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A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation

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Summary

Previously, a promoter was identified in *Lactococcus lactis* that is specifically induced by chloride. Here, we describe the nucleotide sequence and functional analysis of two genes transcribed from this promoter, *gadC* and *gadB*. GadC is homologous to putative glutamate-γ-aminobutyrate antiporters of *Escherichia coli* and *Shigella flexneri* and contains 12 putative membrane-spanning domains. GadB shows similarity to glutamate decarboxylases. A *L. lactis gadB* mutant and a strain that is unable to express both *gadB* and *gadC* was more sensitive to low pH than the wild type when NaCl and glutamate were present. Expression of *gadCB* in *L. lactis* in the presence of chloride was increased when the culture pH was allowed to decrease to low levels by omitting buffer from the medium, while glutamate also stimulated *gadCB* expression. Apparently, these genes encode a glutamate-dependent acid resistance mechanism of *L. lactis* that is optimally active under conditions in which it is needed to maintain viability. Immediately upstream of the chloride-dependent *gadCB* promoter P_{gad}, a third gene encodes a protein (GadR) that is homologous to the activator Rgg from *Streptococcus gordonii*. *gadR* expression is chloride and glutamate independent. A *gadR* mutant did not produce the 3 kb *gadCB* mRNA that is found in wild-type cells in the presence of NaCl, indicating that GadR is an activator of the *gadCB* operon.

Introduction

Lactococcus lactis is a Gram-positive mesophilic non-differentiating microorganism. It gains energy from the fermentation of sugars to (mainly) lactic acid, resulting in the acidification of its environment. This is one of the ways in which the organism can prevent food spoilage and one of the reasons why *L. lactis* is widely used in the dairy industry. Depending on sugar availability and the buffering capacity of the environment, lactococcal cells will spend a large part of their growth cycle at low pH. The question as to how *L. lactis* can survive such harsh conditions has excited increased interest in recent years. The limiting pH for growth in milk is 4.3 (Piard and Desmazeaud, 1991). Resting cells are much better adapted to low pH than actively dividing cells (Hartke *et al.*, 1994). Acid resistance can be improved significantly by pre-exposure to mild (pH 5–6) acid conditions (Hartke *et al.*, 1996). Similar results were obtained when HCl was used instead of lactic acid (Rallu *et al.*, 1996). Similarly, pre-exposure to UV radiation can improve cell survival at low pH (Hartke *et al.*, 1995). Enhanced survival at low pH induced by starvation or mild acid conditions points to the existence and the activation of acid adaptation mechanisms. This notion is supported by the recent observation of Rallu *et al.* (1996) that *L. lactis* MG1363 is very sensitive to low pH when protein synthesis is blocked during an adaptation step at mild acid conditions. Thirty-three acid-induced polypeptides were detected in a two-dimensional analysis of lactococcal cells exposed to pH 5.5 (Hartke *et al.*, 1996). A major role in the maintenance of a constant cellular pH (pH_i) is fulfilled by the F_0F_1-(H^+)-ATPase (Kobayashi *et al.*, 1986). *L. lactis* mutants with a reduced F_0F_1-ATPase activity were shown to be more acid sensitive (Tomita *et al.*, 1996). The arginine deiminase pathway (ADI) was found to be active at very low pH. As one of the end products of this pathway is ammonia, ADI activity results in an increase in the extracellular pH (pH_o) and, therefore, in enhanced survival of low-pH conditions (Marquis *et al.*, 1987; Casiano-Colón and Marquis, 1988). Amino acid antiport in combination with amino acid decarboxylation can be seen as a more general system for pH homeostasis, as an acid is exchanged for a more basic amine and because, in addition, a proton is consumed intracellularly by the decarboxylation (Molenaar *et al.*, 1993). In *Escherichia coli* and *Shigella flexneri*, a putative glutamate–γ-aminobutyrate (GABA) antiporter has been shown to confer acid resistance in media supplemented with glutamate (Hersh *et al.*, 1996; Waterman and Small, 1996). The genes for these antiporters were linked to genes encoding glutamate decarboxylases. The *E. coli* Cad system consists of a lysine–cadaverine antiporter

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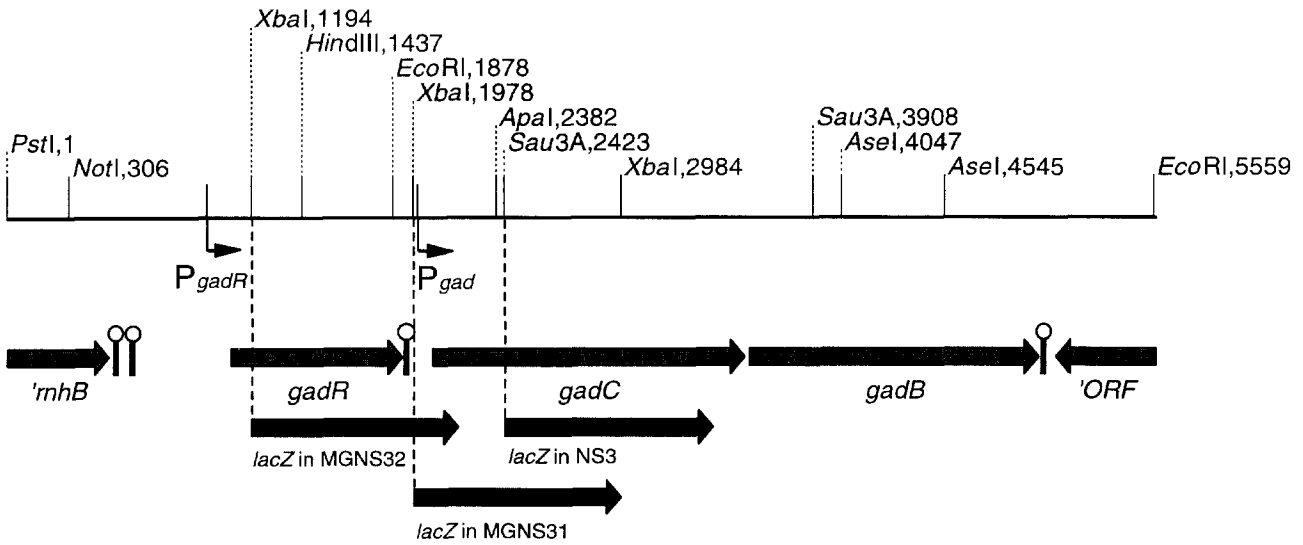

Fig. 1. Gene organization in the NS3 locus. Lollipop, inverted repeat; P, promoter. Relevant restriction enzyme sites are indicated. The black arrows indicate the positions of *lacZ* fusions in the chromosome of the indicated strains (*lacZ* not drawn to scale).

