Multi-stress resistance in *Lactococcus lactis* is actually escape from purine-induced stress sensitivity

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Multi-stress resistance is a widely documented and fascinating phenotype of lactococci where single mutations, preferentially in genes involved in nucleotide metabolism and phosphate uptake, result in elevated tolerance to multiple stresses simultaneously. In this report, we have analysed the metabolic basis behind this multi-stress-resistance phenotype in *Lactococcus lactis* subsp. *cremoris* MG1363 using acid stress as a model of multi-stress resistance. Surprisingly, we found that *L. lactis* MG1363 is fully resistant to pH 3.0 in the chemically defined SA medium, contrary to its sensitivity in the rich and complex M17 medium. When salvage of purines and subsequent conversion to GTP was permitted in various genetic backgrounds of *L. lactis* MG1363, the cells became sensitive to acid stress, indicating that an excess of guanine nucleotides induces stress sensitivity. The addition of phosphate to the acid-stress medium increased the stress sensitivity of *L. lactis* MG1363. It is also shown that high intracellular guanine nucleotide pools confer increased sensitivity to high temperatures, thus showing that it is indeed a multi-stress phenotype. Our analysis suggests that an increased level of guanine nucleotides is formed as a result of an improved conversion of guanosine in the salvage pathway. Based upon our findings, we suggest that *L. lactis* MG1363 is naturally multi-stress resistant in habitats devoid of any purine source. However, any exogenous purine that results in increased guanine nucleotide pools renders the bacterium sensitive to environmental stresses.

INTRODUCTION

Multi-stress resistance is a widely documented phenotype of *Lactococcus lactis* (Budin-Verneuil *et al.*, 2005, 2007; Duwat *et al.*, 1995a, 1999; Hartke *et al.*, 1994, 1996; Rallu *et al.*, 2000), which has been investigated thoroughly using transposon mutants selected on M17 by resistance to different stresses (Duwat *et al.*, 1999, 1997; Rallu *et al.*, 2000). One type of combination was heat-stress treatment of a stress-sensitized (*recA*) mutant (Duwat *et al.*, 1999), while another employed simultaneous heat and acid stress (Rallu *et al.*, 2000). The multi-stress-resistant mutants have been divided into three major classes: (i) those with mutations in genes that were connected to purine nucleotide metabolism (*guaA*, *hpt*, *deoB*, *tktA* and *relA* genes), (ii) those with mutations implicated in phosphate transport (*pstB* and *pstS*) and (iii) those with mRNA stability (*pnpA*) (Duwat *et al.*, 1999; Rallu *et al.*, 2000).

In relation to our ongoing characterization of the purine nucleotide metabolism in *L. lactis* MG1363 (Beyer *et al.*, 2003; Jendresen *et al.*, 2011, 2012, 2014; Kilstrup *et al.*, 2005; Kilstrup & Martinussen, 1998; Martinussen *et al.*, 2003; Nilsson & Kilstrup, 1998), we are interested in understanding the role of purine metabolism in multi-stress resistance. Fig. 1 shows the parts of the purine metabolism in *L. lactis* that are relevant for the study. IMP is formed in the *de novo* pathway from 5-phosphoribosyl 1-pyrophosphate (PRPP). From IMP, the synthesis branches into an ATP and a GTP branch. All mutants leading to multi-stress resistance in M17 broth are related to the GTP branch or to the interplay between phosphate and nucleotide metabolism. The rich broth M17 medium (Terzaghi & Sandine, 1975) is the preferred medium for growing *L. lactis*. It has acceptable buffering capacity around pH 7, due to the presence of glycophosphate, and an abundance of peptides, amino
acids, vitamins, nucleotides, nucleosides and bases from yeast and beef extract that satisfy the demands of most multiple auxotrophic lactic acid bacteria.

