* **59:**121-147.

Csonka, L.N., T.P. Ikeda, S.A. Fletcher, and S. Kustu. 1994. The accumulation of glutamate is necessary for optimal growth of *Salmonella typhimurium* in media of high osmolarity but not induction of the *proU* operon. *J. Bacteriol.* **176:**6324-6333.

Dattananda, C.S., K. Rajkumari, and J. Gowrishankar. 1991. Multiple mechanisms contribute to the osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and of a negative regulatory element within the first structural gene. *J. Bacteriol.* **173:**7481-7490.

Davies, F.L., H.M. Underwood and M.J. Gasson. 1981. The value of plasmid profiles for strain identification in lactic streptococci and the relationship between *Streptococcus lactis* 712, ML3 and C2. *J. Appl. Bacteriol.* **51:**325-337.

Davis, M.J., P.J. Coote and C.P. O' Byrne. 1996. Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology* . **142:**2975-2982.

Deborde, C. 1998. Etude du metabolisme carbone primaire de bacteries propioniques laitieres par resonance magnetique nucleaire *in vivo* ^{13}C. Thèse Doctorat. L'Ecole Nationale Superieure Agronomique de Rennes.

Delorme, C., J.J. Godon, S.D. Ehrlich and P. Renault. 1994. Mosaic structure of large regions of the *Lactococcus lactis* subsp. *cremoris* chromosome. *Microbiology.* **140:**3053-3060.

Deuerling, E., B. Paeslack and W. Schumann. 1995. The *ftsH* gene of *Bacillus subtilis* is transiently induced after osmotic and temperature upshift. *J. Bacteriol.* **177**:4105-4112.

Dinnbier, U. E. Limpenstiel, R. Schmid and E.P. Bakker. 1988. Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. *Arch Microbiol.* **150**:348-357.

Dinsmore, P.K., D.A. Romero and T.R. Klaenhammer. 1993. Insertional mutagenisis in *Lactococcus lactis* subsp. *lactis* mediated by IS*946*. *FEMS Microbiol. Lett.* **107**:43-48.

Dosch, D.C., G.L. Helmer, S.H. Sutton, F.F. Salvacion and W. Epstein. 1991. Genetic analysis of potassium transport loci in *Escherichia coli:* evidence for three constitutive systems mediating uptake of potassium. *J. Bacteriol.* **173**:687-696.

Doyle, D.A., J.M. Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbuis, S.L. Cohen, B.T. Chait and R. MacKinnon. 1998. The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* **280**:69-77.

Dunlap, V.J., and L.N. Csonka. 1985. Osmotic regulation of L-proline transport in *Salmonella typhimurium. J. Bacteriol.* **163**:296-304.

Epstein, W. 1986. Osmoregulation by potassium transport in *Escherichia coli. FEMS Microbiol. Rev.* **39**:73-78.

Epstein, W. and B.S. Kim. 1971. Potassium transport loci in *Escherichia coli. J. Bacteriol.* **108**:639-644.

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Epstein, W. and S.G. Schultz. 1965. Cation transport in *Escherichia coli.* V. Regulation of cation content. *J. Gen. Physiol.* **49:**221-234.

Epstein, W., V. Whitelaw and J. Hesse. 1978. A K^+ transport ATPase in *Escherichia coli. J. Biol. Chem.* **253:**6666-6668.

Eshoo, M.W. 1988. *lac* fusion analysis of the *bet* genes of *Escherichia coli*: regulation by osmolarity, temperature, oxygen, choline and glycine betaine. *J. Bacteriol.* **170:**5208-5215.

Flahaut, S., A. Benachour, J.-C. Giard, P. Boutibonnes and Y. Auffray. 1996. Defense against lethal treatments and de novo protein synthesis induced by NaCl in *Enterococcus faecalis* ATCC 19433. *Arch. Microbiol.* **165:**317-324.

Flahaut, S., A. Hartke, J.-C. Giard and Y. Auffray. 1997. Alkaline stress response in *Enterococcus faecalis*: adaptation, cross-protection, and changes in protein synthesis. *Appl. Environ. Microbiol.* **63:**812-814.

Foucaud, C., E.R.S. Kunji, A. Hagting, J. Richard, W.N. Konings, J. Desmazeaud and B. Poolman. 1995. Specificity of peptide transport systems in *Lactococcus lactis*: evidence for a third system which transports hydrophobic di- and tripeptides. *J. Bacteriol.* **177:**4652-4657.

Free, A. and C.J. Dorman. 1997. The *Escherichia coli stpA* gene is transiently expressed during growth in rich medium and is induced in minimal medium and by stress conditions. *J. Bacteriol.* **179:**909-918.

Frymier, J.S., T.R. Reed, S.A. Fletcher and L.N. Csonka. 1997. Characterisation of transcriptional regulation of the *kdp* operon of *Salmonella typhimurium. J. Bacteriol.* **179:**3061-3063.

Galinski, E. and H.G. Trüper. 1982. Betaine, a compatible solute in the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochloris. FEMS Microbiol. Lett.* **13:**357-360.

Galinski, E.A., H.P. Pfeiffer and H.G. Truper. 1985. 1,4,5,6-tetrahydro-2-methyl-4 pyrimidinecarboxylic acid: a novel cyclic amino acid from halophilic phototrophic bacteria of the genus *Ectothiorhodospira halochloris. Eur. J. Biochem.* **149:**135-139.