(CadB) and a lysine decarboxylase (CadA). Expression of CadAB is induced at low pH in the presence of lysine (Meng and Bennett, 1992). In a study of regulatory mechanisms involved in acid resistance in *L. lactis*, a number of acid-resistant mutants was found (Rallu *et al.*, 1996). The identity of the affected genes suggests that a relationship exists between the stringent response control circuit and acid resistance and confirms the involvement of the ADI pathway in acid resistance.

In a previous study, a chloride-dependent chromosomal promoter of *L. lactis* was identified (Sanders *et al.*, 1997) using a random *lacZ* integration strategy. One integrant expressed increased levels of *lacZ* with increasing Cl^- concentrations in the culture medium. Expression from this promoter was cation independent, and no expression was detectable in the presence of other anions or at a high osmotic pressure. Here, we report on the genetic analysis of the regions surrounding this promoter and show that a regulator is involved in Cl^--dependent gene expression. The promoter drives the Cl^--dependent expression of a putative amino acid antiporter and glutamate decarboxylase that together operate in glutamate-dependent acid stress resistance in *L. lactis*.

Results

Nucleotide sequence of the chloride-inducible NS3 locus

In a previous study (Sanders *et al.*, 1997), a chloride-dependent chromosomal promoter (P_{gad}) was identified in *L. lactis* using a *lacZ* integration strategy. The nucleotide sequence of the chromosomal region surrounding the *lacZ* integration site in the strain in question, *L. lactis* NS3, was determined, and the data are presented in Figs 1 and 2. Upstream of P_{gad}, an open reading frame (ORF) could encode a protein of 276 residues with a calculated molecular weight of 32 990. Its deduced amino acid sequence is homologous (25% identity and 18% similarity) to Rgg, a positive regulator involved in the expression of glucosyltransferase in *Streptococcus gordonii* (Sulavik *et al.*, 1992; Sulavik and Clewell, 1996). This ORF was named *gadR* because of its regulatory function (see below). GadR also shows 19% identity with PlcR, a positive regulator of the *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C gene (Lereclus *et al.*, 1996). Interestingly, *gadR* is highly similar, but not identical, to a previously identified ORF with unknown function associated with *pip* in *L. lactis* C2 (Geller *et al.*, 1993). *gadR* is preceded by a possible ribosome-binding site and is immediately followed by a 21 bp inverted repeat (IR; $\Delta G[25°C] = -35.0\,kcal\,mol^{-1}$; Tinoco *et al.*, 1973; see Fig. 2). The putative product of a truncated ORF that stops 594 bp upstream of *gadR* showed 43% identity to the C-terminal end of RNaseHII of *E. coli*, an enzyme involved in the specific degradation of the ribonucleotide moiety of DNA/RNA hybrids (Itaya, 1990). *L. lactis rnhB* is followed by a 9 bp IR ($\Delta G[25°C] = -7.6\,kcal\,mol^{-1}$) and a 8 bp IR ($\Delta G[25°C] = -10.6\,kcal\,mol^{-1}$) that may function as rho-independent transcriptional terminators (Fig. 1). Two other weak inverted repeats are present in the long non-coding region between *rnhB* and *gadR* (not shown).

The original *Sau*3A fusion site in the *lacZ* integrant *L. lactis* NS3 is located in an ORF of 503 codons immediately

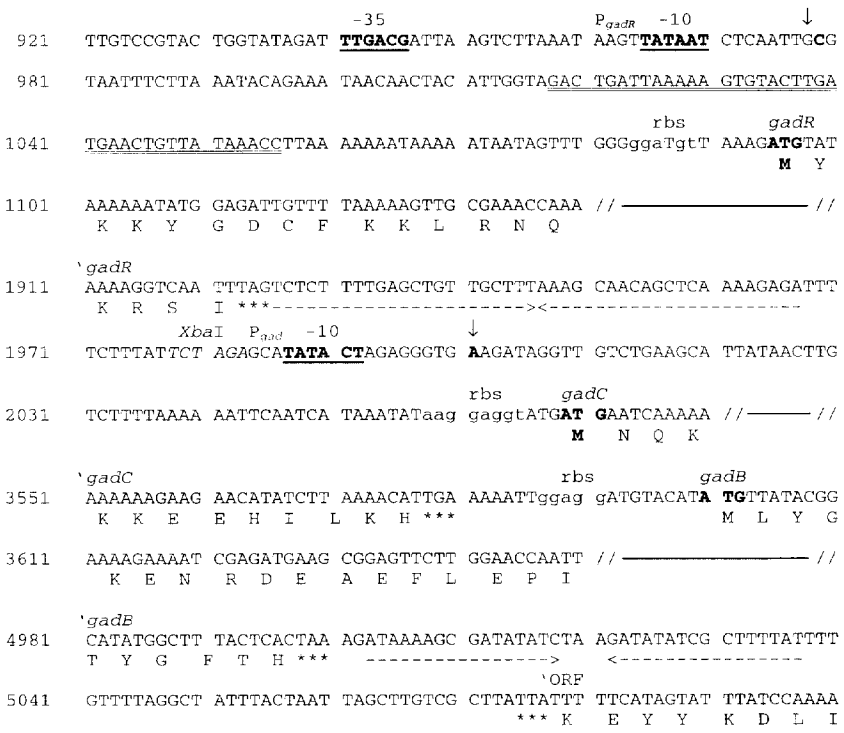

Fig. 2. Nucleotide sequence and expression signals of the intergenic regions in the NS3 locus. Basepair numbering is as in Fig. 1. Parts of the deduced amino acid sequences of the genes are given below the sequence. Facing arrows, inverted repeats; −10 and −35 promoter sequences are underlined and in boldface; vertical arrows, transcription start points; rbs, ribosome-binding site (nucleotides indicated in lower case). Stop codons are indicated with asterisks; start codons are in bold face. Relevant restriction enzyme sites are indicated above the sequence. The position to which primer NS3-12 anneals is double underlined.