We have repeatedly observed that multi-stress-resistance phenotypes are not very reproducible using M17 broth. Therefore, in this study we chose to use the chemically defined SA medium (Jensen & Hammer, 1993) as the basis for the analysis of the multi-stress phenotypes. After switching to defined medium, we surprisingly found that the WT L. lactis MG1363 was inherently stress resistant and that sensitivity could be induced by exogenous purine addition. In the light of these observations, the complex phenotypes in M17 of the stress-resistant mutants described above were not easily understood, but they could be reproduced in a chemically defined medium where the uptake and metabolism of guanosine (GR) were limited. We also found that L. lactis MG1363 is naturally stress resistant during growth in milk. In summary, we suggest that L. lactis strains have acquired multi-stress resistance in their natural habitats, which are characterized by a lack of purines, by evolving a genetic setup that warrants reduced intracellular guanine nucleotide pool sizes.

**METHODS**

**Strains and growth conditions.** The strains used in this paper are listed in Table 1. Cultures were grown in M17 (Terzaghi & Sandine, 1975) supplemented with 1% glucose (GM17), in SA medium (Jensen & Hammer, 1993) supplemented with 1% glucose and modified by exchanging sodium acetate with lipoic acid to 2 μg ml⁻¹ (GSAL), or skimmed milk supplemented with 1% glucose and the same amino acids mix as the GSAL medium. When required, the following combinations of nucleosides/nucleobases were added: GR at 40 μg ml⁻¹; adenosine (AR) at 40 μg ml⁻¹; GR at 10 μg ml⁻¹ + AR at 400 μg ml⁻¹; GR at 40 μg ml⁻¹ + AR at 400 μg ml⁻¹; hypoxanthine (Hx) at 15 μg ml⁻¹. Cells were incubated at 30 °C unless indicated otherwise.

**Construction of chromosomal deletions of the guaA and guaB genes.** Strains containing chromosomal gene deletions were constructed by double homologous recombination with DNA produced by overlap extension PCR. Separate upstream and downstream fragments were amplified in the first PCR round using UF + UR primers and DF + DR primers, respectively (Table 2) and chromosomal DNA from L. lactis MG1363 as a template. The UR primers for amplification of the upstream fragment of the guaA and guaB genes contained sequences that could pair with the downstream fragments, permitting the amplification of combined fragments by overlap extension. The combined products for guaA and guaB contained ORFs consisting of the first five codons fused in-frame to the last five. After cloning of the fragments into plasmid pGhost8 using restriction enzymes Xhol and BamHI, deletions were generated in strain MG1363 (MCD1001 and MSD1002) by double crossing over as described elsewhere (Biswas et al., 1993). After selection for loss of the plasmid at 37 °C on GM17 plates containing 1% NaCl, the correct size and nucleotide sequence of the deleted region of the resulting strains were verified by sequence analysis of the products from PCR amplifications performed directly on bacterial colonies using plasmid-specific primers.

**Analysis of acid-stress resistance.** Cells that had grown exponentially for more than eight generations in either GSAL or milk were inoculated into freshly prepared medium at an OD₄₅₀ between 0.01 and 0.04. Please note that for growth in milk the optical density could not be measured; therefore, pH served as a measurement of cell growth. The growth was followed by OD₄₅₀ measurements until OD 0.1, or pH 6 when the acid stress was started. A total of 100 μl of the culture was transferred to 0.9 ml of acid-stress medium at pH 2.8 or 2.5. The acid-stress medium was identical in composition to the GSAL growth medium (when indicated 20 mM Pi was added to the acid-stress medium). The number of c.f.u. was determined after 0, 10, 20, 30 and 60 min. Cells were diluted in physiological salt water (0.9% NaCl) and 100 μl of the appropriate dilutions was spread on agar plates. The plates were incubated overnight at 30 °C. In experiments with decoyinine the same procedure was followed as for the acid stress, except that the culture was exposed to 5 μg decoyinine ml⁻¹ for 30 min in GM17 before and during acid stress.

**RESULTS**

**MG1363 is stress resistant in GSAL medium but an exogenous purine source induces sensitivity**

M17 broth has an extremely complex and largely unknown composition due to inclusion of beef and yeast extracts, and batch-to-batch variations are significant. This results in day-to-day variations of phenotypes in relation to multi-stress resistance, hampering the use of M17 for in-depth studies (data not shown). These difficulties prompted us to analyse the phenotypes in the chemically defined GSAL.
medium in which we previously had indications of elevated levels of resistance compared to growth on glucose-supplemented M17 (Kilstrup & Hammer, 2000). We observed that GSAL plates supported growth of MG1363 at much higher temperatures than GM17 plates, but that the addition of 1 % salt enabled MG1363 to grow at high temperatures on GM17 plates (Kilstrup & Hammer, 2000). To elucidate the background behind the multi-stress resistance in GSAL we chose to focus on acid stress, as it previously has been shown that stress-resistant mutants isolated after challenge with one type of stress showed resistance to other stress forms (Duwat et al., 1999; Rallu et al., 2000).