Garvie, E.I., J.A.E. Farrow and B.A. Phillips. 1981. A taxonomic study of some strains of streptococci which grow at 10°C but not at 45°C including *Streptococcus lactis* and *Streptococcus cremoris. Zbl. Bakt. Hyg., I. Abt. Orig.* C **2:**151-165.

Gasson, M.J. 1983. Plasmid components of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154:**1-9.

Gauthier, M.J., G.N. Flatau, D. Le Rudulier, R.L. Clément and M-P. Combarro. 1991. Intracellular accumulation of potassium and glutamate specifically enhances survival of *Escherichia coli* in seawater. *Appl. Environ. Microbiol.* **57:**272-276.

Gentry, D.R., V.J. Hernandez, L.H. Nguyen, D.B. Jensen and M. Cashel. 1993. Synthesis of the stationary-phase signa factor σ^s is positively regulated by ppGpp. *J. Bacteriol.* **175:**7982-7989.

Georgopoulos, C. 1992. The emergence of the chaperone machines. *Trends in Biochemical Sciences* **17**:295-299.

Gerhardt, P.N., L.T. Smith and G.M. Smith. 1996. Sodium-driven, osmotically activated glycine betaine transport in *Listeria monocytogenes* membrane vesicles. *J. Bacteriol.* **178**:6105-6109.

Giæver, H.M., O.B. Styrvold, I. Kaasen and A.R. Strøm. 1988. Biochemical and genetic characterisation of osmoregulatory trehalose synthesis in *Escherichia coli. J. Bacteriol.* **170**:2841-2849.

Glaasker, E. 1998. Osmotic regulation of transport processes in *Lactobacillus plantarum.* Ph.D thesis University of Groningen, The Netherlands.

Glaasker, E., W.N. Konings and B. Poolman. 1996a. Osmotic regulation of intracellular solute pools in *Lactobacillus plantarum. J. Bacteriol.* **178**:575-582.

Glaasker, E., W.N. Konings and B. Poolman. 1996b. Glycine betaine fluxes in *Lactobacillus plantarum* during osmostasis and hyper- and hypo-osmotic shock. *J. Biol. Chem.* **271**:10060-10065.

Glasemacher, J. A. Siebers, K. Altendorf and P. Schönheit. Low-affinity potassium uptake system in the archaeon *Methanobacterium thermoautotrophicum*: overproduction of a 31-kilodalton membrane protein during growth on low potassium medium. *J. Bacteriol.* **178**:728-734.

Godon, J.J., C. Delorme, S.D. Ehrlich and P. Renault. 1992. Divergence of genomic sequences between *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris. Appl. Environ. Microbiol.* **58:**4045-4047.

Goodson, M. and R.J. Rowbury. 1989. Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. *Lett. Appl. Microbiol.* **8:**77-79.

Gousbet, G., A. Trautwetter, S. Bonnassie, L.F. Wu and C. Blanco. 1996. Characterisation of the *Erwinia chrysanthemi* osmoprotectant transporter *ousA*. *J. Bacteriol.* **178:**447-455.

Gowrishankar, J. 1985. Identification of osmoresponsive genes in *Escherichia coli*: evidence for the participation of potassium and proline transport systems in osmoregulation. *J. Bacteriol.* **164:**434-445.

Graham, J.E. and B.J. Wilkinson. 1992. *Staphylococcus aureus* osmoregulation: roles for choline, glycine betaine, proline, and taurine. *J. Bacteriol.* **175:**2400-2406.

Grothe, S., R.L. Krogsrad, D.J. McLellan, J.L. Milner and J.M. Wood. 1986. Proline transport and osmotic stress response in *Escherichia coli* K-12. *J. Bacteriol.* **166:**253-259.

Haardt, M., B. Kempf, E. Faatz and E. Bremer. 1995. The osmoprotectant proline betaine is a major substrate for the binding protein-dependent transport syatem ProU of *Escherichia coli* K-12 *Mol. Gen. Genet.* **246:**783-786.

Hafer, J., A. Siebers and E.P. Bakker. 1989. The high-affinity K^+-translocating ATPase complex from *Bacillus acidocaldarius* consists of three subunits. *Mol. Microbiol.* **3**:487-495.

Harold, F.M. and J.R. Baarda. 1967. Inhibition of potassium transport by sodium in a mutant of *Streptococcus faecalis. Biochemistry.* **6**:3107-3110.

Hartke, A. S. Bouché, X. Gansel, P. Boutibonnes and Y. Auffray. 1994. Starvation-induced stress resistance in *Lactococcus lactis* subsp. *lactis* IL1403. *Appl. Environ. Microbiol.* **60**:3474-3478.

Hartke, A., J. Frère, P. Boutibonnes and Y. Auffray. 1997. Differential induction of the chaperonin GroEL and the co-chaperonin GroES by heat, acid and UV-irradiation in *Lactococcus lactis* subsp. *lactis. Curr. Microbiol.* **34**:23-26.

Hartke, A., S. Bouché, J-C. Giard, A. Benachour, P. Boutibonnes and Y. Auffray. 1996. The lactic acid stress response of *Lactococcus lactis* subsp. *lactis. Curr. Microbiol.* **33**:194-199.

Hartke, A., S. Bouché, J-M. Laplace, A. Benachour, P. Boutibonnes and Y. Auffray. 1995, UV-inducible proteins and UV-induced cross-protection against acid, ethanol, H_2O_2 or heat treatments in *Lactococcus lactis* subsp. *lactis. Arch. Microbiol.* **163**:329-336.