downstream of P_{gad}. This ORF was named *gadC*, as its deduced amino acid sequence (Fig. 3) is homologous to GadC of *S. flexneri* (51% identity and 17% similarity; Waterman and Small, 1996) and *E. coli* GadC (XasA; Hersh *et al.*, 1996). The GadC proteins are homologous to a number of amino acid antiporters, including the lysine–cadaverine antiporter CadB of *E. coli*. Lactococcal GadC has a deduced molecular weight of 55 369, a pI of 9.73 and is highly hydrophobic. The hydrophobic residues are clustered in 12 domains (Fig. 3), whose locations coincide with those of the hydrophobic domains in *S. flexneri* GadC (as predicted by a number of topology-predicting computer programs). This suggests that GadC is an integral membrane protein. A conserved domain found in glutamate-transporting proteins is also present in *L. lactis* GadC (Fig. 3; Waterman and Small, 1996). Two ATG codons are present at the 5′ end of *gadC*. On the basis of the average spacing between ribosome-binding sites and start codons in *L. lactis* (4–12 bp; Van de Guchte *et al.*, 1992), the second was tentatively indicated as the *gadC* start codon. *gadC* is separated by 19 bp from another ORF of 466 codons of which the deduced protein (with a molecular weight of 53 849 and a pI of 4.87) is homologous to glutamate decarboxylases. Highest homology is found with glutamate decarboxylase from *Synechocystis* spp. (48% identity and 15% similarity; Kaneko *et al.*, 1996). It is also highly similar to the glutamate decarboxylases, GadA and GadB, of *E. coli* (43% and 44% identity respectively; Smith *et al.*, 1992). In *E. coli* and *S. flexneri*, *gadB* is linked to the *gadC* genes. The lactococcal gene was therefore named *gadB*. Whereas in *E. coli* and *S. flexneri* the putative antiporter genes are preceded by *gadB*, the gene order in *L. lactis* is *gadCB*. No possible transcription signals could be identified in or near the 19 bp intergenic region between *gadC* and *gadB*, which suggests that they form an operon. Downstream of *gadB*, a 16 bp IR ($\Delta G[25°C] = -23$ kcal mol^{-1}) followed by a stretch of Ts may function as a rho-independent transcription terminator (Fig. 2). This IR is followed by an ORF in the opposite orientation, and it may therefore function as a dual transcription terminator of both *gadCB* and this ORF of unknown function.

Chromosomal map location

Two rare restriction enzyme sites, *Not*I and *Apa*I, are separated by only 2065 bp in the nucleotide sequence of the NS3 locus (Fig. 1). In the genetic map of *L. lactis* MG1363 (Le Bourgeois *et al.*, 1995), there is only one position at which *Not*I and *Apa*I are that closely linked, namely between *ldh* and *leu-ilv*. Therefore, the *gad* operon is located between these two gene clusters.

GadR regulates the expression of gadCB positively

Its homology with known positive regulators and the close proximity of *gadR* to P_{gad} suggests that GadR is involved in the Cl^--dependent expression of *gadCB*. To investigate

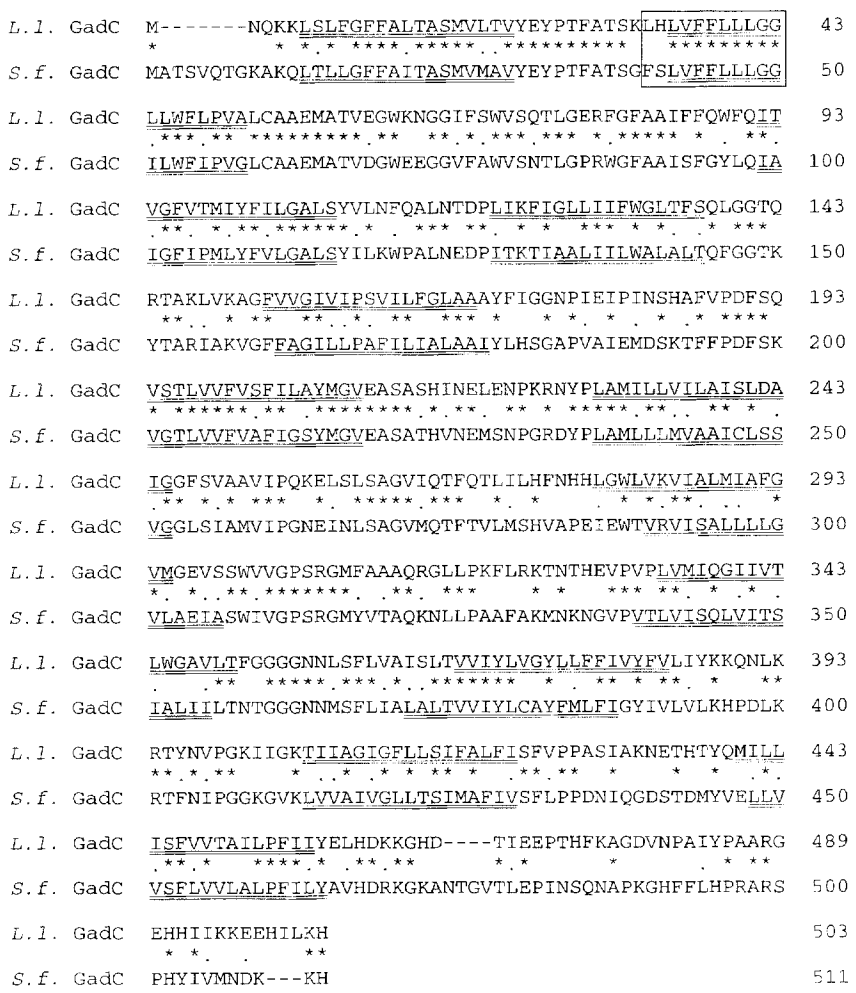

Fig. 3. Alignment of the deduced translation products of *gadC* from *L. lactis* (*L.l.* GadC) and *S. flexneri* (*S.f.* GadC). Asterisks, identical amino acids; periods, similar amino acids. Putative membrane-spanning domains are double underlined. Residues of a putative glutamate-binding box (Waterman and Small, 1996) are boxed.

