Heat Resistance and Salt Hypersensitivity in *Lactococcus lactis* Due to Spontaneous Mutation of *llmg_1816* (*gdpP*) Induced by High-Temperature Growth

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During construction of several gene deletion mutants in *Lactococcus lactis* MG1363 which involved a high-temperature (37.5°C) incubation step, additional spontaneous mutations were observed which resulted in stable heat resistance and in some cases salt-hypersensitive phenotypes. Whole-genome sequencing of one strain which was both heat resistant and salt hypersensitive, followed by PCR and sequencing of four other mutants which shared these phenotypes, revealed independent mutations in *llmg_1816* in all cases. This gene encodes a membrane-bound stress signaling protein of the GdpP family, members of which exhibit cyclic dimeric AMP (c-di-AMP)-specific phosphodiesterase activity. Mutations were predicted to lead to single amino acid substitutions or protein truncations. An independent *llmg_1816* mutant ($\Delta 1816$), created using a suicide vector, also displayed heat resistance and salt hypersensitivity phenotypes which could be restored to wild-type levels following plasmid excision. *L. lactis* $\Delta 1816$ also displayed improved growth in response to sublethal concentrations of penicillin G. High-temperature incubation of a wild-type industrial *L. lactis* strain also resulted in spontaneous mutation of *llmg_1816* and heat-resistant and salt-hypersensitive phenotypes, suggesting that this is not a strain-specific phenomenon and that it is independent of a plasmid integration event. Acidification of milk by the *llmg_1816*-altered strain was inhibited by lower salt concentrations than the parent strain. This study demonstrates that spontaneous mutations can occur during high-temperature growth of *L. lactis* and that inactivation of *llmg_1816* leads to temperature resistance and salt hypersensitivity.

Lactococcus lactis is a starter culture bacterium that encounters a variety of stresses, including high and low temperature, osmotic, acid, oxidative, and cell envelope stressors, during the cheesemaking process (24). The result of these stressors is damage to DNA, proteins, lipid membranes, peptidoglycan, and other cellular components, which consequently lead to reduction of growth and acidification rates and, when severe enough, cell death (24). To combat these challenges, *Lactococcus* has accumulated a variety of specific and nonspecific stress resistance mechanisms, some of which are known and have been characterized (25), while many others remain unstudied.

Traditional methods for investigating the roles of genes in stress resistance in *Lactococcus* have been through the use of both targeted and random gene mutagenesis approaches. In these studies, mutants with altered resistance to heat, acid, UV light, and oxidative stress have been generated and characterized (4, 5, 17, 26). *Lactococcus* has also been found to be able to adapt to stress through spontaneous mutation. Hydrogen peroxide was used to select three spontaneous oxidative (SpOx) stress-resistant mutants in a milk-adapted strain derived from *L. lactis* MG1363. In particular, SpOx3 viability was 40-fold higher than the parent after 2 days in stationary phase following growth in milk, and it also had greater resistance to hydrogen peroxide (20). This study provides clear evidence that *Lactococcus* can readily evolve and adapt to a stressful environment. These spontaneous mutants are considered nongenetically modified (non-GM) and may be more acceptable for use in food applications.

Although the location of spontaneous mutations in SpOx mutants have not been identified, it has become feasible in recent

years to use next-generation sequencing technologies to carry this out. As an example of this approach, next-generation sequencing was recently utilized to identify the genetic variations responsible for observed differences in phenotype between *L. lactis* MG1363 and the derivative strain NZ9000 (9). In total, six single nucleotide polymorphisms (SNPs) were observed in NZ9000 compared to MG1363. However, with the addition of transcriptome analysis, the observed phenotypic differences in sugar metabolism were ascribed to two SNPs in the *ptcC* promoter, which impacts regulation of cellobiose (glucose disaccharide) transport. Selection for these spontaneous mutations was suggested to arise in order to allow improved growth on glucose-containing media (GM17).

During the routine construction of several gene deletion mutants in *L. lactis* MG1363, we observed the appearance of other spontaneous mutations which led to significant phenotypic changes, namely, heat resistance and in some cases salt hypersensitivity. The objective of this study was therefore to identify the location of these spontaneous mutations and the reason for their appearance. It was found that high-temperature incubation selects for spontaneous mutations in the stress signaling protein-

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encoding gene *llmg_1816* and that this phenomenon is not dependent upon the presence of a plasmid and is not strain specific.

