

Title	A plasmid-encoded putative glycosyltransferase is involved in hop tolerance and beer spoilage in Lactobacillus brevis					
Authors	Feyereisen, Marine;Mahony, Jennifer;O'Sullivan, Tadhg;Boer, Viktor;van Sinderen, Douwe					
Publication date	2020-01-21					
Original Citation	Feyereisen, M., Mahony, J., O'Sullivan, T. and van Sinderen, D. (2020) 'A Plasmid-Encoded Putative Glycosyltransferase Is Involved in Hop Tolerance and Beer Spoilage in Lactobacillus brevis', Applied and Environmental Microbiology, 86(3), e02268-19, (10 pp). doi: 10.1128/AEM.02268-19					
Type of publication	Article (peer-reviewed)					
Link to publisher's version	https://aem.asm.org/content/86/3/e02268-19 - 10.1128/ AEM.02268-19					
Rights	Open access - http://creativecommons.org/licenses/by/4.0/© 2020 American Society for Microbiology.					
Download date	2025-07-31 17:49:33					
Item downloaded from	https://hdl.handle.net/10468/9955					



University College Cork, Ireland Coláiste na hOllscoile Corcaigh AEM Accepted Manuscript Posted Online 22 November 2019 Appl. Environ. Microbiol. doi:10.1128/AEM.02268-19 Copyright © 2019 American Society for Microbiology. All Rights Reserved.

### 1 Title: A plasmid-encoded putative glycosyltransferase is involved in hop tolerance and

- 2 beer spoilage in *Lactobacillus brevis*.
- 3 Running title: A glycosyltransferase involved in beer spoilage in *Lb. brevis*
- 4 Marine Feyereisen<sup>1</sup>, Jennifer Mahony<sup>1,2</sup>, Tadhg O'Sullivan<sup>3</sup>, Viktor Boer<sup>3</sup> and Douwe van
- 5 Sinderen\*<sup>1,2</sup>
- 6 <sup>1</sup>School of Microbiology, University College Cork, T12 YT20 Cork, Ireland;
- 7 116221209@umail.ucc.ie (M.F.); J.Mahony@ucc.ie (J.M.)
- 8 <sup>2</sup>APC Microbiome Ireland, University College Cork, T12 YT20 Cork, Ireland
- <sup>3</sup>HEINEKEN Global Innovation and Research, Heineken Supply Chain B.V, 2382 PH,
- 10 Zoeterwoude, The Netherlands; Tadhg.OSullivan@heineken.com (T.O.S.), Viktor Boer
- 11 viktor.boer@heineken.com (V.B.)
- 12 \*Correspondence: d.vansinderen@ucc.ie; Tel.: +353 21 4901365
- 13
- 14 Keywords: Lactic acid bacteria, plasmid, resistance, HorA, cell wall polysaccharide, phage.

Downloaded from http://aem.asm.org/ on February 7, 2020 at IRIS

### 15 Abstract

16 Lactobacillus brevis beer-spoiling strains harbor plasmids that contain genes such as horA, 17 *horC* and *hitA*, which are known to confer hop tolerance. The *Lb. brevis* beer-spoiling strain 18 UCCLBBS124, which possesses four plasmids, was treated with novobiocin resulting in the 19 isolation of UCCLBBS124 derivatives exhibiting hop-sensitivity and an inability to grow in 20 beer. One selected derivative was shown to have lost a single plasmid, designated here as 21 UCCLLBS124\_D, which harbors the UCCLBBS124\_pD0015 gene, predicted to encode a 22 glycosyltransferase. Hop tolerance and growth in beer was restored when 23 UCCLBBS124 pD0015 was introduced in one of these hop-sensitive derivatives on a 24 plasmid. We hypothesize that this gene modifies the surface composition of the 25 polysaccharide cell wall conferring protection against hop compounds. Furthermore, 26 introduction of this gene in trans in Lb. brevis UCCLB521, a strain that cannot grow in and 27 spoil beer, was shown to furnish the resulting strain with the ability to grow in beer while its 28 expression also conferred phage-resistance. This study underscores how the acquisition of 29 certain mobile genetic elements plays a role in hop tolerance and beer spoilage for strains of 30 this bacterial species.

### 31 Importance

32 Lactobacillus brevis is a member of the lactic acid bacteria and is often reported as the 33 causative agent of food or beverage spoilage, in particular that of beer. Bacterial spoilage of 34 beer may result in product withdrawal or recall with concomitant economic losses for the 35 brewing industry. A very limited number of genes involved in beer spoilage have been 36 identified and primarily include those involved in hop resistance such as horA, hitA, and 37 *horC*. However, since none of these genes are universal, it is clear that there are likely (many) 38 other molecular players involved in beer spoilage. Here, we report on the importance of a 39 plasmid-encoded glycosyltransferase associated with beer spoilage by Lb. brevis that is

- 40 involved in hop tolerance. The study highlights the complexity of the genetic requirements to
- 41 facilitate beer spoilage and the role of multiple key players in this process.

