Increased expression of Mg^{2+} transport proteins enhances the survival of *Salmonella enterica* at high temperature

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Mg^{2+} homeostasis is important for *Salmonella* pathogenesis. In *Salmonella enterica*, the transcription of the *mgtA* gene, which encodes a Mg^{2+} transporter, is regulated by a Mg^{2+} -sensing riboswitch [Cromie MJ, Shi Y, Latifi T, Groisman EA (2006) *Cell* 125:71–84]. In a genetic analysis of the determinants of thermotolerance in *S. enterica* serovar Typhimurium, we isolated the *chr-1* mutation that increased the resistance of exponential phase cells to killing by high temperature. This mutation is a single base change in the *mgtA* riboswitch that causes high-level constitutive expression of *mgtA*. We showed that another *mgtA* riboswitch mutation, ΔUTR^{re-100} , which had been constructed by Cromie et al., also confers similar increased thermotolerance. Surprisingly, the *chr-1* mutation is located at a position that would not be predicted to be important for the regulatory function of the riboswitch. We obtained physiological evidence suggesting that the *chr-1* mutation increases the cytosolic free Mg^{2+} concentration. High-level expression of the heterologous MgtE Mg^{2+} transport protein of *Bacillus subtilis* also enhanced the thermotolerance of *S. enterica*. We hypothesize that increased Mg^{2+} accumulation might enhance thermotolerance by protecting the integrity of proteins or membranes, by mitigating oxidative damage or acting as an inducer of thermoprotective functions.

heat tolerance | riboswitch | Mg^{2+} homeostasis | heat shock

As a pathogen that can grow in a free living state in various environments and in hosts ranging from ectothermic reptiles to warm-blooded mammals and birds whose temperature may be elevated by fever (1), *Salmonella* possesses sophisticated mechanisms for thermal adaptation. *Salmonella* is the most frequent causative agent of foodborne bacterial infections in the United States (2), and because heat treatment is the most common and economical means for inactivating food pathogens better understanding of the regulation of thermotolerance in this organism has important applications in food safety.

The best-studied mechanism of thermal adaptation is the heat shock response. This response involves the transient induction of thermoprotective proteins by exposure to sublethal high temperatures, which enables organisms to survive subsequent treatments with higher temperatures that would be otherwise lethal (3). Thermotolerance can be regulated by a number of other factors, including stationary phase (4) and acidic or alkaline pH (5, 6).

Another environmental condition that regulates thermoresistance is water activity (a_w). Exposure to elevated osmolality enhances the thermotolerance of bacteria in two ways: by elevating their upper limit of growth temperature (7, 8) and increasing their viability at lethal high temperatures (9, 10). Our laboratory observed that the thermoprotective effect of high osmolality on the survival of *Salmonella enterica* serovar Typhimurium is reversed by glycine betaine (11). Glycine betaine is an osmoprotectant, which can alleviate the growth inhibitory effects of high osmolality (12). Along with ameliorating the osmotic inhibition of growth, this compound suppresses a number of other responses brought about by osmotic stress, such as

the accumulation of K^+ , the synthesis of trehalose, and the shrinkage of the cytoplasmic volume (13).

To identify components of the apparatus mediating the osmotic control of thermotolerance, we wanted to isolate mutants of *S. enterica* in which the high osmolality-dependent stimulation of thermotolerance was no longer antagonized by glycine betaine. In this selection, we obtained *chr* (constitutively heat resistant) mutations that conferred increased thermotolerance not only at high osmolality in the presence of glycine betaine but also at low osmolality. Surprisingly, one of these mutations, *chr-1*, resulted in high-level constitutive expression of the *mgtA* (Mg^{2+} transport) gene. Thus, these results revealed that Mg^{2+} has a hitherto unknown function in the regulation of thermotolerance.

Mg^{2+} , the second-most abundant cation in bacteria, has multiple functions: it is a cofactor in ATP-dependent phosphorylations and numerous other enzymatic reactions, it stabilizes ribosomes, it influences nucleic acid-protein interactions and RNA folding, and it is required for membrane integrity (14). Typical of the exquisite homeostatic control observed for the intracellular concentrations of most cations (15), the Mg^{2+} content of the cells is maintained at an essentially invariant level by tight regulation of uptake, efflux, and binding to macromolecules. As a result, there is <5-fold change in the intracellular Mg^{2+} content in response to five orders of magnitude variation in the external Mg^{2+} concentration (16, 17).