MATERIALS AND METHODS

Bacterial strains, chemicals, and enzymes. *L. lactis* subsp. *cremoris* MG1363 (kindly provided by Scott Chandry, CSIRO, Werribee, Australia) was grown using M17 (Oxoid, Basingstoke, United Kingdom) supplemented with 0.5% glucose (GM17) and was incubated at 30°C or 37.5°C as required. The commercial strain *L. lactis* subsp. *cremoris* ASCC892185 (kindly provided by Dairy Innovation Australia Limited, Werribee, Australia) was normally grown in M17 supplemented with 0.5% lactose and at either 30°C or 34°C as required. *L. lactis* MG1363 strains containing freely replicating pGh9 derivatives were grown at their replication permissive temperature of 30°C in the presence of 5 µg/ml erythromycin (Sigma-Aldrich, St. Louis, MO) (1, 12). *L. lactis* strains containing chromosomally integrated pGh9 derivatives were grown at 37.5°C in the presence of 3 µg/ml erythromycin (Sigma-Aldrich, St. Louis, MO). *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA) containing pGh9:cm (pPNG904) (10) was grown in heart infusion (Oxoid, Basingstoke, United Kingdom) medium containing 20 µg/ml chloramphenicol (Oxoid, Basingstoke, United Kingdom) at 30°C. *E. coli* DH5α strains containing other pGh9 derivatives were grown in heart infusion medium containing 300 µg/ml erythromycin (Sigma-Aldrich, St. Louis, MO) at 30°C.

Construction of the marker-free *llmg_1768*, *llmg_0514*, *llmg_0342*, and *llmg_1593* deletion mutants. Two-step single crossover homologous recombination was employed to generate marker-free deletion mutant strains. Upstream (US) and downstream (DS) flanking regions of each gene were amplified by PCR from the *L. lactis* MG1363 chromosome using the primer pairs in Table S1 in the supplemental material. The US and DS fragments were digested with XbaI, ligated together, and cloned into pGh9:cm to generate pGh9Δ1768, pGh9Δ0514, pGh9Δ0342, and pGh9Δ1593, which were then transformed into *L. lactis* MG1363 by electroporation as described previously (28).

Freely replicating pGh9 derivatives in *L. lactis* MG1363 were integrated into the chromosome by temperature shift from 30°C to 37.5°C (13), and successful integration was confirmed by PCR using the chromosome-specific primers (see Table S1 in the supplemental material). The pGh9 derivative vectors were then removed from the chromosome by temperature shift from 37.5°C to 30°C in the absence of erythromycin selection (13). Marker-free *L. lactis* Δ1768, Δ0514, Δ0342, and Δ1593 mutants were confirmed using PCR (see Table S1).

Complementation of the *llmg_1768* and *llmg_0514* mutants using two-step single crossover homologous recombination. Fragments containing full-length *llmg_1768* and *llmg_0514* genes and flanking regions (see Table S1 in the supplemental material) were cloned into pGh9:cm and were transformed into *L. lactis* Δ1768 and Δ0514 deletion mutants, respectively. The plasmids were then integrated and excised from the chromosome using the same temperature shift approach as for mutant construction. Those clones that contained the reintroduced gene were confirmed by PCR (see Table S1).

Construction of strains *L. lactis* 0342Rwt and 1593Rwt. During construction of Δ0342 and Δ1593 mutants, excision of pGh9Δ0342 and pGh9Δ1593 from the chromosome (see Table S1 in the supplemental material) yielded clones with the intended deletion mutation, while the remaining clones reverted to a wild-type genotype still containing the target gene. Clones of the later type were named 0342Rwt and 1593Rwt (for revertant wild-type) and were derived from experiments to create the Δ0342 and Δ1593 mutants, respectively. The presence of *llmg_0342* and *llmg_1593* in 0342Rwt and 1593Rwt, respectively, was confirmed by PCR.

Construction of the disrupted *llmg_1816* mutant *L. lactis* Δ1816. An 804-bp internal fragment of *llmg_1816* was amplified using primers shown in Table S1 in the supplemental material from the *L. lactis* MG1363 chromosome and cloned into pRV300 (8). This plasmid was used to transform fresh competent *L. lactis* MG1363 cells by electroporation (28). Plasmid integration within *llmg_1816* was confirmed by PCR using the

primers shown in Table S1 in the supplemental material. Note that during this process, *L. lactis* was always grown at 30°C.