Hayes, F., C. Daly and G.F. Fitzgerald. 1990. Identification of the minimal replicon of *Lactococcus lactis* subsp. *lactis* UC317 plasmid pCI305. *Appl. Environ. Microbiol.* **56**:202-209.

Hecker, M., C. Heim, U. Völker and L. Wölfel. 1988. Induction of stress proteins by sodium chloride treatment in *Bacillus subtilis. Arch. Microbiol.* **150:**564-566.

Hecker, M., W. Schumann and U. Völker. 1996. Heat shock and general stress response in *Bacillus subtilis. Mol. Microbiol.* **19:**417-428.

Hengge Aronis, R. 1996. Regulation of gene expression during entry into stationary phase. In F.C. Neidhardt *et al.* (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington DC USA.

Hengge-Aronis, R., R. Lange, N. Henneberg and D. Fischer. 1993. Osmotic regulation of *rpoS*-dependent genes in *Escherichia coli. J. Bacteriol.* **175:**259-265.

Hengge-Aronis, R., W. Klein, R. Lange, M. Rimmele and W. Boos. 1991. Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary phase thermotolerance in *Escherichia coli. J. Bacteriol.* **173:**7918-7924.

Higgins, C.F., C.J. Dorman, D.A. Stirling, L. Waddell, I.R. Booth, G. May and E. Bremer. 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli. Cell* **52:**569-584.

Hill, C., C. Daly and G.F. Fitzgerald. 1987. Development of high-frequency delivery system for transposon Tn*919* in lactic streptococci: random insertion in *Streptococcus lactis* subsp. *diacetylactis* 18-16. *Appl. Environ. Microbiol.* **53:**74-78.

Hill, C., C. Daly and G.F. Fitzgerald. 1991. Isolation of chromosomal mutations of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* 18-16 after introduction of Tn*919*. *FEMS Microbiol. Lett.* **81**:135-140.

Hinton, J.C.D., D.S. Santos, A. Seirafi, C.S.J. Hulton, G.D. Pavitt and C.F. Higgins. 1992. Expression and mutational analysis of the nucleoid-associated protein H-NS of *Salmonella typhimurium. Mol. Microbiol.* **6**:2327-2337.

Hui Bon Hoa, G., E. Bégard, P. Beaudry, P. Maurel, M. Grumberg-Manago and P. Douzou. 1980. Analysis of cosolvent and divalent cation effects on association equilibrium and activity of ribosomes. *Biochemistry* **19**:3080-3087.

Huisman, G. and R. Kolter. 1994. Sensing starvation: a homoserine lactone-dependent signalling pathway in *Escherichia coli. Science.* **265**:537-539.

Hutkins, R.W., W.F. Ellefson, and E.R. Kashket. 1987. Betaine transport imparts osmotolerance on a strain of *Lactobacillus acidophilus. Appl. Environ. Microbiol.* **53**:2275-2281.

Imhoff, J.F. 1986. Osmoregulation and compatible solutes in eubacteria. *FEMS Microbiol. Rev.* **39**:57-66.

Jebbar, M., C. von Blohn and E. Bremer. 1997. Ectoine functions as an osmoprotectant in *Bacillus subtilis* and is accumulated via the ABC transport system OpuC. *FEMS Microbiol. Lett.* **154**:325-330.

Jenkins, D.E., J.E. Schultz and A. Matin. 1988. Starvation-induced cross-protection against heat or H_2O_2 challenge in *Escherichia coli. J. Bacteriol.* **170:**3910-3914.

Jenkins, D.E., S.A. Chaisson and A. Matin. 1990. Starvation -induced cross protection against osmotic challenge in *Escherichia coli. J. Bacteriol.* **172:**2779-2781.

Jørgensen, F., P.J. Stephens and S. Knøchel. 1995. The effect of osmotic shock and subsequent adaptation on the thermotolerance and cell morphology of *Listeria monocytogenes. J. Appl. Bacteriol.* **79:**274-281.

Kakinuma, Y. 1993. K^+ transport in *Enterococcus hirae.* In E.P. Bakker (ed) Alkali cation transport systems in prokaryotes. CRC Press Inc. Boca Raton FL USA.

Kanaya, S., M. Oobatake and Y. Liu. 1996. Thermal stability of *Escherichia coli* ribonuclease HI and its active site mutants in the presence and absence of the Mg^{2+} ion. *J. Biol. Chem.* **271:**32729-32736.

Kappes, R., B. Kempf and E. Bremer. 1996. Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterisation of OpuD. *J. Bacteriol.* **178:**5071-5079.

Kappes, R.M. and E. Bremer. 1998. Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and γ-butyrobetaine via the ABC transport system OpuC. *Microbiology* **144:**83-90.

Kashket, E.R. and S.L. Barker. 1977. Effects of potassium ions on the electrical and pH gradients across the membrane of *Streptococcus lactis* cells. *J. Bacteriol.* **130:**1017-1023.

Kempf, B., and E. Bremer. 1995. OpuA: an osmotically regulated binding-protein dependent transport system for the osmoprotectant glycine betaine in *Bacillus subtilis*. 1995. *J. Biol. Chem.* **270:**16701-16713.

Kets, E.P., P.J.M. Teunissen and J.A.M. de Bont. 1996. Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. *Appl. Environ. Microbiol.* **62:**259-261.

Kets, E.P.W. and J.A.M. de Bont. 1997. Effect of carnitines on *Lactobacillus plantarum* subjected to osmotic stress. *FEMS Microbiol. Lett.* **146:**205-209.