#### 42 Introduction

43 Lactobacillus brevis is a major threat for commercial and amateur brewers as strains 44 of this species are the predominant bacterial contaminants associated with beer spoilage (1). 45 Such Lb. brevis strains can grow in beer despite the presence of ethanol, low pH and the 46 depletion of oxygen and nutrients (2). Moreover, hop compounds added to beer for bitter 47 flavor development during the fermentation process also exert antibacterial activity through 48 the presence of iso- $\alpha$ -acids (1, 2). Lb. brevis beer-spoiling (BS) strains appear to have 49 acquired chromosomally- or plasmid-derived genetic content to survive and grow in beer (2). 50 Lb. brevis resistance to ethanol (up to 10 %) and pH lower than the optimal growth 51 conditions (pH 4-6) seems to be associated with chromosomal genes, possibly due to the 52 general stressors they represent (3, 4). However, Lb. brevis BS strains are also known to 53 harbor plasmids that are associated with their beer-spoilage phenotype and more specifically 54 with hop tolerance (5-8). Plasmid-derived genes that underpin hop-resistance in Lb. brevis 55 include horA, horC, hitA and  $orf5_{ABBC45}$  (1, 2). The genes horA and horC encode multidrug transporter proteins driven by ATP and proton motive force (PMF), respectively, and were 56 identified as being involved in *iso*- $\alpha$ -acid extrusion from the bacterial cell (5, 7). The gene 57 58 hitA encodes a transmembrane protein involved in the transport of divalent cations such as  $Mn^{2+}$  in exchange of protons released from hop bitter acids (8). The orf5<sub>ABBC45</sub> gene was 59 identified in Lb. brevis BS strain ABBC45 which was unable to grow in beer after it had lost 60 a plasmid carrying this gene. The  $orf 5_{ABBC45}$  gene encodes a predicted transmembrane protein 61 62 resembling a PMF-dependent multidrug transporter, which is presumed to be responsible for 63 *iso*- $\alpha$ -acid export (9).

However, these genes are not always indicative of BS ability as the presence of such
genes can be found among *Lb. brevis* strains that are unable to grow and consequently spoil
beer (designated here as NBS strains) (10). Indeed, *horA* is present in the *Lb. brevis* NBS

Applied and Environmental Microbiology

AEM

67 strain UCCLB556 (10). Moreover, genes identified as conferring hop-resistance are not 68 always simultaneously present in BS strains, e.g. the BS strain UCCLBBS124 carries 69 plasmids harboring horA and horC, however it does not possess hitA (10). Analysis of BS 70 strain Lb. brevis BSO 464 has highlighted the importance of plasmids and genes on mobile 71 genetic elements for bacterial growth in beer and beer spoilage ability (6). Recently, a gene 72 predicted to encode a glycosyltransferase was identified among BS strains responsible for 73 excess  $\beta$ -glucan formation (11). This gene is also present on the genome of *Lb. brevis* BS 74 strain UCCLBBS124, while it is absent in that of BS strain UCCLBBS449 (10). This 75 indicates that beer spoilage is not uniquely governed by the presence of a few genes, but 76 rather a combination of genes acting in concert to confer beer resistance to the strain. It also 77 suggests that other plasmid-encoded genes involved in beer spoilage are yet to be discovered. 78 In the present study we generated plasmid-cured derivatives of Lb. brevis BS strain 79 UCCLBBS124 using novobiocin. This approach has been successfully employed previously 80 to cure plasmids from lactic acid bacteria (LAB) isolates (6, 12). Plasmid-cured derivatives 81 were assessed for their ability to grow in the presence of hop and in beer. A derivative that 82 showed inability to grow in beer was selected and analyzed to ascertain which plasmids were 83 responsible for this phenotype. Bioinformatic analysis of the genetic content of such plasmids 84 revealed candidate genes required for growth in beer. These genes were used in 85 transformation experiments to revert the NBS phenotype. 86

87

5

### 88 Results and Discussion

89 Derivatives with impaired growth in beer reveal loss of plasmid UCCLBBS124\_D

90 The beer-spoiling Lb. brevis strain UCCLBBS124 (abbreviated here as UCC124) possesses 91 four plasmids carrying genes of interest for bacterial beer spoilage (Table 3). Following 92 exposure to novobiocin, surviving Lb. brevis UCC124 cells were plated and fifty isolated 93 colonies (10) were randomly selected for further analysis. Thirty four of these fifty colonies 94 displayed impaired growth in beer. PCR-based identification of the hop-resistance gene horA 95 revealed the loss of this gene, located on plasmid UCCLBBS124 D (abbreviated here as 96 UCC124 D) in 33 out of the 34 isolates. One derivative, designated here as MB569, was 97 selected for genome sequencing, after which its sequence was compared to that of the WT, 98 confirming that plasmid UCC124 D had been lost from strain MB569.

99 Tolerance of MB569 to iso-α-acids, ethanol and low pH

100 The inability of strain MB569 to grow in beer highlights the apparent importance of plasmid 101 UCC124\_D in conferring a beer spoilage phenotype on strain UCC124 (Figure 1). Beer is a 102 harsh environment incorporating a number of stresses such as low pH, lack of nutrients, and 103 the presence of ethanol and hop compounds. In order to understand which of these stresses 104 imposed a negative impact on growth of MB569, the WT strain and MB569 were grown in 105 MRS broth and mimicking conditions encountered in beer, e.g. pH4, 5.4 % ethanol, and 30 106 ppm *iso*- $\alpha$ -acids. Strain MB569 was shown to be capable of growth in MRS broth at neutral 107 pH and at pH 4, while it can also grow in the presence of ethanol comparable to the WT 108 strain (Figure 1). However, MB569 is incapable of growth in the presence of *iso-* $\alpha$ -acids 109 unlike the WT strain UCC124 (Figure 1). This indicates that plasmid-cured derivative 110 MB569 has lost the ability to spoil beer due to its sensitivity to the antimicrobial compounds 111 present in hops. Therefore, based on this phenotype and the finding that MB569 lacks 112 plasmid UCC124\_D (when compared to its parental strain), it indicates that this plasmid is 113 linked to hop tolerance and thus contributing to the ability of strain UCC124 to cause beer 114 spoilage.