this possibility, *gadR* was inactivated by single cross-over integration of pNS3i3, resulting in strain MGNS3i3. The effect on the transcription of *gadC* was analysed by Northern hybridization using the 5′ end of *gadC* as a probe. A 3 kb mRNA was detected only in RNA isolated from *L. lactis* MG1363 grown in the presence of NaCl (Fig. 4). No signal was found with RNA from MGNS3i3 grown in either the absence or the presence of NaCl, indicating that GadR is indispensable for transcription from P_{gad}. The size of the mRNA corresponded to the size of *gadC* plus *gadB*. The smaller hybridizing RNAs are probably breakdown products of the 3 kb transcript. The same pattern of hybridization was observed in blots using RNA from independent isolations. The 1.6 kb hybridizing band may be a transcript encoding *gadC* only. The faint bands seen in MGNS3i3 and in MG1363 without NaCl may be caused by background expression from P_{gad} or by aspecific hybridization. Alternatively, these bands may be an artifact of the Northern blot procedure related to the position of rRNA (Huang *et al.*, 1992; Sulavik and Clewell, 1996).

Expression of gadCB *is enhanced at low pH and by glutamate*

Expression of lactococcal *gadC* was studied in *L. lactis* NS3, the strain carrying a single-copy *gadC*::*lacZ* fusion in its chromosome (Fig. 1). The experiment was done several times, and a typical example is given in Fig. 5. A modified M17 medium (mM17) was used, in which β-glycerophosphate was omitted to lower the buffering capacity of the medium. mM17 also lacked soytone. β-Galactosidase activity in NS3 was still induced in this modified medium in the presence of 0.3 M NaCl and 2% β-glycerophosphate buffer, albeit at a level fivefold lower than that in standard G1/2M17 (data not shown). In the experiments described in Fig. 5, NaCl (0.3 M) was present in all cultures. In the absence of buffer and depending on the presence of glutamate, the pH of the cultures dropped to values of 4.0–4.5 in the stationary phase, while cultures growing in buffered mM17 reached a pH of 5.5, irrespective of added glutamate. *lacZ* expression in buffered mM17

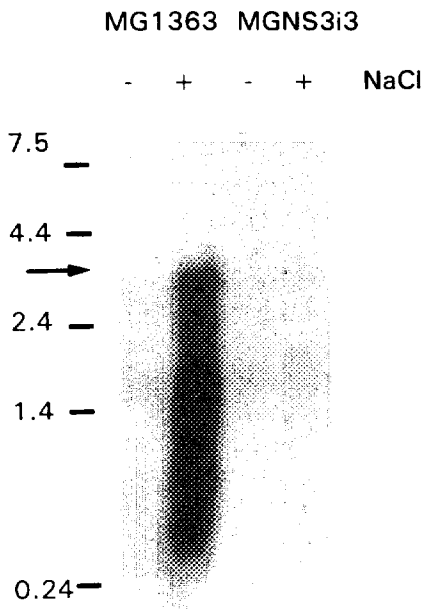

Fig. 4. Autoradiograph of a Northern blot of 30 μg of total RNA from *L. lactis* MG1363 and *L. lactis* MGNS3i3 (*gadR*) grown in the absence (−) or presence (+) of 0.5 M NaCl. The probe was a [α-^{32}P]-dCTP-labelled 450 bp *Xba*I–*Sau*3A fragment encoding the 5′ end of *gadC*. The sizes of an RNA marker are given in the left margin. The arrow indicates the position of the 3 kb *gadCB* transcript.

was low in the early stages of exponential growth and increased to an optimum in the late logarithmic phase of growth (Fig. 5A). Expression of *lacZ* was increased 10-fold in mM17 containing no buffer (Fig. 5B). The addition of 5 mM glutamate to cultures stimulated *lacZ* expression, but optimal expression was obtained with 50 mM glutamate. Glutamate at 50 mM resulted in a 1.5- to twofold additional increase in the expression of *lacZ*, both in unbuffered and in buffered mM17 broth (Fig. 5). No β-galactosidase activity was detectable in the absence of NaCl, whether or not glutamate was present and independent of the culture pH (data not shown). Therefore, expression of *gadCB* in batch culture is highest at the onset of the stationary growth phase in the presence of NaCl and glutamate, and at low pH.

gadR *is constitutively expressed*

Expression of *gadR* was studied in strain MGNS32 carrying a single-copy *gadR*::*lacZ* transcriptional fusion (see Fig. 1) under the same growth conditions as used for NS3. β-Galactosidase activity in exponentially growing MGNS32 in buffered mM17 was 6.0 U mg^{-1} and decreased to 4.0 U mg^{-1} in the stationary growth phase. In the absence of buffer, 1.5 U mg^{-1} was measured during exponential growth, and this dropped to 0.5 U mg^{-1} in the stationary phase. β-Galactosidase activity was independent of the presence or absence of 0.3 M NaCl and was also the same in the presence and absence of 50 mM glutamate. In other words, NaCl-dependent and glutamate-dependent expression from P_{gad} is not regulated by variations in the level of transcription of *gadR*. Another *lacZ* fusion, located immediately downstream of the 21 bp IR, was used to show that transcription of *gadR* is effectively terminated by this IR. No β-galactosidase activity could be detected in strain MGNS31 carrying this fusion (see Fig. 1). Transcription of *gadR* starts from a G-residue 117 bp upstream of the AUG start codon (Figs 1 and 6). The *gadR* transcription start point is the same in the absence or presence of NaCl (data not shown). The *gadR* promoter consists of canonical −35 and −10 hexanucleotides separated by 18 bp (Fig. 2).