Construction of strain *L. lactis* 1816Rwt. *L. lactis* Δ1816 was subcultured for approximately 50 generations in GM17 broth without erythromycin (Er) to allow for plasmid excision. The culture was then serially diluted and plated onto GM17 agar. Colonies were replica plated onto GM17 agar with and without 3 µg/ml erythromycin. Erythromycin-sensitive (Er^S) colonies were selected and further screened by PCR for the absence of plasmid using the primers in Table S1 in the supplemental material. A plasmid-negative Er^S clone was selected and designated *L. lactis* 1816Rwt. Note that during this process, *L. lactis* was always grown at 30°C.

Construction of a transposon library using pGh9::ISS1 in *L. lactis* MG1363. The temperature-sensitive insertional mutagenesis vector pGh9::ISS1 was transformed into *L. lactis* MG1363 by electroporation (28). An ISS1 insertional mutant library was constructed by incubation of transformants at 37.5°C on GM17 agar containing 2 µg/ml erythromycin as previously described (13). Single colonies were restreaked onto GM17 agar containing 2 µg/ml erythromycin and grown for 48 h at 37.5°C. These clones were screened for heat resistance and salt sensitivity as described below.

Selection of natural spontaneous heat-resistant and salt-hypersensitive mutants from the industrial strain *L. lactis* ASCC892185. *L. lactis* ASCC892185 was grown in LM17 broth overnight at 30°C, subcultured by a 1:100 dilution into fresh LM17 broth, and incubated at 30°C for 2.5 h. This early-log-phase culture was shifted to 34°C for a further 2.5 h and then serially diluted and plated onto replica LM17 agar plates, one of which was incubated at 30°C overnight and the other incubated at 34°C for 3 days. Counts were taken, and 20 of the largest colonies growing at 34°C were restreaked onto LM17 agar and incubated for an additional 3 days at 34°C to obtain single isolated colonies. These isolates were transferred to 10 ml LM17 broth, grown overnight at 30°C, and stored in glycerol at −80°C for later use.

Monitoring growth and acidification of *L. lactis* ASCC892185 in milk. Cultures were grown in LM17 at 30°C overnight. Aliquots of 1 ml were harvested by centrifugation (16,000 × g, 5 min) and resuspended in 1 ml of full-cream UHT milk (Devondale, Murray Goulburn Cooperative Co. Ltd., Australia), and then each aliquot was used to inoculate 50 ml of full-cream UHT milk in a 50-ml tube. Inoculated milk samples were incubated at 30°C for 4 h before sodium chloride addition to final concentrations of 0%, 1.5%, 2%, 2.5%, 3%, 3.5%, and 4%. All samples were incubated at 30°C for a further 20 h. All samples were measured for pH at hourly intervals up to 12 h and then again after 24 h. All variables were tested in duplicate.

Dilution drop plate assay for screening strains for high-temperature resistance and salt sensitivity. *L. lactis* MG1363 derivatives were grown in GM17 at 30°C such that all cultures reached early log phase at the same time. At an optical density at 600 nm (OD₆₀₀) of ~0.3, all cultures were serially diluted 10-fold to 10^{−5}, and 5 µl of each dilution was spotted onto two GM17 agar plates and one GM17 agar plate containing an additional 0.25 M sodium chloride. These plates were incubated at 30°C, except for one GM17 agar plate which was incubated at 37.5°C. All agar plates were incubated for 24 h. The same approach was used to screen derivatives of the commercial strain *L. lactis* ASCC892185, except lactose was added to M17 in place of glucose, incubation was carried out at 34°C for determining heat resistance, and sodium chloride was added at 0.3 M for determining salt resistance. This assay was based on methods previously described (26) and was replicated 3 times.