Kets, E.P.W., E.A. Galinski and J.A.M. de Bont. 1994. Carnitine: a novel compatible solute in *Lactobacillus plantarum. Arch. Microbiol.* **162:**243-248.

Kilstrup, M., S. Jacobsen, K. Hammer, and F.K. Vogensen. 1997. Induction of heat shock proteins DnaK, GroEL and GroES by salt stress in *Lactococcus lactis. Appl. Environ. Microbiol.* **63:**1826-1837.

Kim, S.G.K. and C.A. Batt. 1993. Cloning and sequencing of the *Lactococcus lactis* groESL operon. *Gene.* **127:**121-126.

Ko, R., L.T. Smith, and G.M. Smith. 1994. Glycine betaine confers enhanced osmotolerance and cryotolerance on *Listeria monocytogenes. J. Bacteriol.* **176:**426-431.

Koch, A.L. 1995. Bacterial growth and form. Chapman and Hall. London.

Koch, A., M.C. Higgins and R. Doyle. 1981. Surface tension-like forces determine bacterial cell shapes: *Streptococcus faecium. J. Bacteriol.* **117:**97-100.

Koo, S.-P. and I.R. Booth. 1994. Quantitive analysis of growth stimulation by glycine betaine in *Salmonella typhimurium. Microbiology.* **140:**617-621.

Koo, S.P., C.F. Higgins and I.R. Booth. 1991. Regulation of compatible solute accumulation in *Salmonella typhimurium*: evidence for a glycine betaine efflux system. *J. Gen. Microbiol.* **137:**2617-2625.

Koujima, I., H. Hayashi, K. Tomochika, A. Okabe, and Y. Kanemasa. 1978. Adaptational change in proline and water content of *Staphylococcus aureus* after alteration of environmental salt concentration. *Appl. Environ. Microbiol.* **35:**467-470.

Kroll, R.G. and I.R. Booth. 1983. The relationship between intracellular pH, the pH gradient and potassium transport in *Escherichia coli. Biochem. J.* **216:**709-716.

Kullik, I. and P. Giachino. 1997. The alternative sigma factor σ^B in *Staphylococcus aureus*: regulation of the *sigB* operon in response to growth phase and heat shock. *Arch. Microbiol.* **167:**151-159.

Kunin, C.M. and J. Rudy. 1991. Effect of NaCl induced osmotic stress on intracellular concentrations of glycine betaine and potassium in *Escherichia coli, Enterococcus faecalis* and staphylococci. *J. Lab. Clin. Med.* **118:**217-224.

Kunji, E.R.S., I. Mierau, A. Hagting, B. Poolman and W.N. Konings. 1996. The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoek* **70:**187-221.

Laimins, L.A., D.B. Rhoads and W. Epstein. 1981. Osmotic control of *kdp* operon expression in *Escherichia coli. Proc. Natl. Acad Sci. USA.* **78:**464-468.

Laimins, L.A., D.B. Rhoads, K. Altendorf and W. Epstein. 1978. Identification of the structural proteins of an ATP-driven potassium transport system in *Escherichia coli. Proc. Natl. Acad. Sci.* USA. **75:**3216-3219.

Lamark, T., I. Kaasen, M. Eshoo, P. Falkenberg, J. McDougall and A.R. Strøm. 1992. DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli. Mol. Microbiol.* **5:**1049-1064.

Lamark, T., O.B. Styrvold and A.R. Strom. 1992. Efflux of choline and glycine betaine from osmoregulating cells of *Escherichia coli. FEMS Microbiol. Lett.* **96:**149-154.

Lamark, T., T.P. Røkenes, J. McDougall and A.R. Strøm. 1996. The complex *bet* promoters of *Escherichia coli*: Regulation by oxygen (ArcA), choline (BetI), and osmotic stress. *J. Bacteriol.* **178:**1655-1662.

Landfald, B. and A.R. Strøm. 1986. Choline-glycine betaine pathway confers a high level of osmotolerance in *Escherichia coli. J. Bacteriol.* **165:**849-855.

Lange, R. and R. Hengge-Aronis. 1991. Identification of a single regulator of stationary -phase gene expression in *Escherichia coli. Mol Microbiol.* **5:**49-59.

Lange, R. and R. Hengge-Aronis. 1994. The cellular concentration of the σ^S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation and protein stability. *Genes Dev.* **8:**1600-1612.

Lange, R., D. Fischer, and R. Hengge-Aronis. 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the ss subunit of RNA polymerase in *Escherichia coli. J. Bacteriol.* **177:**4676-4680.

Larsen, P.I., L.K. Sydnes, B. Landfald and A.R. Strøm. 1987. Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid and trehalose. *Arch. Microbiol.* **147:**1-7.

Le Bourgeois, P., M. Lautier, L. Van Den Berghe, M.J. Gasson and P. Ritzenthaler. 1995. Physical and genetic map of the *Lactococcus lactis* subsp. *cremoris* MG1363 chromosome: comparison with that of *Lactococcus lactis* subsp. *lactis* IL1403 reveals a large genome inversion. *J. Bacteriol.* **177:**2840-2850.

Le Rudelier, D. and L. Bouillard. 1983. Glycine betaine, an osmotic effector in *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae. Appl. Environ. Microbiol.* **46:**152-159.

Leenhouts, K.J., J. Kok and G. Venema. 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis. Appl. Environ. Microbiol.* **55:**394-400.

Leyer, G.J. and E.A. Johnson. 1993. Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium. Appl. Environ. Microbiol.* **59:**1842-1847.