When the acid resistance of the WT strain \textit{L. lactis} MG1363 was analysed after growth in GSAL medium we found that the strain, in contrast to GM17-grown cultures, was fully resistant to pH 3.0 for at least 60 min (data not shown). At pH 2.8 the strain had a slightly decreased viability after 60 min, but survived fully until 30 min (Fig. 2, ●). This high resistance was repeatedly found in more than 10 independent experiments with cultures in exponential growth phase, after the cells had been grown exponentially for more than eight generations to avoid entry into the stationary growth phase, which is known to increase the general resistance of \textit{Lactococci} (Hartke et al., 1994).

Since stress resistance has been linked to genes involved in purine nucleotide metabolism (Duwat et al., 1999), and since one of the principal differences between GM17 and GSAL is the absence of purine nucleotide precursors in the latter, it prompted us to test whether addition of purines could induce stress sensitivity. Indeed, addition of the purine base Hx to GSAL medium rendered the bacteria acid sensitive (Fig. 2, ●), showing that exogenous purine supplements induce a stress-sensitive phenotype. Other purine precursors like AR and GR gave similar results (data not shown). Additional phosphate during the acid challenge accentuated the Hx-induced sensitivity (Fig. 2, h).

\begin{table}
\centering
\caption{Strains used in this study}
All strains are derivatives of \textit{L. lactis} subsp. \textit{cremoris} MG1363.
\begin{tabular}{|l|l|l|}
\hline
\textbf{Strain} & \textbf{Relevant genotype} & \textbf{Reference} \\
\hline
MG1363 & WT & Gasson (1983) \\
MG1363/pGhost8 & \textit{tet} 28 °C & Biswas \textit{et al.} (1993) \\
MK138 & \textit{purD:pGhost9::IS1} 37 °C & Kilstrup & Martinussen (1998) \\
MK185 & \textit{purD::IS1} & This work* \\
MSD1001 & \textit{guaA} & This work \\
MSD1002 & \textit{guaB} & This work \\
MB140 & \textit{upp12, tdk1, pup1} & Martinussen & Hammer (1995) \\
\hline
\end{tabular}
\end{table}

*MK185 was derived from MK138 by curing the pGhost9::IS1 plasmid by homologous recombination as described previously (Kilstrup \textit{et al.}, 1998).

\begin{table}
\centering
\caption{Primers for deletion by overlap extension for \textit{guaA} and \textit{guaB} genes}
\begin{tabular}{|l|l|l|}
\hline
\textbf{Primer} & \textbf{Nucleotide sequence (5′→3′)} & \textbf{Description} \\
\hline
MK493 & TAACTTTTATATGACAGATACAAACAGTTGGACATCGAAAATTATTCCCTCC & \textit{guaB} deletion, UR \\
MK494 & CCCTGAGACAGCATCTGTTCTAGGGAGTGTTATCGATGAGAG & \textit{guaB} deletion, UF \\
MK495 & TATCTGTACAAATTGTTTA & \textit{guaB} deletion, DF \\
MK496 & CGGGAATCCGCTTTAAGATTCCGAAAAC & \textit{guaB} deletion, DR \\
MK497 & CATGGTTTTGTGTCAGGACATAGTGCAGGACAGGAC & \textit{guaA} deletion, UR \\
MK498 & CCGTGAGACGTATCCAGATGGACCTCGTTG & \textit{guaA} deletion, UF \\
MK499 & GAGTGGCAATGAATACAAAAC & \textit{guaA} deletion, DF \\
MK500 & CGCGATCAGAATAGTTAGTTAGTGGAGAAG & \textit{guaA} deletion, DR \\
\hline
\end{tabular}
\end{table}

DF, Downstream forward; DR, downstream reverse; UF, upstream forward; UR, upstream reverse.
neither Hx nor adenine (A) could support growth. Both Hx and A are converted to IMP (Fig. 1), but the subsequent conversion to GMP is blocked in either of the mutants. In accordance with our expectations the guaB mutant was able to use xanthine (X) as a purine source, since xanthine phosphorybosyltransferase encoded by xpt converts X into XMP that subsequently is converted into GMP. The guaA mutant could not utilize X due to the block in the conversion of XMP to GMP (Fig. 1).