Resistance to cell-wall-active antimicrobials. This assay was carried out using a disk diffusion assay based on previous methods (11). Briefly, the bottom layer of each agar plate was composed of 15 ml GM17 containing 1.5% agar. The top layer was composed of 0.1 ml of the indicated strain harvested at mid-log phase (OD₆₀₀ = 0.4) mixed with 5 ml of GM17 containing 0.75% agar. Sterile 8-mm-diameter filter paper disks (Advantec, Dublin, CA) were loaded with 20 µg penicillin G, 20 µg vancomycin,

2 µg nisin, or 1 mg lysozyme (all sourced from Sigma-Aldrich, St. Louis, MO). The diameter of each growth inhibition zone was measured after overnight growth at 30°C.

Whole-genome sequencing, reference genome assembly, and comparative genome analysis. *L. lactis* MG1363 and the $\Delta 1768$ mutant were grown under nonstress conditions (GM17, 30°C) for genomic DNA extraction. This was achieved by standard methods (7) using chloroform-isoamylalcohol (24:1) in place of phenol-chloroform. High-purity DNA was used for library preparation using the Illumina TruSeq DNA library preparation protocol. Briefly, 1 µg of DNA was fragmented using Covaris shearing followed by an end-repair reaction (3'→5', exonuclease and polymerase fill in) to generate blunt ends. An A-tailing reaction was used to create 3' A overhangs for complementary ligation with 3' T overhangs on the Illumina adaptors. Ligated DNA fragments were size selected by gel electrophoresis and gel excision. Sample genomic DNA libraries were pooled for paired-end sequencing on the Illumina HiSeq 2000 instrument according to the manufacturer's protocols. Base calling was processed with Illumina RTA software version 1.10.36. Demultiplexing and conversion to the FastQ format was performed with CASAVA version 1.7. Reads of 100 bp were paired and assembled to the reference *L. lactis* MG1363 genome (27) using Geneious Pro 5.4.5 (3). Average read depths for MG1363 and the $\Delta 1768$ mutant were 846-fold and 425-fold, respectively. Criteria for SNP identification were >100-fold coverage with >90% variant frequency as used previously (9).

RNA isolation, cDNA synthesis, and qPCR. RNA was harvested from 3 replicate cultures. Early-log-phase ($OD_{600} = 0.2$) cultures were first subjected to RNA stabilization with RNA protect (Qiagen, Hilden, Germany) and harvested by centrifugation (16,000 × g, 5 min), and RNA was extracted using the RNeasy minikit (Qiagen, Hilden, Germany). DNA contamination was removed using 2U DNase I (NEB, Ipswich, MA). The quality and integrity of RNA was confirmed by electrophoresis. cDNA synthesis reactions were standardized to contain 0.5 µg of RNA (including no reverse transcriptase controls to ensure the absence of DNA contamination) and were then reverse transcribed (where necessary) using the Superscript III first-strand synthesis supermix (Invitrogen, Carlsbad, CA). Platinum SYBR green quantitative PCR (qPCR) SuperMix-UDG (Invitrogen, Carlsbad, CA) was used for qPCR. The *busA* gene and reference genes (*alaS* and *tufA*) were amplified using the primers shown in Table S1 in the supplemental material. Duplicate reactions were used for each sample, including negative controls. The Rotor-Gene Q qPCR machine (Qiagen) was used for qPCR. Data were analyzed by relative quantification using REST 2009 (16).

RESULTS

The *L. lactis* $\Delta 1768$, $\Delta 0514$, $\Delta 0342$, and $\Delta 1593$ mutants all possess a high-temperature-resistant phenotype which develops following the high-heat incubation stage of mutant construction. Mutants with deletions in genes encoding two Spx-like proteins (Llmg_0514 and Llmg_1768), a putative methionine ABC transporter ATP binding protein (Llmg_0342), and a putative cysteine ABC transporter substrate binding protein (Llmg_1593) were generated using the temperature-sensitive plasmid vector pGh9. Complemented and revertant wild-type strains were also constructed, and subsequently all strains were screened for stress resistance phenotypes. The relative resistances of *L. lactis* strains compared to that of *L. lactis* MG1363 to high temperature can be seen in Fig. 1. It was found that all mutant, complemented, and revertant wild-type strains were heat resistant (Fig. 1). It is unlikely that deletion of these four functionally different genes would lead to heat resistance, and due to the fact that complementation and revertant wild-type strains also possessed this phenotype, it was suspected that a spontaneous mutation was occurring during the mutant construction process. We decided to examine the heat resistance of intermediate strains in the mutation process, such as

strains containing freely replicating plasmid and those obtained after the first plasmid integration step. Heat resistance was observed only following plasmid integration (Fig. 1). This coincides with the requirement of incubation at 37.5°C for plasmid integration.