Lin, Y. and J.N. Hansen. 1995. Characterisation of a chimeric *proU* operon in a subtilin-producing mutant of *Bacillus subtilis* 168. *J. Bacteriol.* **177:**6874-6880.

Lou, Y. and A.E. Yousef. 1996. Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. *J. Food. Prot.* **59:**465-471.

Louis, P. and E.A. Galinski. 1997. Characterisation of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli. Microbiology.* **143**:1141-1149.

Lysenko, E., T. Ogura and S.M. Cutting. 1997. Characterisation of the *ftsH* gene of *Bacillus subtilis. Microbiology* . **143:**971-978.

MacLeod, R.A. and E.E. Snell. 1948 The effect of related ions on the potassium requirement of lactic acid bacteria. *J. Biol. Chem.* **176:**39-52.

Maguin, E., H. Prévost, S.D. Ehrlich and A. Gruss. 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J. Bacteriol.* **178:**931-935.

Malin, G. and A. Lapidot. 1996. Induction of synthesis of tetrahydropyrimidine derivitives in *Streptomyces* strains and their effect on *Escherichia coli* in response to osmotic and heat stress. *J. Bacteriol.* **178:**385-395.

Manna, D. and J. Gowrishankar. 1994. Evidence for involvement of proteins HU and RpoS in transcription of the osmoresponsive *proU* operon in *Escherichia coli. J. Bacteriol.* **176:**5378-5384.

May, G., E. Faatz, M. Villarejo and E. Bremer. 1986. Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K12. *Mol. Gen. Genet.* **205:**225-233.

McLaggan, D., J. Naprstek, E.T. Buurman and W. Epstein. 1994. Interdependance of K^+ and glutamate accumulation during osmotic adaptation of *Escherichia coli. J. Biol. Chem.* **269:**1911-1917.

Measures, J.C. 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. *Nature.* **257:**298-400.

Menzel, R. and J. Roth. 1980.Identification and mapping of a second proline permease in *Salmonella typhimurium. J. Bacteriol* **141:**1064-1070.

Meury, J. 1988. Glycine betaine reverses the effects of osmotic stress on DNA replication and cellular division in *Escherichia coli. Arch. Microbiol.* **149:**232-239.

Meury, J. and M. Kohiyama. 1991. Role of heat shock protein DnaK in osmotic adaptation of *Escherichia coli. J. Bacteriol.* **173:**4404-4410.

Meury, J., A. Robin and P. Monnier-Champeix. 1985. Turgor controlled K^+ fluxes and their pathways in *Escherichia coli. Eur. J. Biochem.* **151**:613-619.

Michels, M. and E.P. Bakker. 1987. Low-affinity potassium uptake system in *Bacillus acidocaldarius. J. Bacteriol.* **169:**4335-4341.

Miller, K.J., S.C. Zelt and J.-H. Bae. 1991. Glycine betaine and proline are the principle compatible solutes of *Staphylococcus aureus. Curr. Microbiol.* **23:**134-137.

Milner, J.L., D.J. McLellan and J.M. Wood. 1987. Factors reducing and promoting the effectiveness of proline as an osmoprotectant in *Escherichia coli* K12. *J. Gen. Microbiol.* **133:**1851-1860.

Minton, K.W., P. Karmin, G.M. Hahn and A.P. Minton. 1982. Nonspecific stabilization of stress-susceptible proteins by stress-resistant proteins: a model for the biological role of heat shock proteins. *Proc. Natl. Acad. Sci. USA.* **79:**7107-7111.

Mojica, F.J. and C.F. Higgins. 1997 In vivo supercoiling of plasmid and chromosomal DNA in an *Escherichia coli hns* mutant. *J. Bacteriol.* **179:**3528-3533.

Molenaar, D. A. Hagting, H. Alkema, A.J.M. Driessen and W.N. Konings. 1993. Characteristics and osmoregulatory roles of uptake systems for proline and glycine betaine in *Lactococcus lactis. J. Bacteriol.* **175:**5438-5444.

Mulvey, M.R., J. Switala, A. Borys and P.C. Loewen. 1990. Regulation of transcription of *katE* and *katF* in *Escherichia coli. J. Bacteriol.* **172:**6713-6720.

Munro, P.M., M.J. Gauthier, V.A. Breittmayer and J. Bongiovanni. 1989. Influence of osmoregulation processes on starvation survival of *Escherichia coli* in seawater. *Appl. Environ. Microbiol.* **55:**2017-2024.

Nagata, S., K. Adachi and H. Sano. 1996. NMR analyses of compatible solutes in a halotolerant *Brevibacterium* sp. *Microbiology* **142**:3355-3362.

Ni Bhriain, N., C.J. Dorman, and C.F. Higgins. 1989. An overlap between osmotic and anaerobic stress responses: a potential role of DNA supercoiling in the coordinate regulation of gene expression. *Mol. Microbiol.* **3:**933-944.

O' Driscoll, B. C.G.M. Gahan and C. Hill. 1996. Adaptive acid tolerance response in *Listeria monocytogenes:* isolation of an acid tolrant mutant which demonstrates increased virulence. *Appl. Environ. Microbiol.* **62:**1693-1698.

O' Reilly, M. and K.M. Devine. 1997. Expression of AbrB, a transition state regulator from *Bacillus subtilis* is growth phase dependent in a manner resemblng that of Fis, the nucleoid binding protein from *Escherichia coli. J. Bacteriol.* **179:**522-529.

O' Sullivan, E. 1996. The response of *Lactococcus lactis* subsp. *cremoris* 712 to acid stress. Ph.D thesis. National University of Ireland, Dublin.