In *S. enterica*, Mg^{2+} is taken up by three transporters: MgtA and MgtB, which are P-type ATPases, and CorA, which is a channel (18). The *mgtA* and *mgtB* genes can be induced >2,000-fold by limitation for Mg^{2+} (19). The transcriptional activation of these two genes depends on the PhoPQ two-component system, which influences the expression of $\approx 5\%$ of the genes of *S. enterica* (20). PhoQ is a membrane-bound signal sensor kinase, which phosphorylates PhoP protein at low external concentrations of Mg^{2+} , Ca^{2+} , and Mn^{2+} (21–23). The phosphorylated PhoP is the transcriptional activator of genes in the PhoPQ regulon. At high concentrations of Mg^{2+} , Ca^{2+} , or Mn^{2+} , PhoQ promotes the dephosphorylation of PhoP and down-regulates the transcription of genes in the PhoPQ regulon. PhoQ responds to other regulatory signals in addition to divalent cations, because the repression caused by high concentrations of Mg^{2+} , Ca^{2+} , or Mn^{2+} is strongly antagonized by low concentrations of certain antimicrobial peptides (21, 24). Acidic pH can also overcome the repression by divalent cations, although there is a discrepancy in the literature concerning whether pH signal is sensed by PhoQ (24) or by a PhoPQ-independent mechanism (22). Mg^{2+} homeostasis is important for *Salmonella* pathogenesis, as

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Fig. 1. The thermotolerance of the strains TL1 (wild type; *A*) and TL3290 (*chr-1*; *B*) was determined at 53 °C in exponentially growing cells in M63 + 10 mM glucose containing the indicated supplements, as described in *Materials and Methods*. GB, 1 mM glycine betaine.

suggested by the fact that the PhoPQ (25, 26) and the CorA proteins (16) are essential virulence determinants and the *mgtA* and *mgtB* genes are induced during infection in mice (27) and in cultured epithelial cells (28). Because macrophages have an acidic cytoplasm that is believed to be low in Mg^{2+} , it has been proposed that *S. enterica* uses the PhoPQ system to detect that it is in an intracellular environment and needs to induce virulence genes (24, 29). Recently, it has been shown that high-level expression of the Rob protein, which is a transcriptional activator of genes involved in resistance to antibiotics, heavy metals, superoxide, and organic solvents, results in the induction of the *mgtA* gene from a PhoP-independent promoter that is 43 nt downstream of the major PhoP-dependent promoter. However, the role of this protein in the regulation of expression of *mgtA* is unclear, because deletion of *rob* does not affect the expression of *mgtA* at low or high Mg^{2+} concentrations (30).

Superimposed on the regulation of transcription initiation by the PhoPQ system, which is responsive to the external Mg^{2+} concentration, the *mgtA* gene is subject to an additional control mechanism that is mediated by an Mg^{2+} -sensing riboswitch. This regulatory element, which is specified by a 264-nt UTR at the 5' end of the *mgtA* mRNA, has been proposed to adapt two different stem and loop conformations at high vs. low intracellular Mg^{2+} , where the structure formed at high Mg^{2+} acts as a transcription terminator at a site between the promoter and the *mgtA* coding sequences (31). Transcription from the *mgtA* promoter requires the Mg^{2+} -regulated PhoPQ system for initiation (23, 27), but the riboswitch amplifies the response to Mg^{2+} by directing the RNA polymerase to stop or to proceed at the terminator at adequate intracellular or limiting Mg^{2+} concentrations, respectively. Mutations that destabilize the terminator structure reduce the efficiency of transcription termination at high Mg^{2+} concentrations, but they do not eliminate the need for an upstream promoter for transcription initiation (31).

In this work, we show riboswitch mutations resulting in increased expression of the *mgtA* gene confer increased heat tolerance in *S. enterica*.