Hypersensitivity to salt occurs in some heat-resistant strains. All strains were screened for the ability to grow on media containing an additional 0.25 M sodium chloride. Salt hypersensitivity appeared during construction of both *L. lactis* $\Delta 1768$ and $\Delta 0342$ mutants but not for $\Delta 1593$ and $\Delta 0514$ mutants (Fig. 1). As with the heat resistance development mentioned above, the development of salt hypersensitivity coincided with plasmid integration and 37.5°C incubation (Fig. 1A and C). These results suggested that a spontaneous mutation(s) was occurring, caused by either the plasmid integration event or 37.5°C incubation, leading to heat resistance and in some cases salt hypersensitivity.

Whole-genome sequencing of the heat-resistant and salt-hypersensitive *L. lactis* $\Delta 1768$ strain. To determine the genetic change(s) that had occurred in the $\Delta 1768$ strain compared to the parent strain MG1363, we carried out whole-genome sequencing on both strains. The parent *L. lactis* MG1363 used in this study contains several variations from those previously published (9, 27); however, these will be described in more detail elsewhere. As well as the intentional deletion of *llmg_1768*, the $\Delta 1768$ strain contained seven SNPs in three different loci (Table 1) in comparison to the parent MG1363 strain used in this study. Five SNPs were identified within or immediately downstream of *rdrA* (*llmg_1767*). Their location immediately adjacent to the intentionally deleted gene *llmg_1768* provided reason to believe that they were more likely linked to the *llmg_1768* deletion event rather than contributors to heat resistance and/or salt hypersensitivity. A synonymous SNP was present in *rpoB* (*llmg_1982*), and a nonsynonymous SNP was found in *llmg_1816*, resulting in an A573D substitution of the encoded protein. Despite being annotated as encoding an RecJ-like protein, based on similarity of the predicted protein sequence, *llmg_1816* encodes a membrane-bound stress signaling protein of the GdpP family (COG3887), which has cyclic dimeric AMP (c-di-AMP)-specific phosphodiesterase activity. Due to its predicted role in stress signaling and it being the only nonsynonymous mutation that was not in the vicinity of the *llmg_1768* deletion, the role of *llmg_1816* in heat and salt stress resistance was further examined.

Salt-hypersensitive strains all contain mutations in *llmg_1816*. We amplified and sequenced the *llmg_1816* gene from the $\Delta 0342$ mutant and identified a thymine insertion which would result in a truncation (K122Stop) of Llmg_1816 (Fig. 2). The same thymine insertion was also identified in the related strains *L. lactis* pGh9 $\Delta 0342$ [integrated] and 0342Rwt. Similarly, PCR and sequencing was used to confirm the C→A mutation at nucleotide position 1718 in *llmg_1816* of the $\Delta 1768$ mutant and the related strains *L. lactis* pGh9 $\Delta 1768$ [integrated] and 1768c. Several other strains containing chromosomally integrated pGh derivatives (using pGh9 $\Delta 1768$ and pGh9:ISS1) were constructed and screened for heat resistance and salt hypersensitivity. It was found that all three pGh derivative integrants characterized which were heat resistant and salt hypersensitive contained either an SNP or a DNA duplication mutation in *llmg_1816* (Fig. 2).

Disruption of *llmg_1816* confirms its role in salt hypersensitivity and heat resistance. Seeing that the above-mentioned *llmg_1816*-altered strains contain additional pGh-derived muta-

FIG 1 Relative growth in response to normal growth conditions (30°C), high temperature (37.5°C), and the addition of 0.25 M sodium chloride. Panel A includes strains constructed in the deletion and complementation of *llmg_1768*. a, *L. lactis* MG1363; b, *L. lactis* pGh9Δ1768 (freely replicating); c, *L. lactis* pGh9Δ1768 (integrated); d, *L. lactis* Δ1768; e, *L. lactis* 1768c (complemented). Strains with equivalent modifications for mutation and complementation or wild-type reversion of *llmg_0514* (B), *llmg_0342* (C), and *llmg_1593* (D) were also screened for phenotypes under these conditions. Note that in panel B, the 37.5°C plate was incubated anaerobically to compensate for sensitivity to oxidative stress observed for *L. lactis* Δ0514.