O'Sullivan, E. and S. Condon. 1997. Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis. Appl. Environ. Microbiol.* **63:**4210-4215.

Ohwada, T. and S. Sagisaka. 1987. An immediate and steep increase in ATP concentration in response to reduced turgor pressure in *Escherichia coli. Arch. Biochem. Biophys.* **259:**157-163.

Osuna, R., D. Lienau, K.T. Hughes and R.C. Johnson. 1995. Sequence, regulation and functions of *fis* in *Salmonella typhimurium. J. Bacteriol.* **177:**2021-2052.

Overdier, D.G. and L.N. Csonka. 1992. A transcriptional silencer downstream of the promoter in the osmotically controlled *proU* operon of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **89:**3140-3144.

Overdier, D.G., E.R. Olson, B.D. Erikson, M.M. Ederer, and L.N. Csonka. 1989. Nucleotide sequence of the transcriptional control region of the osmotically regulated *proU* operon of *Salmonella typhimurium* and identification of the 5' endpoint of the *proU* mRNA. *J. Bacteriol.* **171:**4694-4706.

Owen-Hughes, T.A., D.D. Pavitt, D.S. Santos, J.M. Sidebotham, C.S.J. Hulton, J.C.D. Hinton, and C.F. Higgins. 1992. The chromatin-associated protein H-NS interacts with curved DNA to influence topology and gene expression. *Cell* **71:**255-265.

Panoff, J.M., B. Thammavongs, J.M. Laplace, A. Hartke, P. Boutibonnes and Y. Auffray. 1995. Cryotolerance and cold adaptation in *Lactococcus lactis* subsp. *lactis* IL1403. *Cryobiology* **32:**516-520.

Patchett, A., A.F. Kelly and R.G. Kroll. 1992. Effect of sodium chloride on the intracellular solute pools of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **58:**3959-3963.

Patchett, A., A.F. Kelly and R.G. Kroll. 1994. Transport of glycine betaine by *Listeria monocytogenes*. *Arch. Microbiol.* **162:**205-210.

Peddie, B.A., J. Wong-She, K. Randall, M. Lever and S.T. Chambers. 1998. Osmoprotective properties and accumulation of betaine analogues by *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **160:**25-30.

Perego, M. and J.A. Hoch. 1988. Sequence analysis and regulation of the *hpr* locus, a regulatory gene for protease production and sporulation in *Bacillus subtilis*. *J. Bacteriol.* **170:**2560-2567.

Peter, H., A. Burkovski, and R. Krämer. 1996. Isolation, characterisation, and expression of the *Corynebacterium glutamicum bet*P gene, encoding the transport system for the compatible solute glycine betaine. *J. Bacteriol.* **178:**5229-5234.

Pirt, S.J. 1975. Principles of microbe and cell cultivation. Blackwell Scientific Publications Oxford.

Polarek, J.W., G. Williams and W. Epstein. 1992. The products of the kdpDE operon are required for expression of the Kdp ATPase of *Escherichia coli*. *J. Bacteriol.* **174:**2145-2151.

Pourkomailan, B. and I.R. Booth. 1992. Glycine betaine transport in *Staphylococcus aureus*: evidence for two transport systems and for their possible roles in osmoregulation. *J. Gen. Microbiol.* **138:**2515-2518.

Pourkomailan, B. and I.R. Booth. 1994. Glycine betaine transport by *Staphylococcus aureus*: evidence for feedback regulation of the activity of the two transport systems. *Microbiology* **140:**3131-3138.

Raefeli-Eshkol, D. and Y. Avi-Dor. 1968. Studies on halotolerance in a moderately halophilic bacterium. Effect of betaine on salt resistance of the respiratory system. *Biochem J.* **109:**687-691.

Rallu, F., A. Gruss and E. Maguin. 1996. *Lactococcus lactis* and stress. *Antonie van Leeuwenhoek.* **70:**243-251.

Ramirez, R.M and M. Villarejo. 1991. Osmotic signal transduction to *proU* is independent of DNA supercoiling in *Escherichia coli. J. Bacteriol.* **173:**879-885.

Ramirez, R.M., W.S. Prince, E. Bremer and M. Villarejo. 1989 *In vitro* reconstitution of osmoregulated expression of *proU* of *Escherichia coli. Proc. Natl. Acad. Sci. USA* **86:**1153-1157.

Reed, R.H. and W.D.P. Stewart. 1985. Evidence for a turgor-sensitive K^+ influx in the cyanobacterium *Anabena variabilis* ATCC 29413 and *Synechocystis* PCC 6714. *Biochim. Biophys. Acta* **812:**155-162.

Rhoads, D.B., F.B. Waters and W. Epstein. 1976. Cation transport in *Escherichia coli.* VIII. Potassium transport mutants. *J. Gen. Physiol.* **67:**325-341.

Røkenes, T.P., T. Lamark and A.R. Strøm. 1996. DNA binding properties of the BetI repressor protein of *Escherichia coli*: the inducer choline stimulates BetI-DNA complex formation. *J. Bacteriol.* **178:**1663-1670.

Romero, D.A. and T.R. Klaenhammer. 1992. IS*946* mediated integration of heterologous DNA into the genome of *Lactococcus lactis* subsp. l*actis. Appl. Environ. Microbiol.* **58:**699-702.

Roth, W.G. M.P. Leckie and D.N. Deitzler. 1985a. Osmotic stress drastically inhibits active transport of carbohydrates by *Escherichia coli. Biochem. Biophys. Res. Comm.* **126:**434-441.