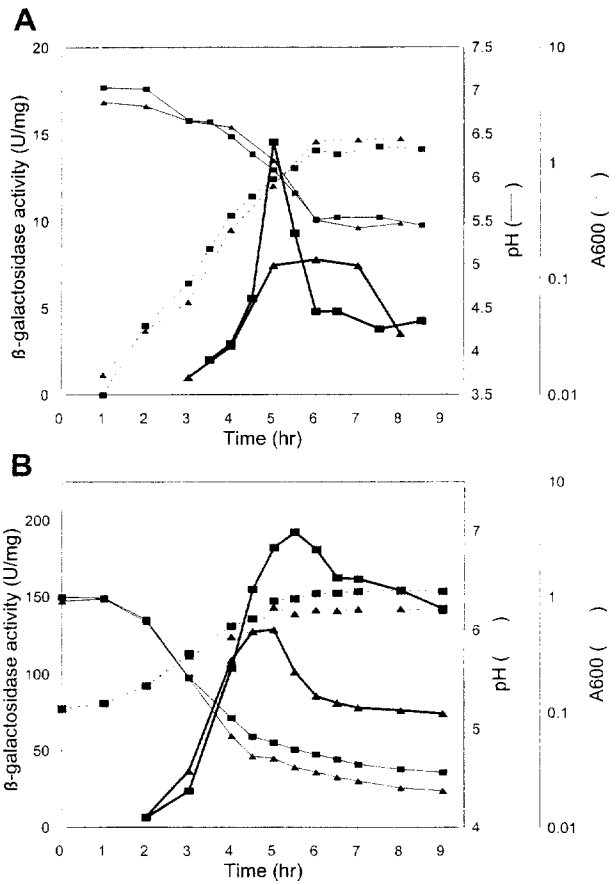

Fig. 5. pH- and glutamate-dependent β-galactosidase expression in *L. lactis* NS3 (*gadC*::*lacZ*). Strain NS3 was grown in mM17 medium with (A) or without (B) 2% β-glycerophosphate. β-Galactosidase activity (U mg^{-1}) was followed during growth (fat solid lines) either in the presence of 0.3 M NaCl (▲) or in the presence of 0.3 M NaCl plus 50 mM glutamic acid (■). Note that the scales of the left *y*-axes in A and B are different. The optical densities and pH values of the cultures are indicated with dotted and thin lines respectively.

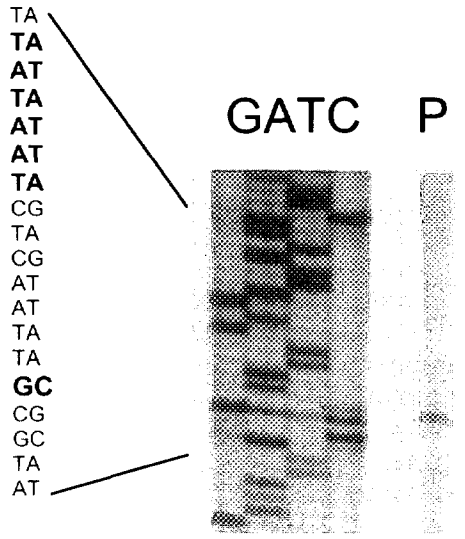

Fig. 6. Primer extension using 5 μg of RNA isolated from *L. lactis* and primer NS3-12 (see Fig. 2). The sequence ladder was obtained using the same primer and plasmid pNS3d as a template. The −10 hexanucleotide of the promoter and the transcription start site of *gadR* are indicated in bold in the sequence shown on the left.

gadCB *confers acid resistance*

The ability of *L. lactis* MG1363 to survive acid stress was tested in the presence and in the absence of *gadCB* expression. When MG1363 was grown in 1/2M17 to an OD_{600} of 0.5 (pH 6.5) and acid challenged with lactic acid at pH 3.5 in MS15 medium for 2 h, the viability of the cells decreased dramatically (10^6-fold, see Table 1). Viability was almost unchanged upon incubation for 2 h in MS15 at pH 6.5. Lactic acid was much more deleterious than hydrochloric acid in this respect. The addition of 1 mM glutamate during challenge did not affect the viability at pH 3.5. When *gadCB* expression was induced during growth with 0.3 M NaCl, however, lactic acid challenge in the presence of 0.3 M NaCl and 1 mM glutamate reduced viability only 200-fold. Under the latter conditions, omission of glutamate reduced viability 10^6-fold to the same level as seen in cells in which *gadCB* was not expressed. As the level of *gadCB* expression is only slightly affected by the presence of glutamate (see Fig. 5), these results indicate that glutamate is essential for conferring acid resistance.

To confirm the direct involvement of *gadCB* in acid resistance, the *gadR* mutant, *L. lactis* MGNS3i3 (which does not express *gadCB*, see Fig. 4), was subjected to acid challenge. Survival of this strain in the presence of NaCl and glutamate was reduced to a level similar to that of the wild type in the absence of NaCl and glutamate (Table 2). An insertion mutant in *gadC* (strain MGNS3i4) and an insertion mutant in *gadB* (strain MGNS3i7) were even more acid sensitive in the presence of NaCl and glutamate. The former mutation has, most probably, a polar effect on *gadB*, which would result in the absence of both GadC and GadB. The acid resistance of MGNS3i4 and MGNS3i7 was also lower than the wild type in the absence of NaCl and glutamate. These data show that *gadCB*, which is transcribed from the chloride-dependent promoter P_{gad}, is involved in glutamate-dependent acid resistance in *L. lactis*.

Discussion

A new type of acid resistance mechanism, recently described for *S. flexneri* and *E. coli*, has been found and characterized in *L. lactis*. It consists of a putative glutamate–GABA antiporter, GadC, and the glutamate decarboxylase, GadB. In a model by Waterman and Small (1996) for *S. flexneri* GadBC, the putative membrane protein, GadC, is proposed to be involved in the antiport of glutamate and GABA, while the glutamate decarboxylase GadB converts internalized glutamate to GABA with the simultaneous consumption of a proton and production of one molecule of CO_2. According to this model, the net result of the combined action of GadBC is the removal of a proton from the cytosol, which would, thus, lead to an increase in the internal pH. In addition, extracellular glutamate is exchanged for the more alkaline GABA. An alternative function for *gadCB* may be the generation of metabolic energy. Recently, Higuchi *et al.* (1997) have shown the generation of ATP by glutamate metabolism in resting Lactobacillus cells. This was assumed to occur through a similar mechanism of coupled electrogenic

Table 1. Acid resistance of *L. lactis* MG1363 under different conditions of growth and challenge.[a] Acid resistance was measured at pH 3.5 and at pH 6.5. To set the challenge medium to pH 3.5 either lactic acid or HCl was used.