115 Identification and functional annotation of genes present on plasmid UCC124\_D

116 Plasmid UCC124\_D is 21 kb in size and is predicted to encompass 16 genes. Interestingly, a 117 7 kb region of this plasmid, contains six genes that are uniquely present among the plasmids 118 of Lb. brevis BS strains (Table 4) (10). In order to assess the possible role of these genes in 119 beer spoilage, the BS plasmid-specific genes UCCLBBS124\_pD0014 (abbreviated here as 120 UCC124\_D14), encoding a predicted cytosine deaminase, UCCLBBS124\_pD0015 (renamed 121  $gtf_{D15}$ ), encoding a predicted glycosyltransferase, UCCLBBS124 pD0016 (designated here as 122 UCC124\_horA), which encodes HorA (Table 4), were individually cloned into plasmid 123 pNZ44 prior their transformation into NZ9000. The resulting plasmids were then introduced 124 into strain MB569 to determine the ability of the obtained recombinant strains to grow in beer 125 (where MB569 itself is unable to do so). Genes with locus tags UCCLBBS124\_pD0017 126 (abbreviated as UCC124\_D17), UCCLBBS124\_pD0018 (abbreviated as UCC124\_D18) and 127 UCCLBBS124\_pD0019 (abbreviated as UCC124\_D19) and encoding acyl-sn-glycerol-3-128 phosphate acyltransferases and a glycosyltransferase (Table 4) were cloned together as a 129 cluster (as present in plasmid UCC124\_D) in pNZ44 prior their introduction into NZ9000 130 and, subsequently, MB569.

131 Introduction of the genes UCC124 D14, UCC124 horA, UCC124 D17, UCC124 D18 and 132 UCC124\_D19 in MB569 did not enable any obvious improvement of growth in the presence 133 of *iso*- $\alpha$ -acid (30 ppm) or beer (when compared to strain MB569) (data not shown).

134 Interestingly, expression of  $gtf_{D15}$  in MB569 was shown to confer a positive effect on its 135 ability to grow in MRS broth containing 30 ppm iso- $\alpha$ -acids, with a significant (P value < 136 0.05) growth increase after 72 h compared to the non-complemented strain or MB569 137 carrying the control plasmid pNZ44 (Figure 2A). When Lb. brevis MB569 pNZ44:gtf<sub>D15</sub> was 138 cultivated in beer, it also exhibited an ability to grow in beer that was significantly better than 139 that of MB569 itself (P value < 0.05) (Figure 2B). Provision of  $gtf_{D15}$  in trans in MB569 did 140 not restore its growth in beer to the same level as the WT strain (i.e. the strain from which 141 MB569 was derived), but nonetheless allowed survival and growth in beer for this 142 recombinant strain across 96 h. MB569 and MB569 pNZ44 are still able to survive in the 143 presence of *iso*- $\alpha$ -acids or beer after culture for 72 h (Figure 2A and 2B) which might be due to the presence of plasmid UCCLBBS124\_C carrying the gene horC (Table 3). The  $gtf_{D15}$ 144 145 gene is predicted to encode a glycosyltransferase based on BLAST analysis and a HHPred 146 analysis (13) predicted the protein to belong to the glycosyl transferase family 8 associated with cell wall glycosylation (99.9 % probability and E-value  $< 10^{-28}$ ). Further sequence 147 148 scrutiny suggests that the Gtf<sub>D15</sub> protein is a membrane-associated protein (TMHMM Server 149 2.0 (14)) with a predicted signal peptide in its N-terminus that may act as a membrane anchor 150 for the protein (http://phobius.sbc.su.se/ (15)). These predictions suggest that GtfD15 is a cell 151 envelope-associated protein that confers protection against certain environmental stressors 152 such as hop compounds.

153 Introduction of gtf<sub>D15</sub> in NBS Lb. brevis strains allows growth in beer

The introduction of  $gtf_{D15}$  in MB569 was shown to significantly improve growth of the strain in MRS broth containing hop compounds (30 ppm *iso-* $\alpha$ -acids) and in beer indicating the importance of this gene for beer spoilage by *Lb. brevis* strain UCC124. In order to assess the potential growth-promoting effect of this gene for an NBS strain when inoculated in beer,  $gtf_{D15}$  when cloned into pNZ44 (pNZ44: $gtf_{D15}$ ) was introduced into the NBS *Lb. brevis* strain UCCLB521 (renamed here as UCC521) (Table 1). Remarkably, the presence of pNZ44: $gtf_{D15}$ in the NBS strain *Lb. brevis* UCC521 permitted the strain to grow significantly better (P Accepted Manuscript Posted Online

Applied and Environmental Microbioloay

AEM

value < 0.05) in MRS broth containing 30 ppm *iso*-α-acids and in beer compared to the strain carrying an empty plasmid which is incapable of survival or growth in these environments (Figure 2C and D). These observations reinforce our results above and highlight the significance of the  $gtf_{D15}$  gene in hop tolerance and beer spoilage. An alternative, though in our opinion less likely explanation is that strains for which we obtained no or reduced CFUs had entered a viable, but non-culturable (so-called VBNC) state as has been previously observed for beer-passaged *Lb. brevis* strains (16).

168 Introduction of pNZ44:gtf<sub>D15</sub> into ATCC 367, another NBS strain, did not allow improved 169 survival or growth in the presence of hop compounds or in beer (data not shown). This 170 suggests that a strain-specific mechanism and involvement of other genes that are absent in 171 ATCC 367 are responsible for increased hop tolerance. Among beer spoilage-related genes, 172 UCC521 possesses the  $orf5_{ABBC45}$  gene previously identified as involved in hop tolerance (9), 173 unlike ATCC 367 which does not harbor known genes involved in beer spoilage. Moreover, 174 UCC521, although a non-beer spoiler strain, was isolated from the brewery environment, 175 unlike ATCC 367 which was isolated from silage (10). UCC521 may have acquired genes 176 (such as  $orf5_{ABBC45}$ ) or plasmids (UCC521 harbors five plasmids) throughout its presence in the brewery environment, which confer hop tolerance when combined with  $gtf_{D15}$ . This 177 178 scenario has previously been observed, indicating that Lb. brevis strains can only survive and 179 grow in beer when multiple beer spoilage-related genes are present in a particular 180 combination (17). Selection pressures of the beer environment determine the genetic content 181 of beer-spoiling strains. The identification of diagnostic marker genes (DMGs) are important 182 in distinguishing BS from NBS strains, as well as predicting the ability of a given strain to 183 grow in beer (17, 18). In the study from Bergsveinson and Ziola, proposed DMGs were not 184 related to hop tolerance and no genes encoding glycosyltransferases were identified as 185 DMGs. However, a glycosyltransferase-encoding gene located on the plasmid of the BS Lb.