Roth, W.G., S.E. Porter, M.P. Leckie, B.E. Porter and D.N. Deitzler. 1985b. Restoration of cell volume and the reversal of carbohydrate transport and growth inhibition of osmotically upshocked *E. coli. Biochem. Biophys. Res. Comm.* **126:**442-449.

Sakaguchi, K. 1960. Betaine as a growth factor for *Pediococcus soyae*. VIII. Studies on the activities of bacteria in soy sauce brewing. *Bull. Agric. Chem. Soc. Jpn.* **24:**489-496.

Salama, M., W. Sandine and S. Giovannoni. 1991. Development and application of oligonucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris. Appl. Environ. Microbiol.* **57:**1313-1318.

Salama, M.S., W.E. Sandine and S.J. Giovannoni. 1993. Isolation of *Lactococcus lactis* subsp. *cremoris* from nature by colony hybridisation with rRNA probes. *Appl. Environ. Microbiol.* **59:**3941-3945.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, Cold Sp[ring Harbour, NY.

Sandine, W.E., P.C. Radich and P.R. Elliker. 1972. Ecology of the lactic streptococci. A review. *J. Milk Food Technol.* **35:**176-184.

Schleifer, K.H. and R. Kilpper-Balz. 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: a review. *System. Appl. Microbiol.* **10:**1-19.

Schleifer, K.H., J. Kraus, C. Dvorak, R. Kilpper-Balz, M.D. Collins and W. Fischer. 1985. Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. *System. Appl. Microbiol.* **6:**183-195.

Schleyer, M., R. Schmid and E.P. Bakker. 1993. Transient, specific and extremely rapid release of osmolytes from growing cells of *Escherichia coli* exposed to hypoosmotic shock. *Arch. Microbiol.* **160:**424-431.

Schweder, T., K.H. Lee, O. Lomovskaya and A. Matin. 1996. Regulation of *Escherichia coli* starvation sigma factor (σ^s) by ClpXP protease. *J. Bacteriol.* **178:**470-476.

Severin, J., A. Wohlfarth and E.A. Galinski. 1992. The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. *J. Gen. Microbiol.* **138:**1629-1638.

Siebers, A. and K.H. Altendorf. 1993. K^+ translocating Kdp-ATPases and other bacterial P-type ATPases. In Alkali cation transport systems in prokaryotes. E.P. Bakker, (Ed). CRC press, Boca Raton Florida USA.

Skowyra, D., and S. Wickner. 1995. GrpE alters the affinity of DnaK for ATP and Mg^{2+}. *J. Biol. Chem.* **270:**26282-26285.

Small, P., D. Blankenhorn, D. Welty, E. Zinser and J.L. Slonczewsi. 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. *J. Bacteriol.* **176:**1729-1737.

Smid, E.J. A.J.M. Driessen, and W.N. Konings. 1989. Mechanism and energetics of dipeptide transport in membrane vesicles of *Lactococcus lactis. J. Bacteriol.* **171:**292-298.

Smid, E.J., and W.N. Konings. 1990. Relationship between utilisation of proline and proline-containing peptides and growth of *Lactococcus lactis. J. Bacteriol.* **172:**5286-5292.

Stimeling, K., J.E. Graham, A. Kaenjenak and B.J. Wilkinson. 1994. Evidence for feedback (trans) regulation of, and two systems for, glycine betaine transport in *Staphylococcus aureus. Microbiology* **140:**3139-3144.

Strauch, M.A. 1995. AbrB modulates expression and catabolite repression of a *Bacillus subtilis* ribose operon. *J. Bacteriol.* **177:**6727-6731.

Strauch, M.A. and J.A. Hoch. 1993. Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. *Mol. Microbiol.* **7:**337-342.

Styrvold, O.B., P. Falkenberg, B. Landfald, M.W. Eshoo, T. Bjørnsen and A.R. Strøm. 1986. Selection, mapping and characterisation of osmoregulatory mutants of *Escherichia coli* blocked in the choline glycine betaine pathway. *J. Bacteriol.* **165:**856-863.

Sutherland, L., J. Cairney, M.J. Elmore, I.R. Booth, and C.F. Higgins. 1986 Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium. *J. Bacteriol.* **168:**805-814.

Talibart, R., M. Jebbar, G. Gousbet, S. Himdi-Kabbab, H. Wróblewski, C. Blanco and T. Bernard. 1994. Osmoadaptation in *Rhizobia*: ectoine-induced salt tolerance. *J. Bacteriol.* **176:**5210-5217.

Terzaghi, B.E. and W.E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29:**807-813.

Teuber, M. 1995. The genus *Lactococcus*. In: The genera of lactic acid bacteria. B.J.B. Wood and W.H. Holzapfel (eds) Blackie Academic and Professional, London.

Thomas, T.D. and R.D. Batt. 1968. Survival of *Streptococcus lactis* in starvation conditions. *J. Gen. Microbiol.* **50:**367-382.

Thomas, T.D., Ellwood, D.C. and M.V.C. Longyear. 1979. Change from homo-to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. *J. Bacteriol.* **138:**109-117.

Tolker-Nielsen, T. and S. Molin. 1996. Role of ribosome degradation in the death of heat-stressed *Salmonella typhimurium. FEMS Microbiol. Lett.* **142:**155-160.

Tombras-Smith, L. 1996. Role of osmolytes in adaptation of osmotically stressed and chill-stressed *Listeria monocytogenes* grown in liquid media and on processed meat surfaces. *Appl. Environ. Microbiol.* **62:**3088-3093.