tions, we created a targeted *llmg_1816*-disrupted strain and a subsequent revertant strain using a suicide plasmid vector. Disruption of *llmg_1816* was carried out in the wild-type background of *L. lactis* MG1363 using pRV300 to create the *L. lactis* Δ1816 mu-

tant strain. A control strain (1816Rwt) was also constructed by removing the interrupting plasmid through subculturing in the absence of erythromycin selection. As expected, the Δ1816 mutant displayed heat resistance and salt hypersensitivity compared to the

TABLE 1 Polymorphisms identified in *L. lactis* Δ1768 compared to *L. lactis* MG1363 used in this study

Reference position	Nucleotide change	Region and/or gene or product	Amino acid change	ORF
1752208	A→G	<i>rdrA</i> (transcriptional regulator of sugar metabolism)	Asn→Ser	<i>llmg_1767</i>
1752286	T→C	<i>rdrA</i> (transcriptional regulator of sugar metabolism)	Ile→Thr	<i>llmg_1767</i>
1752352	T→A	<i>rdrA</i> (transcriptional regulator of sugar metabolism)	Val→Asp	<i>llmg_1767</i>
1752624	T→C	Intergenic (downstream of <i>rdrA</i>)		
1752646	T→C	Intergenic (downstream of <i>rdrA</i>)		
1796041	G→T ^a	<i>llmg_1816</i> (<i>gdpP</i>)	Ala→Asp	<i>llmg_1816</i>
1966834	G→A	<i>rpoB</i>	None	<i>llmg_1982</i>

^a The orientation of *llmg_1816* is opposite to the direction of the reference genome sequence; therefore, the coding sequence of the *llmg_1816* in *L. lactis* Δ1768 contains a C→A substitution at nucleotide position 1718.

FIG 2 Polymorphisms identified in *llmg_1816* in heat-resistant and salt-hypersensitive strains. ^a, mutation found in a heat-resistant and salt-hypersensitive derivative of *L. lactis* ASCC892185.

wild type and 1816Rwt (Fig. 3). The salt hypersensitivity for *L. lactis* Δ 1816 was not as severe as that seen for most of the spontaneous mutants (Fig. 1). This is likely due to low-level plasmid excision in the absence of antibiotic. Erythromycin was omitted in order to compare the wild-type (MG1363), Δ 1816, and 1816Rwt strains on the same agar plate.

Mutation of *llmg_1816* affects growth in the presence of penicillin G. Recent work has shown that *llmg_1816* homologs (*gdpP* and *yybT*) are involved in resistance or tolerance to cell-wall-active antibiotics in *Staphylococcus aureus* and *Bacillus subtilis* (2, 6, 10). *L. lactis* Δ 1816 displayed a zone of inhibition in response to penicillin G similar to those of the wild type (MG1363) and 1816Rwt but, interestingly, had improved growth at sublethal concentrations of penicillin G, as revealed by enhanced growth immediately outside the zone of inhibition (Fig. 4). Sensitivity to vancomycin, nisin, and lysozyme were also tested; however, no significant differences were observed between the wild type and the Δ 1816 mutant (data not shown).

Disruption of *llmg_1816* leads to lower basal expression of *busAA*. The activity of the glycine betaine uptake system (*BusA*) in *L. lactis* has been reported to be a strong determinant of tolerance to osmotic stress (14). One possibility for the salt-hypersensitive phenotype of the *llmg_1816* mutant is that expression of *BusA* is altered. Using qPCR, it was found that expression of *busAA* was 5-fold lower ($P < 0.005$) in *L. lactis* Δ 1816 than in *L. lactis* MG1363 under non-salt-stressed conditions. The addition of salt to a final concentration of 0.5 M and incubation for 30 min resulted in an increase of *busAA* expression by a mean factor of 7.0-fold ($P < 0.005$) in *L. lactis* MG1363 and 94.8-fold ($P < 0.005$) in *L. lactis* Δ 1816 compared to that without salt addition. Therefore, following salt addition, levels of *busAA* transcript in *L. lactis* Δ 1816 cells were 2.9-fold ($P < 0.005$) higher than in *L. lactis* MG1363 cells.