oplied and Environmental Microbiology 186 *brevis* strain BSO 464 which showed more than 99 % nt similarity to  $gtf_{D15}$  was described as 187 unique when compared to a NBS *Lb. brevis* strain KB290 and a BS *Pediococcus damnosus* 188 strain Pc344<sup>T</sup> (17). From these observations and knowing that  $gtf_{D15}$  is highly prevalent in BS 189 *Lb. brevis* strains (Table 4), we propose to include this gene as a DMG to assess the beer 190 spoiling potential of *Lb. brevis* strains.

### 191 Effect on phage sensitivity

192 As demonstrated above  $gtf_{D15}$  was observed to play a role in hop and beer tolerance and is 193 predicted to encode a glycosyltransferase. Since the protein is predicted to be involved in 194 biosynthesis or modification of a cell surface-associated saccharidic polymer, the possible 195 role of this protein in bacteriophage infection was investigated. Lb. brevis strain UCC521 is 196 sensitive to Lb. brevis phages 3-521 and 521B (19). Plaque assays employing these phages 197 and Lb. brevis UCC521 harboring the empty vector pNZ44, or strain UCC521 containing 198 pNZ44:gtf<sub>D15</sub> displayed similar EOP (Efficiency Of Plaquing) values with no significant 199 difference to the WT (Table 5). However, notable differences in plaque morphology were 200 observed, where plaques were faint and hard to distinguish on the bacterial lawn of UCC521 201  $pNZ44:gtf_{D15}$ . Moreover, overnight incubation of the different strains with the two phages led 202 to complete lysis-in-broth of UCC521 and UCC521 containing pNZ44 with an approximately 203 1000-fold increase of phage titre after overnight propagation (Table 5). In contrast, UCC521 pNZ44:gtf<sub>D15</sub> did not show visible lysis and was able to grow after overnight incubation with 204 205 just a ten-fold increase in phage numbers after overnight propagation (Table 5). These results 206 reinforce the role of the protein Gtf<sub>D15</sub> in bacterial protection against diverse environmental 207 hazards such as hop compounds or bacteriophages.

208

209

### 210 Conclusions

211 In this study, we identified a novel genetic component required for beer spoilage and more 212 specifically for hop tolerance. This gene is located on plasmid UCC124\_D of Lb. brevis BS 213 strain UCC124, validating the importance of plasmids to confer a beer spoilage phenotype. 214 Moreover, this gene had been highlighted previously as common among BS strains (10). 215 Genes required for hop tolerance have all been identified on plasmids (5, 7, 8), reinforcing 216 the importance of such mobile genetic elements in adaptation to the specific hurdles imposed 217 by the beer environment. A derivative of UCC124, MB569 showed impaired growth in beer 218 after the loss of plasmid UCC124 D and despite the presence of plasmids UCC124 B and 219 UCC124\_C which carry several genes of interest in beer spoilage. Introduction of  $gtf_{D15}$  in 220 strain MB569 restored the hop tolerance phenotype of the strain which ultimately allowed it 221 to grow in beer. Similar results were observed when the gene was introduced into a NBS 222 strain confirming the notion that  $gtf_{D15}$  is required for the development of hop tolerance and 223 beer spoilage. Furthermore, this gene impacts on phage sensitivity of its host. This gene 224 seems a unique trait shared among BS strains of Lb. brevis and we propose  $gt_{D15}$  as a DMG 225 for the detection of potential bacterial contamination of beer. The gene is predicted to encode 226 a glycosyltransferase and analysis of its topology suggests that it is a membrane-anchored 227 protein involved in the biosynthesis or modification of a cell surface-associated saccharidic 228 polymer. BS strains of Lb. brevis have been shown to increase higher molecular weight 229 lipoteichoic acids (LTA) in their cell wall, in the presence of hop bitter acids, thus believed to 230 confer resistance to the bacteria by enhancing the barrier functions of the cell wall and 231 preventing intrusion of hop compounds (20, 21). Moreover, lipoteichoic acids have been 232 described as phage receptors among lactobacilli phages as seen for Lactobacillus delbrueckii 233 phages LL-H and JCL1032 (22) but also for Lactobacillus plantarum ATCC8014-B2 (23). 234 Therefore, we speculate that this glycosyltransferase is involved in replacing alanine residues

235 with sugar residues on teichoic acids thereby changing their charge and preventing *iso-\alpha-*236 acids to penetrate the membrane as well as affecting phage adsorption and/or DNA injection. 237 This predicted glycosyltransferase shows only limited similarity (36 % amino acid similarity 238 in 20 % query cover) with the glycosyltransferase identified in a previous study as 239 responsible for  $\beta$ -glucan formation (11), and is thus believed to play a different role in beer 240 spoilage. Future studies will focus on defining the mechanism that underpins hop tolerance 241 and on determining how the genes identified to date (5, 7, 8) are linked to each other. 242 Moreover, located on the same plasmid as  $gt_{D15}$  are genes predicted to encode a 243 glycosyltransferase and acyltransferases (Table 2) suggesting a common action on teichoic 244 acids with the acyltransferases involved in the acylation of alanine residues or the lipid 245 moiety of the lipoteichoic acids (24). Follow-up work may therefore focus on determining the 246 precise function of the glycosyltransferase (and other associated genes) in the modification of 247 the cell wall and/or cell surface. Another question to be addressed is if and how hop tolerance 248 is enhanced when these genes are present in a certain combination, and how such tolerance is 249 influenced by their expression level.