Results

Isolation of the Mutations That Confer Increased Heat Resistance. The basis for the isolation of the heat tolerant mutants was the observation that glycine betaine impairs the high osmolality-dependent stimulation of thermotolerance. This result is reproduced in Fig. 1*A*. Osmotic stress imposed by 0.3 M NaCl caused a dramatic increase in survival of the WT strain at 53 °C, which was antagonized by glycine betaine. To isolate the mutants with enhanced heat tolerance, the WT was mutagenized with ethylmethane sulfonate, and derivatives were selected that acquired increased resistance to 53 °C in minimal medium containing 0.3 M NaCl + glycine betaine. This procedure yielded two inde-

pendent mutants, TL3380 (*chr-1*) and TL3360 (*chr-2*), that exhibited similar enhanced thermotolerance not only at high osmolality in the presence of glycine betaine, which was the selected phenotype, but also at low osmolality, which was demonstrated on subsequent characterization (Fig. S1). The two mutations do not have a detectable effect on growth rate at any temperature from 30–45 °C in the presence or absence of 0.3 M NaCl or glycine betaine. So far, we characterized only the *chr-1* mutation, and we do not have additional information about *chr-2* other than that it is not linked to any of the Mg^{2+} transport genes.

Because it is possible that the initial heat-resistant isolates may contain multiple mutations, for further characterization, the *chr-1* mutation was transduced via linkage with the *STM4446::Tn10dCm* insertion into the WT strain. Fig. 1*B* documents that in this new background the *chr-1* mutation resulted in a dramatic increase in thermotolerance in exponential phase cells both in medium of low osmolality and medium containing 0.3 M NaCl + glycine betaine, and it also conferred slightly increased heat tolerance in medium augmented with 0.3 M NaCl without glycine betaine.

The *chr-1* Mutation Is in the Regulatory Riboswitch RNA for the *mgtA*.

The steps for the identification and the confirmation of the sequence of the *chr-1* mutation are described in detail in *SI Materials and Methods*. Briefly, this procedure involved the isolation of the linked *STM4446::Tn10dCm* insertion, three-factor transductional mapping against flanking *treC1::Km^R* and *yjgF2::Tn10* insertions, and amplification and sequencing of a 4881-bp fragment containing the *treR* (trehalose repressor), *mgtA*, and *STM4457* (predicted transposase) genes. The sequence analysis revealed that *chr-1* mutation is a C to T change at position 4,699,521 of *S. enterica* serovar Typhimurium LT2 genome. The affected nucleotide is in the riboswitch of the *mgtA* gene at position +98 of the mRNA (Fig. 2).

The ΔUTR^{re-100} *mgtA* Riboswitch Mutation also Results in Increased Thermotolerance.

Because the *chr-1* mutation was obtained by a positive selection for increased thermotolerance, it might be exceptional in this regard. For the characterization of the *mgtA* riboswitch, Cromie et al. (31) constructed the site-directed mutation ΔUTR^{re-100} , which involved the replacement of nucleotides 148–247 of this regulatory element with an unrelated 84-bp “scar.” We transduced this mutation into our WT strain TL1 (Table S3) and determined its effects on thermotolerance. As shown in Fig. 3, the ΔUTR^{re-100} mutation was similar to *chr-1* in conferring increased thermotolerance in M63 and in M63 + 0.3 M NaCl + glycine betaine. This result demonstrates that the *chr-1* allele is not a special or fortuitous example of a riboswitch mutation that can confer increased thermotolerance. It is note-

Fig. 2. The *chr-1* mutation caused a C to U change at position +98 of the *mgfA* riboswitch. The stem and loop structures formed at low and high Mg^{2+} were proposed by Cromie et al. (31). The beginning and endpoints of the interval from positions 148 to 247 that was replaced by an unrelated sequence of 84 nt in the ΔUTR^{re-100} mutation (31) are indicated by the arrows. [Reproduced with permission from ref. 31 (Copyright 2006, Elsevier).]

worthy that the two mutations were obtained by different approaches: *chr-1* was isolated in our laboratory by positive selection, whereas ΔUTR^{re-100} was constructed by Cromie et al. (31) by reverse genetics, and has not been suspected previously to affect thermotolerance.

