Heat Resistance and Salt Hypersensitivity in *Lactococcus lactis* Due to Spontaneous Mutation of *lmg_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 *lmg_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 *lmg_1816* mutant (Δ1816), 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* Δ1816 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 *lmg_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 *lmg_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 *lmg_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 pteC promoter, which impacts regulation of cellulose (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 ilmG_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 ASC892185 (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). *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA) containing pGh9:Cm (pPNG094) (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 ilmG_1768, ilmG_0514, ilmG_0342, and ilmG_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 transformation with 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 by PCR (see Table S1).

**Complementation of the ilmG_1768 and ilmG_0514 mutants using two-step single crossover homologous recombination.** Fragments containing full-length ilmG_1768 and ilmG_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 latter 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 ilmG_0342 and ilmG_1593 in 0342Rwt and 1593Rwt, respectively, was confirmed by PCR.

**Construction of the disrupted ilmG_1816 mutant *L. lactis* Δ1816.** An 804-bp internal fragment of ilmG_1816 was amplified using primers shown in Table S1 in the supplemental material from the *L. lactis* MG1363 chromosome and cloned into pVR300 (8). This plasmid was used to transform fresh competent *L. lactis* MG1363 cells by electroporation (28). Plasmid integration within ilmG_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+) 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- 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* ASC892185.** *L. lactis* ASC892185 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* ASC892185 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* ASC892185, 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 Δ1768 mutant were grown under nonstress conditions (GM17, 30°C) for genomic DNA extraction. This was achieved by standard methods (7) using chloroform-isooamylalcohol (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 performed 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 Δ1768 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 (OD600 = 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 RNasy 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 busAA 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 Δ1768, Δ0514, Δ0342, and Δ1593 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 generated using the temperature-sensitive plasmid vector 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 performed 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 Δ1768 mutant were 846-fold and 425-fold, respectively. Criteria for SNP identification were >100-fold coverage with >90% variant frequency as used previously (9).

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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 Δ1768 and Δ0342 mutants but not for Δ1593 and Δ0514 strains (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 Δ1768 strain. To determine the genetic change(s) that had occurred in the Δ1768 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 Δ1768 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_182), 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 Δ0342 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 pGh9Δ0342[integrated] and 0342Rwt. Similarly, PCR and sequencing was used to confirm the C−−A mutation at nucleotide position 1718 in llmg_1816 of the Δ1768 mutant and the related strains L. lactis pGh9Δ1768[integrated] and 1768c. Several other strains containing chromosomally integrated pGh derivatives (using pGh9Δ1768 and pGh9ISSI) 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-
tions, we created a targeted \textit{llmg\_1816}-disrupted strain and a subsequent revertant strain using a suicide plasmid vector. Disruption of \textit{llmg\_1816} was carried out in the wild-type background of \textit{L. lactis} MG1363 using pRV300 to create the \textit{L. lactis} /H9004\textsubscript{1816} mutant strain. A control strain (1816Rwt) was also constructed by removing the interrupting plasmid through subculturing in the absence of erythromycin selection. As expected, the /H9004\textsubscript{1816} mutant displayed heat resistance and salt hypersensitivity compared to the wild-type strain.

![Graph showing 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 \textit{llmg\_1768}. a, \textit{L. lactis} MG1363; b, \textit{L. lactis} pGH9\textsubscript{1768} (freely replicating); c, \textit{L. lactis} pGH9\textsubscript{1768} (integrated); d, \textit{L. lactis} \Delta1768; e, \textit{L. lactis} 1768c (complemented). Strains with equivalent modifications for mutation and complementation or wild-type reversion of \textit{llmg\_0514} (B), \textit{llmg\_0342} (C), and \textit{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 \textit{L. lactis} \Delta0514.]

**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 \textit{llmg\_1768}. a, \textit{L. lactis} MG1363; b, \textit{L. lactis} pGH9\textsubscript{1768} (freely replicating); c, \textit{L. lactis} pGH9\textsubscript{1768} (integrated); d, \textit{L. lactis} \Delta1768; e, \textit{L. lactis} 1768c (complemented). Strains with equivalent modifications for mutation and complementation or wild-type reversion of \textit{llmg\_0514} (B), \textit{llmg\_0342} (C), and \textit{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 \textit{L. lactis} \Delta0514.

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\*The orientation of \textit{llmg\_1816} is opposite to the direction of the reference genome sequence; therefore, the coding sequence of the \textit{llmg\_1816} in \textit{L. lactis} \Delta1768 contains a C→A substitution at nucleotide position 1718.
The activity of the glycine betaine uptake system (BusA) in the industrial cheesemaking strain L. lactis MG1363 cannot grow in milk, so we investigated if a wild-cubation or a plasmid integration event. The laboratory strain was a peculiarity of L. lactis MG1363 but, interestingly, had improved growth at sublethal 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 capability just below the zone of inhibition (Fig. 4). Sensitivity to vancomycin, nisin, and lysozyme were also tested; however, no 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 Δ1816 mutant (data not shown).

Mutation of llmg_1816 affects growth in the presence of penicillin G. Recent work has shown that llmg_1816 homologs (gdpP and ybbT) are involved in resistance to tolerance to cell-wall-active antibiotics in Staphylococcus aureus and Bacillus subtilis (2, 6, 10). L. lactis Δ1816 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 capability just below 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 Δ1816 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 Δ1816 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 Δ1816 compared to that without salt addition. Therefore, following salt addition, levels of busAA transcript in L. lactis Δ1816 cells were 2.9-fold (P < 0.005) higher than in L. lactis MG1363 cells.

The industrial cheesemaking strain L. lactis ASC892185 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 incubation. L. lactis ASC892185 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 cultivated in milk, the L. lactis ASC892185 llmg_1816-altered mutant displayed relatively normal growth and acidification, albeit slightly slower than the parent L. lactis ASC892185 (Fig. 5). Lower salt concentrations were shown to inhibit acidification of milk by the L. lactis ASC892185 llmg_1816-altered mutant than the parent L. lactis ASC892185 (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 YbtT) (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...
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* Δ1816 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*, Δ1816 likely affects 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 Δ1816 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* Δ1816 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* Δ1816 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 Δ1816 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 Δ1816 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,
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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