250

251

Downloaded from http://aem.asm.org/ on February 7, 2020 at IRIS

### 252 Materials and Methods

### 253 Bacterial strains and cultivation media

Bacterial strains used in this study are listed in Table 1. *Lb. brevis* strains were grown in
MRS broth (Oxoid Ltd., UK) at 30 °C while *Lactococcus lactis* NZ9000 was grown in M17
broth (Oxoid Ltd., UK) supplemented with 0.5 % glucose. 5 µg/mL chloramphenicol (Cm5)
was added to the medium when indicated.

258 Plasmid curing and plasmid content analysis

259 The overall experimental approach is presented in Figure S1. Plasmid curing of the BS strain 260 UCC124 was achieved using novobiocin treatment (25). A 1 % inoculum of a WT strain 261 overnight culture was used to inoculate 10 mL MRS broth containing 0.25 µg/mL 262 novobiocin. Cultures were incubated at 26 °C for 72 h. After incubation, cells were diluted 263 and plated on MRS agar. After 3 days of incubation at 26 °C, isolated colonies were 264 randomly selected and derivatives with impaired growth in beer (no growth observed after 72 h) were checked for the presence or loss of hop-resistance genes horA, horC and  $orf_{ABBC45}$ 265 266 (Table 2). A derivative showing loss of hop-resistance gene was selected and sequenced 267 using Illumina sequencing technology. Paired-end sequence reads were generated using an 268 Illumina HiSeq2500 system (read length 2 x 250 bp). FASTQ sequence files were generated 269 using the Illumina Casava pipeline version 1.8.3. After Illumina sequencing the obtained 270 sequences were mapped back against the WT reference sequence to detect mutations by 271 single nucleotide polymorphism (SNP) or plasmid content loss. SNP analysis was performed 272 by aligning Illumina raw reads against a reference sequence using Bowtie2 V 2.3.5 (26). The 273 reads were then sorted using Samtools (27) and VarScan v2.3.9 was applied for the detection 274 of variants (28). A minimum allelic variation frequency cut-off of 0.25 was applied.

Downloaded from http://aem.asm.org/ on February 7, 2020 at IRIS

Applied and Environmental Microbiology Genes of interest were amplified by PCR (Table 2) and cloned into the expression vector pNZ44 (29). PCR products and pNZ44 plasmid DNA were digested with the appropriate enzymes (Roche, USA) at 37 °C for at least 4 h, following the manufacturer's instructions (Table 2). A ratio of (3:1) was applied for the ligation of the PCR product with pNZ44 using T4 DNA ligase (Promega, USA). The mixture was incubated at room temperature for at least 4 hours prior to electrotransformation into *L. lactis* NZ9000 competent cells.

282 Preparation of competent cells and electrotransformation

283 Competent cells of L. lactis NZ9000 were prepared as previously described (30). Competent 284 cells of *Lb. brevis* UCC124 were prepared using an adapted version of a previously described 285 protocol (31): An overnight culture was transferred (1 % inoculum) to 10 mL MRS broth 286 containing 1 % glycine and incubated overnight at 30 °C. 5 mL of the overnight culture was 287 transferred to fresh MRS broth containing 1 % glycine (50 mL final volume) and cells were 288 grown to an OD<sub>600nm</sub> of 0.6. Cells were harvested by centrifugation at 4,000  $\times$  g for 15 min at 289 4 °C and washed in ice-cold wash buffer (0.5 M sucrose, 10 % glycerol). The wash step was 290 repeated twice and the cells were finally resuspended in 200 µL wash buffer prior to storage 291 at -80 °C and/or electroporation (see below). All constructs were generated using L. lactis 292 NZ9000 as the cloning host, verified by sequencing after PCR amplification using the 293 primers pnz44F and pnz44R (Table 2) prior to their transfer into Lb. brevis strains. 294 Electrotransformation was performed using freshly prepared competent cells as described 295 above, where 45  $\mu$ L of cells and 5  $\mu$ L of plasmid construct were mixed into a pre-chilled 2 296 mm electroporation cuvette (Cell Projects, Kent, England) and subjected to electroporation at 297 1.5 kV (Lb. brevis) or 2.0 kV (L. lactis), 200 Ω, 25 μF. Following electroporation, 950 μL 298 recovery broth was added (MRS broth supplemented with 0.5 M sucrose and 0.1 M MgCl<sub>2</sub> 299 (*Lb. brevis*) or GM17 broth supplemented with 20 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub> (*L. lactis*)). 300 Cells were recovered at 30 °C for 3 h (*Lb. brevis*) or 2 h (*L. lactis*) prior to spread plating on 301 MRS (*Lb. brevis*) or GM17 (*L. lactis*) agar supplemented with Cm5. Presumed transformants 302 were purified on MRS agar + Cm5 and colonies were checked by sequencing after PCR 303 amplification using the primers pnz44F and pnz44R (Table 2) and applied to growth assays 304 as described below. 305 *Growth assays* 

305 Growth assays

306 Growth profiles of the wild-type strain and its derivative were obtained by transferring an 307 overnight culture (1 % inoculum) to MRS broth, MRS broth supplemented with 30 ppm iso-308  $\alpha$ -acids or beer (fresh Heineken lager 5 % ethanol, pH 4, 23 ppm *iso*- $\alpha$ -acids). Cultures were 309 incubated at 30 °C for 72 hours. One mL of culture was retrieved after 24, 48, 72 and 96 310 hours, diluted in Ringer's solution and plated on MRS agar plates. Plates were incubated at 311 30 °C anaerobically for 48 hours prior to colony counting. The number of viable bacteria of 312 each strain was assessed after CFU/mL calculation. Non-inoculated controls were used in all 313 the experiments as blank measurements. These measurements were then subtracted from each 314 experimental condition to produce the values represented on growth curves. Statistical 315 differences were calculated using unpaired t test method (32).