Tomoyasu, T., T. Yuki, S. Morimura, H. Mori, K. Yamakana, H. Niki, S. Hiraga and T. Ogura. 1993. The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J. Bacteriol.* **175:**1344-1351.

Townsend, D.E. and B.J. Wilkinson. 1992. Proline transport in *Staphylococcus aureus*: a high affinity system and a low affinity system inolved in osmoregulation. *J. Bacteriol.* **174:**2702-2710.

Troller, J.A. and J.V. Stinson. 1981. Moisture requirements for growth and metabolite production by lactic acid bacteria. *Appl. Environ. Microbiol.* **42:**682-687.

Turner, K.W. and T.D. Thomas. 1980. Lactose fermentation in cheddar cheese and the effect of salt. *New Zealand J. Dairy Sci. Technol.* **15:**265-276.

Ueguchi, C., and T. Mizuno. 1993. The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. *EMBO J.* **12:**1039-1046.

Ueguchi, C., M. Kakeda and T. Mizuno. 1993. Autoregulatory expression of the *Escherichia coli hns* gene encoding a nucleoid protein: H-NS functions as a repressor of its own transcription. *Mol. Gen. Genet.* **236:**171-178.

Urbach, E., B. Daniels, M.S. Salama, W.E. Sandine and S.J. Giovannoni. 1997. The *ldh* phylogeny for environmental isolates of *Lactococcus lactis* is consistent with rRNA genotypes but not with phenotypes. *Appl. Environ. Microbiol.* **63:**694-702.

Verheul, A., E. Glaasker, B. Poolman and T. Abee. 1997. Betaine and L-carnitine transport by *Listeria monocytogenes* Scott A in response to osmotic signals. *J. Bacteriol.* **179:**6979-6985.

Verheul. A., F.M. Rombouts, R.R. Beumer and T. Abee. 1995. An ATP-dependent L-carnitine transporter in *Listeria monocytogenes* Scott A is involved in osmoprotection. *J. Bacteriol.* **177:**3205-3212.

Völker, U., H. Mach, R. Schmid and M. Hecker. 1992. Stress proteins and cross-protection by heat and salt stress in *Bacillus subtilis. J. Gen. Microbiol.* **138:**2125-2135.

Walderhaug, M.O., J.W. Polarek, P. Voelkner, J.M. Daniel, J.E. Hesse, K. Altendorf and W. Epstein. 1989. KdpD and KdpE, proteins that control expression of the kdpABC operon are members of the two-component sensor-effector class of regulators. *J. Bacteriol.* **174:**2152-2159.

Walter, R.P. J.G. Morris and D.B. Kell. 1987. The roles of osmotic stress and water activity in the inhibition of the growth, glycolysis and glucose phosphotransferase system of *Clostridium pasteurianum. J. Gen. Microbiol.* **133:**259-266.

Weir, J., M. Predich, E. Dubnau, G. Nair and I. Smith. 1991. Regulation of spoOH, a gene coding for the *Bacillus subtilis* σ^H factor. *J. Bacteriol.* **173:**521-529.

Wengender, P.A. and K.J. Miller. 1995. Identification of a PutP proline permease gene homolog from *Staphylococcus aureus* by expression cloning of the high-affinity proline transport system in *Escherichia coli. Appl. Environ. Microbiol.* **61:**252-259.

Whatmore, A.M. and R.H. Reed. 1990. Determination of turgor pressure in *Bacillus subtilis*: a possible role for K^+ in turgor regulation. *J. Gen. Microbiol.* **136:**2521-2526.

Whatmore, A.M., J.A. Chudek and R.H. Reed. 1990. The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*: a possible role for K^+ in turgor regulation. *J. Gen. Microbiol.* **136**:2521-2526.

Whitaker, R.D. and C.A. Batt. 1991. Characterisation of the heat shock response in *Lactococcus lactis* subsp. *lactis*. *Appl. Environ Microbiol.* **57**:1408-1412.

Winson, M.K., M. Camara, A. Latifi, M. Foglino, S.R. Chhabra, M. Bally, V. Chapon, G.P.C. Salmond, B.W. Bycroft, A. Lazdunski, G.S.A.B. Stewart and P. Williams. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc. Nat. Acad. Sci. USA*. **92**:9427-9431.

Xu, J. and R.C. Johnson. 1995. Identification of genes negatively regulated by Fis: Fis and RpoS Comodulate growth-phase-dependent gene expression in *Escherichia coli*. *J. Bacteriol.* **177**:938-947.

Yamashino, T., C. Ueguchi and T. Mizuno. 1995. Quantative control of the stationary phase-specific sigma factor, σ^S in *Escherichia coli*: involvement of the nucleoid protein H-NS. *EMBO J.* **14**:594-602.

Yan, D., T.P. Ikeda, A.E. Shauger and S. Kustu. 1996. Glutamate is required to maintain the steady-state potassium pool in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA*. **93**:6527-6531.

Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus and G.N. Somero. 1982. Living with water stress: evolution of osmolyte systems. *Science* **217:**1214-1227.

Zeilstra-Ryalls, J., O. Fayet and C. Georgeopoulos. 1991. The universally conserved GroE (Hsp60) chaperonins. *Annu. Rev. Microbiol.* **45:**301-325.

Zhang, A. and M. Belfort. 1992. Nucleotide sequence of a newly-identified *Escherichia coli* gene, *stpA*, encoding an H-NS-like protein. *Nucleic Acids. Res.* **20:**6735.

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