Both mutants were acid sensitive after growth in GSAL medium when GR was added to the medium (data not shown), and since an exogenous source of purines was needed to support growth (Table 3), we could not directly analyse the acid-stress-resistant phenotype in GSAL medium. To analyse the guaA and guaB mutant phenotypes in GSAL medium as reported for guaB::ISS1 mutants in GM17 (Rallu et al., 2000), we first identified the specific supplement that was needed to mimic the acid-stress-related properties of GM17 medium.

### Mutants impaired in GMP formation become resistant in the presence of GR when its flux towards GMP is restricted

A limited but steady supply of GMP in a guaA or guaB mutant could theoretically be obtained in different ways by inhibiting the utilization of GR or G from the medium. We have previously shown that uptake of nucleosides is facilitated by a common uptake system, and that the individual nucleosides act as competitive inhibitors for each other (Martinussen et al., 2003). As shown in Fig. 1, AR, IR and GR are all substrates for the purine nucleoside phosphorylase (Pup) enzyme (Martinussen & Hammer, 1995), and it is very likely that the two different purine nucleosides act as competitive inhibitors for each other for this enzyme. To create a steady GMP starvation we chose to add high concentrations of AR as a competitive inhibitor to reduce GR utilization when the exogenous GR concentration was low. The growth of both guaA and guaB mutants was significantly reduced on GM17 (data not shown). The optimal design of the GMP-limiting medium was, therefore, reached when a significant reduction in the growth rates of guaA and guaB mutants was found, which permitted analysis of their acid-resistance phenotype. After analysis of a series of AR and GR concentrations (data not shown), we arrived at a combination of AR at 400 μg ml<sup>-1</sup> and GR at 10 μg ml<sup>-1</sup> (GSAL-Glim) where the above requirements were met. When GR concentration was raised from 10 to 40 μg ml<sup>-1</sup> still in the presence of 400 μg AR ml<sup>-1</sup>, the normal growth rate was restored.

When tested for acid sensitivity in GSAL-Glim, the WT L. lactis MG1363 was highly sensitive to severe acid stress at pH 2.5 (Fig. 3, ●; Table 4, 10 column), as also observed for GM17 medium (data not shown). In accordance with GM17-grown cultures, the guaB mutant was resistant to severe acid stress at pH 2.5 after growth in GSAL-Glim medium (Fig. 3, ●; Table 4, 10 column), but became sensitive upon addition of additional GR (Table 4, 11 column). The guaA deletion mutant behaved similarly to the guaB mutant (Table 4, 10 and 11 columns), showing that XMP limitation was not important. The limitation of GMP supply was most likely the basis for the resistance,

### Table 3. Growth of guaA and guaB mutants on GSAL plates with various supplements

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Inhibition</th>
<th>Addition to GSAL medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis MG1363</td>
<td>WT</td>
<td>IMP→XMP</td>
<td>+</td>
</tr>
<tr>
<td>MSD1002</td>
<td>ΔguaB</td>
<td>IMP→XMP</td>
<td>-</td>
</tr>
<tr>
<td>MSD1001</td>
<td>ΔguaA</td>
<td>XMP→GMP</td>
<td>-</td>
</tr>
</tbody>
</table>

+, Growth; −, no growth.
but it was not possible to rule out that GR or guanine was the active molecule in the induction of stress sensitivity.