## 316 Phage activity against Lb. brevis strains and transformants

To assess phage sensitivity of Lb. brevis strains, transformants carrying genes of interest were 317 318 compared to the wild-type (WT) strain using plaque assays, as previously described (33). A 319 10 µL volume of the appropriate phage dilution and 200 µL of *Lb. brevis* culture were added 320 to 4 mL of soft agar supplemented with 10 mM CaCl<sub>2</sub>, mixed and poured onto an MRS agar 321 plate supplemented with 10 mM CaCl<sub>2</sub> and 0.5% glycine. Plates were incubated at 30 °C 322 overnight and the resulting plaques were enumerated. Phage titre was determined as plaque-323 forming units per mL (PFU/mL). The ability of phages to propagate and multiply within the 324 host cell was also tested. Lb. brevis strains were grown to early exponential phase (OD<sub>600nm</sub>  $\sim$ 

Applied and Environmental

Microbiology

325 0.25), at which point phages were added to the culture (T0) at a MOI (multiplicity of 326 infection) of 1, along with 10 mM CaCl<sub>2</sub>. The mix was further incubated at 30 °C overnight 327 (T1). The number of phages present in the medium (i.e. following removal of bacterial cells 328 by centrifugation) at T1 was then determined by plaque assay. Phage propagation efficiency 329 on a given host was then determined by dividing the phage titre (PFU/mL) at T1 by the phage 330 titre (PFU/mL) at T0.

331 GenBank accession numbers

*Lb. brevis* UCCLBBS124: CP031169, *Lb. brevis* UCCLBBS124\_A: CP031170, *Lb. brevis*UCCLBBS124\_B: CP031171, *Lb. brevis* UCCLBBS124\_C: CP031172 and *Lb. brevis*UCCLBBS124\_D: CP031173.

Author Contributions: MF performed experiments and genomic analysis. DS, JM, TS and
VB provided materials and strains. MF, JM, TS and DS were involved in project design and
wrote the manuscript. All authors read and approved the final manuscript.

338 Conflicts of Interest: The authors declare that VB and TS are employees of Heineken.

Funding: Marine Feyereisen is the recipient of an Irish Research Council Enterprise
Partnership Scheme postgraduate scholarship (Ref. No. EPSPG/2015/7). Douwe van
Sinderen is supported by a Principal Investigator award (Ref. No. 450 13/IA/1953) through
Science Foundation Ireland (SFI). Jennifer Mahony is in receipt of a Starting Investigator
Research Grant (SIRG) (Ref. No. 15/SIRG/3430) funded by Science Foundation Ireland
(SFI).

345

346

AEN

#### 347 **References**

- Sakamoto K, Konings WN. 2003. Beer spoilage bacteria and hop resistance.
   International Journal of Food Microbiology 89:105-124.
- Suzuki K, Iijima K, Sakamoto K, Sami M, Yamashita H. 2006. A review of hop
   resistance in beer spoilage lactic acid bacteria. Journal of the Institute of Brewing
   112:173-191.
- 353 3. Wang C, Cui Y, Qu X. 2018. Mechanisms and improvement of acid resistance in
  lactic acid bacteria. Archives of microbiology 200:195-201.
- 355 4. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova 356 N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas 357 S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh 358 Y, Benson A, Baldwin K, Lee J, Díaz-Muñiz I, Dosti B, Smeianov V, Wechter W, 359 Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, 360 Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, 361 Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D. 2006. 362 Comparative genomics of the lactic acid bacteria. Proceedings of the national 363 academy of sciences of the United States of America 103:15611-6.
- Sakamoto K, Margolles A, van Veen HW, Konings WN. 2001. Hop resistance in the
  beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-Binding
  Cassette multidrug transporter HorA. Journal of Bacteriology 183:5371-5375.
- Bergsveinson J, Baecker N, Pittet V, Ziola B. 2015. Role of plasmids in *Lactobacillus brevis* BSO 464 hop tolerance and beer spoilage. Applied and environnmental
   microbiology 81:1234-41.

17

- **Accepted Manuscript Posted Online** 370 371 372 373 374 375 376
  - 70 7. Iijima K, Suzuki K, Ozaki K, Yamashita H. 2006. HorC confers beer-spoilage ability
     71 on hop-sensitive *Lactobacillus brevis* ABBC45cc. Journal of Applied Microbiology
     72 100:1282-1288.
  - Hayashi N, Ito M, Horiike S, Taguchi H. 2001. Molecular cloning of a putative
     divalent-cation transporter gene as a new genetic marker for the identification of
     *Lactobacillus brevis* strains capable of growing in beer. Applied Microbiology and
     Biotechnology 55:596-603.
  - Suzuki K, Sami M, Kadokura H, Nakajima H, Kitamoto K. 2002. Biochemical
     characterization of horA-independent hop resistance mechanism in *Lactobacillus brevis*. International journal of food microbiology 76:223-30.
  - Feyereisen M, Mahony J, Kelleher P, Roberts RJ, O'Sullivan T, Geertman J-MA, van
    Sinderen D. 2019. Comparative genome analysis of the *Lactobacillus brevis* species.
    BMC Genomics 20:416.
  - 383 11. Fraunhofer ME, Geissler AJ, Wefers D, Bunzel M, Jakob F, Vogel RF. 2018.
    384 Characterization of beta-glucan formation by *Lactobacillus brevis* TMW 1.2112
    385 isolated from slimy spoiled beer. International Journal of Biological Macromolecules
    386 107:874-881.
  - 387 12. Karthikeyan V, Santosh SW. 2010. Comparing the efficacy of plasmid curing agents
    388 in *Lactobacillus acidophilus*. Beneficial microbes 1:155-8.
  - 389 13. Söding J, Biegert A, Lupas AN. 2005. The HHpred interactive server for protein
    390 homology detection and structure prediction. Nucleic Acids Res 33.
  - 391 14. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting
    392 transmembrane protein topology with a hidden Markov model: application to
    393 complete genomes. Journal of molecular biology 305:567-80.