0.3 M NaCl during growth	Additions to MS15 during challenge	Per cent acid resistance at: pH 3.5 Lactic acid	pH 3.5 HCl	pH 6.5
−	−	5×10^{-4}	1.1	140
−	1 mM glutamate	4×10^{-4}	1.0	130
+	0.3 M NaCl	2×10^{-4}	1.0	60
+	0.3 M NaCl + 1 mM glutamate	0.5	16	60

a. All cultures were pregrown in 1/2M17 broth with or without NaCl to an OD_{600} of 0.5, during which the pH did not drop below 6.5.

Table 2. Acid resistance of *L. lactis gadCB* mutants.

Strain	Genotype	Per cent acid resistance[a] in MS15[b]	Per cent acid resistance[a] in MS15 + NaCl + glu[c]
MG1363	wt	5×10^{-4}	0.5
MGNS3i3	*gadR*	3×10^{-4}	1×10^{-3}
MGNS3i4	*gadC* (GadB$^-$)[d]	7×10^{-5}	9×10^{-5}
MGNS3i7	*gadB*	1×10^{-5}	8×10^{-5}

a. At pH 3.5 with lactic acid.
b. Cultures were pregrown to an OD_{600} of 0.5 in 1/2M17.
c. Cultures were pregrown to an OD_{600} of 0.5 in 1/2M17 with 0.3 M NaCl and challenged in the presence of 0.3 M NaCl and 1 mM glu.
d. Insertions in *gadC* will, most likely, have a polar effect on *gadB*.

antiport and decarboxylation reactions that generate a proton motive force. An increase in the internal pH and, thus, the formation of a proton motive force in *Lactobacillus buchneri* was shown to result from a similar mechanism, involving histidine decarboxylation and histidine/histamine antiport (Molenaar *et al.*, 1993).

Mutants of *L. lactis* carrying either a disrupted *gadC* or *gadB* are acid sensitive, which indicates that GadCB confers acid resistance to this organism. This is in agreement with the acid sensitivity of *E. coli* and *S. flexneri gadC* mutants that were identified in random transposon mutagenesis studies. Remarkably, no *gadB* mutants were reported in these studies, despite the essential role of glutamate decarboxylation in the proposed acid resistance mechanism. In the case of *E. coli*, this can be explained by the presence of two homologous glutamate decarboxylases (Smith *et al.*, 1992). An insertion in lactococcal *gadB* resulted in a severe loss of acid resistance, indicating that glutamate decarboxylation is an indispensable step in GadCB-mediated acid resistance in *L. lactis* and supporting the proposed model. These data also indicate that *L. lactis* has no second glutamate decarboxylase that is sufficiently active under the tested conditions to take over the role of GadB. We also presume that, in the lactococcal GadCB, the antiporter is essential to provide acid resistance, as interruption of the gene by Campbell integration resulted in acid sensitivity. However, as *gadC* and *gadB* are part of one operon, the integration in *gadC* might have a polar effect on the expression of *gadB* and thus confer an acid-sensitive phenotype. The GadB- and GadCB-negative mutants were more acid sensitive than the wild type, even in the absence of NaCl. This can be explained by a background level of *gadCB* expression in the wild type in 1/2M17 caused, most probably, by traces of chloride present in this medium (4 mM in 1/2M17, unpublished information). In addition, 1/2M17 contains some glutamate that could be processed by GadCB in the wild type. The observed difference in sensitivity to hydrochloric acid and lactic acid is a result of the permeability of the membrane for the protonated weak organic acid (Kashket, 1987; Cook and Russel, 1994).

Cells containing NaCl-induced GadC and GadB are still acid sensitive when glutamate is absent, indicating that glutamate is an essential component of the acid resistance mechanism in *L. lactis*. The same has been observed for GadBC-mediated acid resistance in *E. coli* and *S. flexneri* (Hersh *et al.*, 1996; Waterman and Small, 1996).

In the presence of chloride, a 3 kb mRNA was formed that hybridized with the 5′ end of *gadC*. This observation and the absence of a putative terminator downstream of *gadC* suggests that *gadC* and *gadB* form an operon and that the putative terminator downstream of *gadB* is functional. In *S. flexneri* and *E. coli*, *gadB* and *gadC* are also genetically linked, but it is not known whether they are expressed together. It is also not known how the genes are regulated in these organisms. In *L. lactis*, chloride-dependent expression of *gadCB* is activated by GadR. *gadR* expression levels are independent of the NaCl and glutamate concentration of the medium, indicating that Cl^--dependent and glutamate-dependent expression of *gadCB* is not controlled via transcriptional regulation of its activator gene *gadR*. The observed lower expression of *gadR::lacZ* in non-buffered medium compared with buffered medium would contradict the role of *gadR* as a positive regulator in Cl^--dependent *gadCB* expression. However, this result is probably an artifact caused by the instability of β-galactosidase at low pH (Tenu *et al.*, 1971), as the lactococcal cytosol acidifies in an acid environment (Cook and Russel, 1994). GadR may interact directly with P_{gad} or indirectly via other (unknown) factors. It is tempting to speculate, owing to its location close to the −35 position of P_{gad} (see Fig. 2), that the 21 bp IR plays a role in transcription initiation from P_{gad} (Sanders *et al.*, 1997). Chloride may affect *gadCB* expression by an allosteric effect on the possible regulator–DNA complex, as was shown *in vitro* for LacR (Brenowitz and Jamison, 1993). Although other possibilities cannot be dismissed, it is conceivable that *gadR* acquires affinity for P_{gad} through a conformational change under conditions of low pH and the presence of chloride ions.

Expression of lactococcal *gadCB* is higher in non-buffered media than in pH-stabilized media. This is of

significance, as *gadCB* functions in low pH resistance. A similar observation was made for *S. flexneri* glutamate-dependent acid resistance. *S. flexneri* required adaptation at mild acid conditions to acquire full resistance to subsequent lethal acid pH (Lin *et al.*, 1995). The expression of one or both *E. coli* glutamate decarboxylases is induced at acidic pH as well (Yoshida *et al.*, 1993). The best studied acid resistance mechanism is *E. coli cadBA*. This lysine-dependent system consists of a lysine–cadaverine antiporter (CadB) and a lysine decarboxylase (CadA). *cadBA* expression is induced by the presence of lysine at low pH (Meng and Bennett, 1992). *cadBA* is regulated by CadC, a membrane protein with an intracellular DNA-binding region and periplasmic pH and lysine signalling sites (Dell *et al.*, 1994). In addition, the level of CadBA is controlled by LysP and cadaverine (Neely *et al.*, 1994). *gadCB* expression in *L. lactis* is influenced by both low pH and medium components. Medium-dependent expression may be caused by varying levels of glutamate, the amino acid conferring acid resistance through GadCB. The addition of glutamate to growing cultures resulted in higher β-galactosidase levels in *L. lactis* NS3 (*gadC*::*lacZ*). Therefore, glutamate may be a component of the inducing signal, by analogy with lysine in *E. coli cadBA* induction. Whether or not glutamate is essential for Cl^--dependent *gadCB* expression is unknown, as in all Cl^- induction experiments (performed in 1/2M17), a basal amount of glutamate was present.