When the challenge was executed at a more moderate acid-stress level at pH 2.8, we did not see as good a correlation with the GM17 phenotypes. The addition of GR to the guaA or guaB mutants did not render the strains fully sensitive at pH 2.8 (Table 4, 7 column). We found, however, that the presence of additional phosphate during the acid stress at pH 2.8 resulted in phenotypes closer to those obtained with extra GR to 40 μg ml⁻¹ (open symbols). At the middle of the exponential growth phase, cells were removed and diluted into prewarmed medium of identical composition buffered at pH 2.5. At 0, 10, 20 and 30 min, samples were plated on GM17 plates. Survival was measured as the number of c.f.u. relative to c.f.u. at 0 min. Error bars for each data point show the positive SD within triplicate experiments with separate cultures.

**Search for the stress-sensitivity-inducing metabolite: evidence against G and GR**

In the analysis above we concluded that the sensitivity-inducing metabolite is G, GR or a G nucleotide (GMP, GDP or GTP), but since any of these metabolites may be low in GSAL medium and elevated in the presence of GR, the nature of the inducing substance could not be stated with certainty. Therefore, we tried to gain evidence for or against GR and G metabolites as the stress-sensitivity-inducing substance. The Pup enzyme cleaves GR, IR and AR into ribose-1-phosphate and their bases G, Hx and A, respectively. The previously selected pup mutant MB140 (Martinussen & Hammer, 1995) was analysed for its acid resistance at pH 2.8. As expected, the pup mutant was resistant after growth in GSAL medium and the addition of Hx whose conversion to GMP is independent of Pup induced the sensitive phenotype (Table 4, columns labelled 1 to 4). Addition of GR and AR that require the Pup enzyme for conversion to GMP did not induce sensitivity (Table 4, columns labelled 5 to 8). The failure of GR to induce sensitivity shows that GR is not the stress-sensitivity-inducing metabolite. Moreover, the stress sensitivity induced by Hx addition in various strains also disqualifies G as the stress-sensitivity-inducing metabolite as no increased G pool size would be expected under these conditions. Altogether, these results indicate that the stress-sensitivity-inducing metabolite is a G nucleotide, which is consistent with previous results showing that the addition of Hx to a WT strain significantly increases the GTP pool size (Martinussen et al., 2003).

**The G nucleotide-dependent stress sensitivity is a multi-stress sensitivity**

To rule out the possibility that the G-nucleotide-dependent stress sensitivity is limited to acid stress, we wanted to show that the same dependency on G nucleotides was also found in heat-stress sensitivity. Therefore, the WT strain L. lactis MG1363 and the derivatives MB140 (pup, upp, tdk) and MCD1001 (guaA) were propagated on defined media in the absence and presence of purine nucleosides. Due to the purine phosphorylase deficiency of MB140, purine nucleosides cannot be metabolized by this strain (Martinussen & Hammer, 1995). The plates were incubated at both 30 °C and 38 °C, and the growth was scored by colony size after 20 and 40 h incubation, respectively. The results are shown in Table 5. In general, the colonies on plates incubated at 30 °C were larger even though they had been incubated for a much shorter duration of time, so it is not possible to directly compare the colony sizes. Growth of all strains was inhibited at 38 °C on the rich GM17 medium. It is evident that the growth of the WT MG1363 was better in the absence of purines at 38 °C, whereas the opposite was observed at the optimal temperature of 30 °C due to the well-known stimulatory effect of purines (Jendresen et al., 2012). In MB140, which has lost the ability to metabolize purine nucleosides due to purine phosphorylase deficiency, the increased heat-sensitivity by purine nucleoside addition was absent. To verify that it is indeed the G nucleotides that confer the heat sensitivity, the GR-requiring strain MSD1001 (guaA) was challenged by growth at 38 °C under conditions where the G-nucleotide pools were low due to a
Table 4. Survival of bacteria after acid stress following growth in GSAL medium with various supplements