Applied and Environ<u>mental</u>

Microbiology

Applied and Environmental

Microbiology

397 16. Liu J, Deng Y, Soteyome T, Li Y, Su J, Li L, Li B, Shirtliff ME, Xu Z, Peters BM. 398 2018. Induction and recovery of the viable but nonculturable state of hop-resistance 399 Lactobacillus brevis. Frontiers in Microbiology 9:2076-2076.

- 400 17. Bergsveinson J, Ziola B. 2017. Comparative genomic and plasmid analysis of beer-401 spoiling and non-beer-spoiling Lactobacillus brevis isolates. Canadian Journal of 402 Microbiology 63:970-983.
- 403 18. Behr J, Geissler AJ, Schmid J, Zehe A, Vogel RF. 2016. The identification of novel 404 diagnostic marker genes for the detection of beer spoiling Pediococcus damnosus 405 strains using the BlAst Diagnostic Gene findEr. PLOS ONE 11:e0152747.
- 406 19. Feyereisen M, Mahony J, Lugli GA, Ventura M, Neve H, Franz CMAP, Noben J-P, 407 O'Sullivan T, Sinderen Dv. 2019. Isolation and characterization of Lactobacillus 408 brevis phages. Viruses 11:393.
- 409 20. Behr J, Gänzle MG, Vogel RF. 2006. Characterization of a highly hop-resistant 410 Lactobacillus brevis strain lacking hop transport. Applied and Environmental 411 Microbiology 72:6483-6492.
- 412 21. Yasui T, Yoda K. 1997. Purification and partial characterization of an antigen specific 413 to Lactobacillus brevis strains with beer spoilage activity. FEMS Microbiology 414 Letters 151:169-176.
- 415 22. Räisänen L, Schubert K, Jaakonsaari T, Alatossava T. 2004. Characterization of 416 lipoteichoic acids as Lactobacillus delbrueckii phage receptor components. Journal of 417 Bacteriology 186:5529-5532.

AEN

Applied and Environ<u>mental</u>

Microbiology

- 418 23. Wolin 419 *plantar* 420 24. Kiriukl
  - 8 23. Wolin M, Douglas LJ. 1971. Cell wall polymers and phage lysis of *Lactobacillus*9 *plantarum*. Biochemistry 10:1551-1555.
  - 420 24. Kiriukhin MY, Neuhaus FC. 2001. d-alanylation of lipoteichoic acid: role of the d421 alanyl carrier protein in acylation. Journal of bacteriology 183:2051-2058.
  - 422 25. Ruiz-Barba JL, Piard JC, Jiménez-Díaz R. 1991. Plasmid profiles and curing of
    423 plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentations.
    424 Journal of applied bacteriology 71:417-421.
  - 425 26. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nature
    426 Methods 9:357-359.
  - Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G,
    Durbin R. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics
    25:2078-2079.
  - 430 28. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, Miller CA, Mardis
    431 ER, Ding L, Wilson RK. 2012. VarScan 2: somatic mutation and copy number
    432 alteration discovery in cancer by exome sequencing. Genome Research 22:568-76.
  - 433 29. McGrath S, Fitzgerald GF, van Sinderen D. 2001. Improvement and optimization of
    434 two engineered phage resistance mechanisms in *Lactococcus lactis*. Applied
    435 environnemental microbiology 67:608-616.
  - 436 30. van Pijkeren J-P, Britton RA. 2012. High efficiency recombineering in lactic acid
    437 bacteria. Nucleic acids research 40:e76-e76.
  - 438 31. Ahrné S, Molin G, Axelsson L. 1992. Transformation of *Lactobacillus reuteri* with
    439 electroporation: studies on the erythromycin resistance plasmid pLUL631. Current
    440 microbiology 24:199-205.
  - 441 32. Kim TK. 2015. T test as a parametric statistic. Korean journal of anesthesiology
    442 68:540-546.

# 443 33. Svensson U, Christiansson A. 1991. Methods for phage monitoring. Bulletin of the 444 international dairy federation 263:29-39. 445 34. Kuipers OP, de Ruyter PGGA, Kleerebezem M, de Vos WM. 1998. Quorum sensing-446 controlled gene expression in lactic acid bacteria. Journal of biotechnology 64:15-21. 447 448 449

450

AEM

Applied and Environmental Microbiology

Microbiology

## 451 **Table 1.** Bacterial strains and plasmids used in this study.

Strain / Plasmid	Description	References
Lb. brevis strains		
UCCLBBS124 (UCC124)	Beer-spoiling strain isolated from spoiled beer keg (Singapore)	(10)
UCCLBBS449	Beer-spoiling strain isolated from unpasteurized spoiled beer (The Netherlands)	(10)
MB569	Non-beer spoiling strain derivative of UCCLBBS124	This study
UCCLB521 (UCC521)	Non-beer spoiling strain isolated from brewery environment (The Netherlands)	(10)
MB569 pNZ44	MB569 carrying pNZ44	This study
MB569 pNZ44:gtf <sub>D15</sub>	MB569 carrying pNZ44 with $gtf_{D15}$	This study
UCCLB521 pNZ44:gtf <sub>D15</sub>	UCCLB521 carrying pNZ44 with $gtf_{D15}$	This study
L. lactis strains		
NZ9000	Transformation host	(34)
Plasmids		
pNZ44	Transformation vector, chloramphenicol resistance gene	
pNZ44:gtf <sub>D15</sub>	pNZ44 harboring <i>gtf<sub>D15</sub></i>	This study

# 453 **Table 2.** PCR primers used in this study. Incorporated restriction sites are indicated in capital

454 letters.