The industrial cheesemaking strain *L. lactis* ASCC892185 undergoes high-temperature incubation-induced *llmg_1816* mutation, leading to heat resistance and salt hypersensitivity. We next sought to determine if spontaneous mutation of *llmg_1816* was a peculiarity of *L. lactis* strain MG1363 and also if spontaneous mutation of *llmg_1816* was due to high-temperature incubation or a plasmid integration event. The laboratory strain *L. lactis* MG1363 cannot grow in milk, so we investigated if a wild-type industrial cheesemaking *L. lactis* strain would also undergo spontaneous mutation of *llmg_1816* during high-temperature in-

FIG 3 Relative growth of *L. lactis* MG1363 (a), *L. lactis* Δ 1816 (b), and *L. lactis* 1816Rwt (c) under normal growth conditions (GM17, 30°C), high temperature (GM17, 37.5°C), and salt stress conditions (GM17 and 0.25 M NaCl, 30°C).

cubation. *L. lactis* ASCC892185 was incubated at 34°C, a temperature just below its upper growth limit, yielding two stable spontaneous mutants that were both heat resistant and salt hypersensitive. Sequence analysis of *llmg_1816* in these mutants revealed the same G→T substitution, indicating that they are likely siblings. The consequence of this mutation is truncation of *llmg_1816* at amino acid 472 (E472Stop) (Fig. 2). When cultured in milk, the *L. lactis* ASCC892185 *llmg_1816*-altered mutant displayed relatively normal growth and acidification, albeit slightly slower than the parent *L. lactis* ASCC892185 (Fig. 5). Lower salt concentrations were shown to inhibit acidification of milk by the *L. lactis* ASCC892185 *llmg_1816*-altered mutant than the parent *L. lactis* ASCC892185 (Fig. 5).

DISCUSSION

This study demonstrates that spontaneous mutation can occur in *L. lactis* during high-temperature incubation conditions. Through several lines of evidence, we confirmed that mutation of *llmg_1816* causes dual heat resistance and salt hypersensitivity phenotypes. Based on similarity of the predicted amino acid sequence, *llmg_1816* encodes a membrane-bound stress signaling protein of the GdpP family (COG3887). GdpP family proteins, which have been recently characterized in *B. subtilis* (previously called YybT) (19) and *S. aureus* (2), act to reduce cellular levels of the secondary messenger molecule c-di-AMP. These proteins are membrane bound via two N-terminal transmembrane helices (15) and contain a Per-ARNT-Sim (PAS) domain that binds heme to modulate activity of the DHH/DHHA1 domains (18) and a highly modified GGDEF domain that hydrolyzes ATP (19). DHH and DHHA1 domains provide specific c-di-AMP phosphodiesterase activity which is strongly inhibited by the stringent response signaling molecule ppGpp (19). In *B. subtilis*, c-di-AMP is reported to be synthesized by the DNA-scanning protein DisA, whose diadenylate cyclase (DAC) activity is inhibited by DNA damage (15, 29). The *B. subtilis* proteins YbbP and YojJ also contain DAC domains (23) and have been shown to be biologically active (11); however, their cellular role is not fully understood. In *S. aureus*, DacA is considered to be the sole protein responsible for c-di-AMP synthesis (2); however, the mechanisms of its regulation remain unstudied. Similarly, *L. lactis* MG1363 contains only one protein (*llmg_0448*) with similarity to diadenylate cyclase proteins, which also remains unstudied.

The mechanism of c-di-AMP-controlled regulation remains elusive; however, it has been shown to play a role in several cellular

FIG 4 Sensitivity of *L. lactis* MG1363 (A), *L. lactis* Δ 1816 (B), and *L. lactis* 1816Rwt (C) to penicillin G using the disk diffusion assay.