<table>
<thead>
<tr>
<th>Supplement/strain</th>
<th>Relative survival*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10 11</td>
</tr>
<tr>
<td></td>
<td>pH 2.8  pH 2.5</td>
</tr>
<tr>
<td></td>
<td>pH 2.8  pH 2.5</td>
</tr>
<tr>
<td>Supplement†</td>
<td></td>
</tr>
<tr>
<td>AR400</td>
<td>– – – – + + + + + – + +</td>
</tr>
<tr>
<td>GR10</td>
<td>– – – – + + – – – – – –</td>
</tr>
<tr>
<td>GR40</td>
<td>– – – – – – + + + – –</td>
</tr>
<tr>
<td>HX15</td>
<td>– – + + – – – – – – –</td>
</tr>
<tr>
<td>Pi</td>
<td>– + – – + + – – – + –</td>
</tr>
<tr>
<td>MG1363 (wt)</td>
<td>–0.1 (0) –0.3 (–0.2) –3.2 (–0.7) –4.0 (–3.9) –1.9 (–1.5) –3.5 (–3.1) –2.5 (–2.0) –3.6 (–3.4) –5.0 (–4.7) –5.3 (–5.0) –5.7 (5.6)</td>
</tr>
<tr>
<td>MCD1001 (guaA)</td>
<td>NG NG NG NG 0 (0.1) 0 (0.1) –0.1 (0) –2.0 (–1.7) NG –0.7 (–0.6) –4.2 (–3.9)</td>
</tr>
<tr>
<td>MCD1002 (guaB)</td>
<td>NG NG NG NG 0 (0.1) 0 (0.1) –0.4 (–0.2) –1.2 (–0.9) NG –0.2 (–0.1) –5.3 (–5.2)</td>
</tr>
<tr>
<td>MB140 (pup)</td>
<td>–0.1 (0) 0 (0.1) –1.9 (–1.7) –2.9 (–2.4) 0 (0.1) –0.2 (–0.1) 0 (0) –0.3 (–0.2) ND –4.7 (–4.5) –5.0 (–4.8)</td>
</tr>
</tbody>
</table>

ND, Not determined; NG, no growth.
*Relative survival in fresh medium identical to the growth medium was calculated as the number of c.f.u. resulting after 30 min of exposure to acidic test medium at pH 2.8 (1 to 8) or pH 2.5 (9 to 11) divided by the number of c.f.u. at 0 min. Values are mean values of at least triplicate experiments with separate cultures. After the mean value, the log_{10}-transform of the mean value plus one SD is shown in parentheses.
†Supplements are indicated as: –, absent; +, present. Pi phosphate (40 μg ml⁻¹) was present during acid stress. Numbers in subscript indicate concentrations in μg ml⁻¹.
steady GMP starvation created by inhibiting the uptake of GR (10 μg ml⁻¹) with an excess of AR (400 μg ml⁻¹). Table 5 shows that G starvation results in significantly better growth at 38 °C of MSD1001, while the opposite is observed at 30 °C. These findings are in accordance with the acid-resistance pattern; thus, showing that the G-nucleotide-dependent stress resistance is a multi-stress resistance.

**L. lactis MG1363 is stress resistant in milk**

To test the biological relevance of our observations obtained in the defined growth medium, we wanted to address the stress phenotype in milk. *L. lactis* MG1363 was propagated in milk, in the presence of amino acids and glucose, and samples were analysed for resistance to acid at pH 2.8 (results not shown). We found that MG1363 is inherently stress resistant during growth in milk. Interestingly, however, we found that Hx did not induce stress sensitivity at pH 2.8 during growth in milk. To investigate whether this was due to an inability of the cells to utilize the supplied Hx, we inoculated the purine auxotrophic strain MK185 (MG1363 purD) into milk supplemented with amino acids and glucose. MK185 was not able to grow and acidify the medium in the absence of purines, while addition of Hx resulted in growth and acidification (data not shown). The conditions preventing induction of stress sensitivity by purine bases in milk, even though they are utilized as purine sources, are highly interesting, but outside the scope of this work.

**DISCUSSION**

We have shown that *L. lactis* MG1363 is inherently multi-stress resistant, and that high levels of GMP, GDP or GTP induce multi-stress sensitivity. In several studies, it was found that stress resistance acquired by mutations after challenge with one type of stress, confers resistance towards other stress forms; thus, supporting the use of acid stress as a representative for multi-stress resistance (Duwat et al., 1995a, b, 1997, 2000; Rallu et al., 1996, 2000). We ruled out the possibilities that the inducing compounds were either GR or G, but we have not yet discussed the possibility that the sensitizing substance is either ppGpp or pppGpp, which are normally produced during the stringent response. This has been suggested elsewhere (Rallu et al., 2000). However, (p)ppGpp levels have previously been measured in MG1363 during growth in GSAL medium, where the bacterium shows full stress resistance. Under these conditions, no (p)ppGpp was detected (Jendresen et al., 2011). *Bacillus subtilis* GTP homeostasis has been shown to be controlled by (p)ppGpp (Kriel et al., 2012), and in a recent study, GTP was identified as a key player in viability and stress resistance (Bittner et al., 2014). Together, these findings show that multi-stress resistance is not directly dependent upon (p)ppGpp, so we can effectively rule out (p)ppGpp as the directly sensitizing compound.