S. flexneri gadC is expressed in the stationary phase and is under the control of σ^S (Waterman and Small, 1996). In *L. lactis*, expression of *gadCB* is initiated in the late exponential phase, as was the case in *S. flexneri*, and highest expression was observed at the onset of the stationary phase. This pattern of gene expression was seen in both buffered and non-buffered media. However, growth phase dependency and pH dependency of *gadCB* expression could not be distinguished in the batch cultures that were studied. The decrease in β-galactosidase activity in the stationary growth phase was also observed for independent *lacZ* fusions to other genes (not shown) and could be the result of degradation of the enzyme by intracellular peptidases or, as mentioned before, of inactivation by the low intracellular pH. As *gadCB* expression was monitored with a transcriptional *gadC*::*lacZ* fusion, regulation by chloride, low pH and glutamate is most likely at the transcriptional level. The sensing and regulating mechanism that couples data on pH, glutamate and chloride concentration and, possibly, growth stage to *gadCB* expression remains to be elucidated. It is possible that glutamate levels and acidity are sensed simultaneously as the level of glutamic acid, as this level is pH dependent because of the low pK_a (4.25) of glutamate.

GadCB provides acid resistance under very specific conditions in the three organisms in which it has been studied, namely at neutral pH (*E. coli*; Hersh *et al.*, 1996), in stationary phase (*S. flexneri*; Waterman and Small, 1996) or in the presence of chloride (*L. lactis*). Why *L. lactis gadCB* is expressed in the presence of chloride is completely unclear. High NaCl levels, which present an osmotic stress, could provide cross-protection against severe acid challenges. A system with reverse properties, providing NaCl-dependent acid sensitivity, has been described for *E. coli* (Rowbury *et al.*, 1994). Remarkably, glutamate-dependent acid resistance in *S. flexneri* is enhanced in the presence of NaCl. This is not caused by the higher osmotic pressure of the medium (Waterman and Small, 1996). Although the authors conclude that the sodium ion was involved, it may well be that *gadC* expression in *S. flexneri* is also stimulated by chloride. An analogous stress protection mechanism induced by similar conditions in such diverse organisms as *L. lactis* and *S. flexneri* may point towards a common stressful natural environment. Both organisms can enter the gastrointestinal tract where they are faced with the highly acidic and chloride-rich conditions of the stomach. A high level of hydrochloric acid is produced by gastric cells to hydrolyse food peptides and as a barrier against (pathogenic) bacteria. *L. lactis* is able to pass the gastrointestinal tract (Klijn *et al.*, 1995), albeit in low numbers. The *gadCB* acid resistance system may play a significant role under these conditions. *gadCB* may also be important for the survival of lactococcal cells during cheese production as high levels of both NaCl and glutamate are present in cheese.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 3. *L. lactis* was grown at 30°C in twofold diluted M17 broth (Terzaghi and Sandine, 1975), containing 0.5% glucose and 1.9% β-glycerophosphate (1/2M17); 1/2M17 plates contained 1.5% agar. Modified M17 (mM17) contained no β-glycerophosphate and no soytone. β-Glycerophosphate was added to a final concentration of 2%, where indicated. Erythromycin (Em) and chloramphenicol were used at final concentrations of 5 mg ml^{-1}, while spectinomycin was used at a final concentration of 100 mg ml^{-1}. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) was used at a final concentration of 0.008%. *E. coli* was grown in TY broth at 37°C with vigorous agitation or on TY medium supplemented with 1.5% agar. Em was used at 100 mg ml^{-1} for *E. coli*.

General DNA techniques

DNA work was performed essentially as described by Sambrook *et al.* (1989). DNA was introduced by electrotransformation in *E. coli* (Zabarovsky and Winberg, 1990) and *L. lactis* (Holo and Nes, 1989). The Pharmacia T7 sequencing kit was used for DNA sequencing. Oligonucleotides were synthesized with an Applied Biosystems 392A DNA synthesizer. DNA sequences were analysed using the PC/Gene