processes in Gram-positive bacteria. These include control of sporulation in *B. subtilis* (15) and cell wall homeostasis in both *B. subtilis* and *S. aureus* (2, 11). In both these bacteria, *gdpP* mutants were found to be significantly more resistant to β -lactam antibiotics than the parent strain, suggesting modification of the cell wall architecture (2, 11). Here, using a disk diffusion assay, we found that *L. lactis* Δ 1816 did not have greater resistance to the β -lactam penicillin G but did show enhanced growth at sublethal concentrations. This suggests that in *L. lactis*, *llmg_1816* likely af-

fects cell wall homeostasis to some degree but possibly not to the extent of *B. subtilis* and *S. aureus*. Acid stress resistances of *gdpP* (*yybT*) mutants have previously been reported. Acid resistance screening of an *L. lactis* transposon mutant library identified two *llmg_1816* transposon insertional mutants which were 100-fold more tolerant to acid stress than the wild type (17). Improved resistance to acid stress was also observed for a *B. subtilis* *gdpP* mutant (19). To date, *gdpP* mutations in Gram-positive bacteria have been shown to confer resistance to acid, high temperature, and cell wall stressors, possibly suggesting a general stress resistance mechanism; however, our finding of salt hypersensitivity suggests that *gdpP* mutants are not resistant to all stressors.

The reason for salt hypersensitivity for the *L. lactis* *gdpP* mutant is not clear and is unexpected, because *S. aureus* *gdpP* mutants have an increase in cell wall peptidoglycan cross-linking, which would normally be predicted to lead to greater cell wall strength (2). The majority of research of genes involved in osmotic stress resistance in *Lactococcus* has been centered around the Bus (Opu) glycine-betaine ABC transporter complex (and its repressor BusR) (21, 22). Modified strains with reduced BusA expression (due to overexpression of BusR) showed growth defects in high levels of salt when betaine was supplemented in the media, indicating that betaine transport is important for osmotic stress resistance (22). Betaine transport activity has also been implicated as being a likely reason why *L. lactis* subsp. *lactis* strains are naturally more resistant to osmotic stress than *L. lactis* subsp. *cremoris* strains (14). Like that described previously, we found highly increased expression of *busAA* in response to salt addition (22). Interestingly, basal expression of *busAA* in *L. lactis* Δ 1816 was downregulated compared to that in *L. lactis* MG1363. This is likely to be a contributing factor to the salt-hypersensitive phenotype. Upon salt addition, the *busAA* transcript levels in *L. lactis* Δ 1816 were higher than that for *L. lactis* MG1363. It remains to be determined if changes in *busAA* expression are due to signaling by the likely substrate (c-di-AMP) of *llmg_1816* or in response to other cellular disturbances possibly involving cell envelope alterations.

As shown in Fig. 1, the frequency of salt hypersensitivity was found in 50% of heat-resistant mutants, suggesting that when the selection temperature is high enough to generate stable heat-resistant mutants, then a significant proportion of mutants will contain *llmg_1816* mutations. However, in addition to this, we have also found that the stage of growth or type of media where the first temperature upshift occurs is likely important for salt hypersensitivity development. In unpublished work, where stationary-phase wild-type *L. lactis* MG1363 cells were not exposed to a high-temperature upshift in broth before incubation at high temperature

FIG 5 Growth properties of *L. lactis* ASCC892185 and a spontaneous *llmg_1816* mutant derivative. (A) Growth of wild-type ASCC892185 (a) and *llmg_1816*-altered mutant (b) on LM17 agar at 30°C, 34°C, and 30°C with 0.3 M salt. Acidification profile of *L. lactis* ASCC892185 (B) and the *llmg_1816*-altered derivative (C) when grown in milk at 30°C for 4 h followed by salt addition to a final concentration of 0% (■), 1.5% (▲), 2% (×), 2.5% (△), 3% (□), 3.5% (○), and 4% (◇) compared to the uninoculated control (◆).

on agar, no mutants with severe salt hypersensitivity were found among 20 spontaneous heat-resistant mutants screened. Spontaneous mutation of *llmg_1816* in response to incubation at a temperature approaching its upper limit for growth (34°C) was further demonstrated in the commercial strain *L. lactis* ASCC892185, resulting in variants which were heat resistant and salt hypersensitive. This clearly indicates that this phenomenon is not a peculiarity of the laboratory strain *L. lactis* MG1363. Furthermore, it was shown that an *llmg_1816* mutant derivative of *L. lactis* ASCC892185 is still capable of growing in and acidifying milk but has slower acidification rates than the parental strain following the addition of low-salt concentrations. *L. lactis llmg_1816* mutants may find potential application in production of lower-salt cheeses, as their metabolism could be controlled better using lower salt levels, as opposed to that needed for wild-type strains. The simplicity of generating non-GM *llmg_1816* mutants and their subsequent potentially useful phenotypes may make these mutants tenable for use in industry.

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