The acid-stress-sensitive phenotype and the acid-stress-resistant phenotype of mutants in GM17 medium could be mimicked in a chemically defined medium (GSAL-Glim) where GR utilization was inhibited by high concentrations of AR. Inorganic phosphate was shown to accentuate Hx-induced sensitivity. Inorganic phosphate is not used in the metabolism of Hx leading to GMP, so the importance of phosphate for elevating the levels of the inducer is not direct. We found that the Pup enzyme, which utilizes phosphate as a substrate, is not required for the phosphate effect. However, as phosphate is used in the normal mRNA turnover through phosphorolytic cleavage of mRNA into GDP, ADP, CDP and UDP, this could suggest that high phosphate availability would elevate the intracellular levels of GDP and GTP during mRNA recycling.

The function of G nucleotides as important metabolic signals makes perfect biological sense since GTP is used as an energy donor for the most basic processes like protein synthesis and cell division. In contrast, the induction of stress sensitivity by an excess of G nucleotides appears less understandable. In that regard it is interesting that the stringent response in Gram-positive bacteria modulates transcription initiation by lowering the G-nucleotide pool. High levels of (p)ppGpp inhibit the two enzymes GuaB (IMP dehydrogenase) and Gmk (guanylate synthase) and effectively decrease the rate of GMP and GDP synthesis, resulting in low levels of GTP (Kriel et al., 2012; Pao &

<table>
<thead>
<tr>
<th>Strain</th>
<th>GSAL</th>
<th>GSAL + GR100*</th>
<th>GSAL + GR10 + AR400*</th>
<th>GM17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 °C</td>
<td>38 °C</td>
<td>30 °C</td>
<td>38 °C</td>
</tr>
<tr>
<td>MG1363 (wt)</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>MB140 (pup)</td>
<td>2.5</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>MCD1001 (guaA)</td>
<td>ng</td>
<td>ng</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

*Subscript numbers indicate concentrations in μg ml⁻¹.*

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**Table 5. Growth of bacteria during heat stress in the defined GSA medium with various supplements**

Growth determined as relative colony size on agar plates after 20 and 40 h incubation at 30 and 38 °C. The largest colonies from the two temperature series were set to 5. NG, No growth.
Dyes, 1981). Slow growth and lack of precursors for protein synthesis, thus, induce G-nucleotide depletion, which subsequently lowers the transcription of stringently regulated genes (e.g., rRNA and tRNA) due to a high $K_m$ for the initiating (G) nucleotide (Krásný et al., 2008; Krásný & Gourse, 2004).

In this work, we have shown that the multi-stress sensitivity is not directly mediated by (p)ppGpp. However, that does not exclude the possibility that the multi-stress sensitivity is controlled by the same mechanisms that are utilized by the stringent response. The G-nucleotide-mediated events could easily be the same in the stringent response as in the multi-stress-sensitivity phenotype described in this work. Since the stringent response has been found to induce the synthesis of a large number of stress genes in L. lactis (Dressaire et al., 2008), it is fair to believe that some of these stress proteins are responsible for the multi-stress resistance. Therefore, the most likely hypothesis would be that reduction of the GTP pool in L. lactis increases transcription of several stress genes, thereby conferring increased robustness towards multiple stress conditions. In this study, we have found that L. lactis is inherently multi-stress resistant. Considering the fact that L. lactis grows more slowly due to a slight purine starvation without exogenous purines, it is an intriguing possibility that a fast growth rate was sacrificed by lactic acid bacteria during domestication in the milk medium as a trade-off to gain a higher acid tolerance.

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