Table 3. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
L. lactis		
MG1363	Plasmid-free derivative of NCDO712	Gasson (1983)
LL108	Cm^r, *repA*$^+$ derivative of MG1363	Leenhouts *et al.* (1997)
LL302	MG1363 derivative carrying a single copy of *repA* in *pepX*	Leenhouts *et al.* (1997)
NS3	Em^r, MG1363 (*gadC*::*lacZ*)	Sanders *et al.* (1997)
MGNS3i3	Sp^r, derivative of MG1363 with pNS3i3 inserted in *gadR*	This study
MGNS3i4	Em^r, derivative of MG1363 with pNS3i4 inserted in *gadC*	This study
MGNS3i5	Em^r, derivative of MG1363 with pNS3i5 inserted in between *gadC* and *gadB*	This study
MGNS3i7	Em^r, derivative of MG1363 with pNS3i7 inserted in *gadB*	This study
MGNS31	Em^r, MG1363 with *lacZ* inserted between *gadR* and P_{gad}	This study
MGNS32	Em^r, MG1363 (*gadR*::*lacZ*)	This study
E. coli		
EC1000	Km^r, MC1000 derivative carrying a single copy of the pWV01 *repA* gene in *glgB*	Leenhouts *et al.* (1996)
EC101	Km^r, JM101 derivative carrying a single copy of the pWV01 *repA* gene in *glgB*	Law *et al.* (1995)
Plasmids		
pORI13	Promoterless *E. coli lacZ*, Em^r, Ori$^+$, RepA$^-$ derivative of pWV01	Sanders *et al.* (1997)
pORI19	Em^r, α-*lacZ*, Ori$^+$ of pWV01, RepA	Law *et al.* (1995)
pORI19S	Derivative of pORI19, Sp*r*	H. Smith, unpublished
pVE6007	Cm^r, pWV01 derivative encoding a temperature sensitive RepA protein	Maguin *et al.* (1992)
pNS3	Em^r, *gadC*::*lacZ*, pORI13 carrying a 10 kb *Sau*3A chromosomal DNA fragment	Sanders *et al.* (1997)
pNS31	Em^r, pORI13 carrying a 0.77 kb *Xba*I chromosomal DNA fragment encoding the 3′ end of *gadR*	This study
pNS32	Em^r, *gadR*::*lacZ*, pORI13 carrying a 0.74 kb chromosomal DNA fragment	This study
pNS3d	Em^r, *gadC*::*lacZ*, pORI13 carrying a 2.5 kb *Pst*I–*Sau*3A chromosomal DNA fragment	Sanders *et al.* (1997)
pNS3i3	Sp^r, pORI19S carrying a 240 bp *Xba*I–*Hin*dIII fragment internal to *gadR*	This study
pNS3i4	Em^r, pORI19 carrying a 550 bp *Sau*3A–*Xba*I fragment internal to *gadC*	This study
pNS3i5	Em^r, pORI19 carrying a 0.9 kb *Xba*I–*Sau*3A chromosomal DNA fragment encoding the 3′ end of *gadC* and the 5′ end of *gadB*	This study
pNS3i6	Em^r, pORI19 carrying a 2.4 kb *Xba*I–*Eco*RI chromosomal DNA fragment encoding the 3′ end of *gadC* and *gadB*	This study
pNS3i7	Em^r, pORI19 carrying a 500 bp internal *Ase*I fragment of *gadB*	This study

sequence analysis program (IntelliGenetics). Protein homology searches against the GenBank were carried out using the BLAST program (Altschul *et al.*, 1990). Protein sequence alignments were carried out with the PALIGN program of PC/Gene using the structure genetic matrix. Transmembrane segments were predicted using the method of Klein *et al.* (1985).

β-Galactosidase assays

Cell extracts were prepared by vigorous shaking of cells in the presence of glass beads (van de Guchte *et al.*, 1991). β-Galactosidase activity was determined as described by Miller (1972). Protein concentrations were determined by the method of Bradford (1976) with BSA as the standard.

RNA analysis

RNA was isolated from exponentially growing *L. lactis* cultures (optical density at 600 nm of 0.5) as described previously (van Asseldonk *et al.*, 1993). The synthetic oligonucleotide

NS3-12 (5′-GGTTTATAACAGTTCATCAAGTACACTTTTT-AATCAGTC-3′) was used for primer extension as described previously (Sanders *et al.*, 1997). The primer extension product was analysed on a sequencing gel next to the products of a sequence reaction using the same primer. Northern hybridizations were carried out at 40°C in a buffer containing 50% formamide, 7% SDS, 2% blocking reagent (Boehringer Mannheim), 5× SSC, 50 mM sodium phosphate, pH 7, and 0.1% N-lauryl sarcosine. A 450 bp *Xba*I–*Sau*3A fragment encoding the 5′ end of *gadC* was used as a probe by labelling with [α^{32}P]-dCTP using the Boehringer Mannheim random primed DNA labelling kit.

Cloning of gadCB *of* L. lactis

The region downstream of the *lacZ* fusion site in *L. lactis* NS3 was obtained by inverse polymerase chain reaction (PCR) amplification of a 0.55 kb *Sau*3A–*Xba*I fragment from *L. lactis* MG1363 chromosomal DNA (see Fig. 1). The PCR product was cloned in pORI19 using *E. coli* EC101 as a host, resulting in pNS3i4. In a subsequent inverse PCR, the adjoining 0.9 kb *Xba*I–*Sau*3A chromosomal DNA fragment was amplified and cloned in pORI19 using *L. lactis* LL108 as the cloning host. This construct was named pNS3i5 and used for single cross-over integration, resulting in strain MGNS3i5. Chromosomal DNA of MGNS3i5 was digested with *Eco*RI to clone sequences downstream of the insertion site of pNS3i5 by plasmid rescue. The chromosomal *Eco*RI fragments were circularized by self-ligation, and the ligation mixture was used to transform *L. lactis* LL108. Transformants contained a 4.8 kb plasmid (pNS3i6), which is pORI19 containing a 2.4 kb *Xba*I–*Eco*RI fragment from the chromosome (Fig. 1). The sequence of the 2.4 kb *Pst*I–*Sau*3A DNA fragment located upstream of the *lacZ* insertion site in *L. lactis* NS3 was determined from pNS3d. The nucleotide sequence described in this paper has been assigned GenBank accession number AF005098.

Construction of insertion mutants

An internal *Xba*I–*Hin*dIII fragment of *gadR* was cloned in the integration vector pORI19S using the RepA$^+$ *E. coli* helper strain EC101. The resulting plasmid, pNS3i3, was used to disrupt *gadR* in *L. lactis* MG1363 as described previously (Law *et al.*, 1995). The proper chromosomal location of the integrated plasmid was confirmed by Southern hybridization, and the strain was named MGNS3i3. Plasmid pNS3i7 was constructed by cloning a 500 bp *Asn*I fragment (internal to *gadB*) in pORI19 using *E. coli* EC101 as a host. Single cross-over integration of pNS3i4 and pNS3i7 in the *L. lactis* MG1363 chromosome resulted in strains MGNS3i4 (*gadC*) and MGNS3i7 (*gadB*) respectively.

Acid resistance test

Cells of an exponentially growing culture of *L. lactis* in 1/2M17 with or without 0.3 M NaCl were harvested, washed with water and resuspended in an equal volume of MS15 (Cocaign-Bousquet *et al.*, 1995) without glucose and glutamate but containing the same amount of NaCl as the culture. Glutamate was added to a final concentration of 1 mM where indicated. The pH of MS15 was adjusted to 3.5 with either lactic acid or hydrochloric acid before cell resuspension. After incubation for 2 h at 30°C, the number of viable cells was determined by plating onto 1/2M17 plates. The percentage of acid-resistant cells was calculated from the number of colony-forming units (cfu) after acid treatment divided by the number of cfu at the moment of harvest.

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