452

Primer			GenBank
name	Sequence (5' - 3')	Target	accession no.
horAF	cgcaactgaggctaacttct	horA gene in UCCLBBS124	CP031173
horAR	ggcttgctatgctaggata	horA gene in UCCLBBS124	CP031173
horCF	gtatgcctaagtgacgt	horC gene in UCCLBBS124	CP031172
horCR	cattetetgeetetatae	horC gene in UCCLBBS124	CP031172
orf5F	ctggattgaggtgaggg	orf5 gene in UCCLBBS124	CP031172
orf5R	gctgtaaagggtagtgattg	orf5 gene in UCCLBBS124	CP031172
pNZ44F	aacaattgtaacccatac	pNZ44 promoter	
pNZ44R	gaacgtttcaagccttgg	pNZ44 MCS	
pD14F	aaaaaaCTGCAGgtccgaacagcgttcggatt	Gene UCCLBBS124_pD0014 in UCCLBBS124_D	CP031173
pD14R	aaaaaaTCTAGAttaatcttcgaaatagtt	Gene UCCLBBS124_pD0014 in UCCLBBS124_D	CP031173
pD15F	aaaaaaCCATGGgcggtttggatattttatact	Gene UCCLBBS124_pD0015 in UCCLBBS124_D	CP031173
pD15R	aaaaaaTCTAGAtcactcagttttcaattccc	Gene UCCLBBS124_pD0015 in UCCLBBS124_D	CP031173
pD16F	aaaaaaCTGCAGaggcttgctatgctagg	Gene UCCLBBS124_pD0016 in UCCLBBS124_D	CP031173
pD16R	aaaaaaTCTAGAtcacccgttgctcgt	Gene UCCLBBS124_pD0016 in UCCLBBS124_D	CP031173
pD17-19F	aaaaaaCCATGGggggtagaatggttctgtt	Gene UCCLBBS124_pD0017-19 in UCCLBBS124_D	CP031173
pD17-19R	aaaaaaTCTAGAttattgataatgaccagcaa	Gene UCCLBBS124_pD0017-19 in UCCLBBS124_D	CP031173

Applied and Environmental Microbiology

AEM

Applied and Environmental Microbiology

# **Table 3.** *Lb. brevis* UCC124 plasmids and genes of interest for beer spoilage.

463						
	UCC124 plasmids	Size (bp)	ORFs no.	Accession no.	Gene(s) of interest	References
UCCLBB	S124_A (UCC124_A)	49,560	42	CP031170		
UCCLBE	S124_B (UCC124_B)	23,078	20	CP031171	gtf family 2	(11)
UCCLBE	S124_C (UCC124_C)	22,370	27	CP031172	horB, horC, orf5	(7,9)
UCCLBB	S124_D (UCC124_D)	20,971	16	CP031173	horA	(5)
464						
465						
1.00						

**Table 4.** Presence and absence of genes of UCCLBBS124\_D among *Lb. brevis* BS strains.

Cana	Desdicted function					Th have	uia DS a	traina
Gene	F redicted function				Lo. orevis DS strains			
		UCCLBBS124	UCCLBBS449	UCCLB95	TMW1.2108	TMW1.2111	TMW1.2112	TMW1.2113
UCCLBBS124_pD0014 = UCC124_D14	Cytosine deaminase	+	+	-	+	+	-	+
$UCCLBBS124\_pD0015 = gtf_{D15}$	Glycosyltransferase family 8	+	+	-	+	+	+	+
UCCLBBS124_pD0016 = UCC124_horA	HorA	+	+	-	+	+	-	+
UCCLBBS124_pD0017 = UCC124_D17	Acyl-sn-glycerol-3-phosphate acyltransferase	+	+	-	+	+	+	+
UCCLBBS124_pD0018 = UCC124_D18	Glycosyltransferase family 8	+	+	-	+	+	+	+
UCCLBBS124_pD0019 = UCC124_D19	Acyl-sn-glycerol-3-phosphate acyltransferase	+	+	-	+	+	+	+
1.00								

## 

Applied and Environmental Microbiology

AEM

## 476 **Table 5.** Effect of phages 3-521 and 521B on *Lb. brevis* strain UCC521 and derivatives.

			Lb. brevis strains	1
		UCC521	UCC521 pNZ44	UCC521 pNZ44:gtf <sub>D15</sub>
	EOP (Efficiency Of Plaquing)	1.00	$0.58\pm0.29$	$0.64\pm0.21$
Phage 521B	Plaque morphology	Small clear plaques	Small clear plaques	Faint plaques
	Phage titre after O/N propagation* (PFU/mL)	2.90E+09	2.30E+09	3.00E+07
	EOP	1.00	$1.52\pm0.20$	$1.19\pm0.19$
Phage 3-521	Plaque morphology	Small clear plaques	Small clear plaques	Faint plaques
	Phage titre after O/N propagation* (PFU/mL)	4.30E+09	1.80E+09	4.80E+07

477 \*Overnight propagation (O/N) was realized with a starting phage titre of  $10^6$  PFU/mL (results

478 are average of triplicate assays).

479

Downloaded from http://aem.asm.org/ on February 7, 2020 at IRIS





Applied and Environmental

Microbiology

Downloaded from http://aem.asm.org/ on February 7, 2020 at IRIS



**Figure 2.** Number of viable bacteria (CFU/mL) of the WT BS strain *Lb. brevis* UCC124, the derivative MB569 +/- the empty plasmid pNZ44 and MB569 carrying the gene  $gtf_{D15}$  after growth in (**A**) MRS broth containing 30 ppm *iso*-α-acids for 72 h and (**B**) beer for 96 h (P<0.05). CFU/mL of the WT BS strain *Lb. brevis* UCC124, the NBS UCC521 +/- the empty plasmid pNZ44 and the NBS UCC521 carrying the gene  $gtf_{D15}$  after growth in (**C**) MRS broth containing 30 ppm *iso*-α-acids and (**B**) beer for 96 h (P<0.05).

AEM