The *chr-1* Mutation Renders *mgtA* Expression Constitutive. To test whether the *chr-1* mutation altered the transcriptional control of *mgtA*, we introduced it upstream of a *mgtA-lacZ* fusion and determined its effect on the β -galactosidase activities of cells grown with various concentrations of Mg^{2+} (Table 1). As controls, we used isogenic strains that carried the $\Delta\text{UTR}^{\text{re-100}}$ mutation and the *chr*⁺ allele in combination with the *mgtA-lacZ* fusion. In accord with previous observations (19, 27, 31), in the *chr*⁺ background, the *mgtA-lacZ* fusion was expressed maximally at 0.016 mM Mg^{2+} (the lowest concentration tested) and was progressively down-regulated as the concentration of this cation was raised. The *chr-1* mutation resulted in 8- to 45-fold increase in the expression of *mgtA-lacZ* in the presence of 0.016-16 mM Mg^{2+} compared with that seen in the *chr*⁺ background. In the $\Delta\text{UTR}^{\text{re-100}}$ mutant, *mgtA* was expressed at 30-35% higher level

than in the *chr-1* mutant at all Mg^{2+} concentrations, but the two mutations were similar in that they both rendered the expression of the gene essentially insensitive to the external Mg^{2+} concentration. These results suggest that the enhanced heat tolerance of the *chr-1* mutant is the consequence of increased expression of the *mgtA*⁺ gene. We can rule out the possibility that the riboswitch mutations result in enhanced thermotolerance by elevating the transcription of a gene that is downstream from *mgtA*, because the next downstream gene, *STM4457*, is transcribed in the opposite direction than *mgtA*.

The result that the expression of the *mgtA-lacZ* fusion is essentially insensitive to the Mg^{2+} concentration in the riboswitch mutants is unexpected, because the initiation of transcription at the *mgtA* promoter would be predicted to be subject to residual control by this cation via the PhoPQ system. Different members of the PhoPQ regulon display a wide range of regulation, from 4- to >250-fold induction by Mg^{2+} limitation (23). It is possible that the PhoP-dependent transcription initiation at the *mgtA* promoter might not be very sensitive to the Mg^{2+} concentration, and most of the transcriptional control of the *mgtA* gene is provided by the riboswitch.

Effect of the *chr-1* Mutation on Mg^{2+} Homeostasis. To determine whether the accumulation of Mg^{2+} could be elevated by increasing the expression of *mgtA*, we determined the effect of the ΔUTR^{re-100} mutation on the total cellular concentration of this

Fig. 3. The effect of the ΔUTR^{re-100} mutation on thermotolerance. This experiment with strain TL4391 was carried out at the same time and under the same conditions as those shown in Fig. 1. GB is 1 mM glycine betaine.

Table 1. The effect of riboswitch mutations on the expression of *mgtA-lacZ*

MgSO ₄ , mM	β-galactosidase specific activity ± SD, Miller units		
	<i>chr⁺ mgtA-lacZ</i>	<i>chr-1 mgtA-lacZ</i>	<i>ΔUTR^{re-100} mgtA-lacZ</i>
0.016	83 ± 4	628 ± 62	1,010 ± 126
0.16	72 ± 1	646 ± 18	1,000 ± 18
1.6	28 ± 3	727 ± 25	940 ± 33
16	12 ± 1	538 ± 8	798 ± 102

Strains TL4295 (*chr⁺ mgtA-lacZ*), TL4293 (*chr-1 mgtA-lacZ*), and TL4385 (ΔUTR^{re-100} *mgtA-lacZ*) were grown in Mg²⁺-free M63 containing the indicated concentrations of MgSO₄, and the β -galactosidase activities were determined as described in *Materials and Methods*.

8 Miller units, respectively. The ≈ 2 -fold repression of the *mgtA-lacZ* fusion at 42 °C vs. 30 °C in the riboswitch mutants may be caused by a down-regulation of transcription initiation by the PhoPQ system or to a temperature-dependent functioning of a vestigial riboswitch, but we have not examined this effect further.

The *treR* and the *mgtA* genes are transcribed from partially overlapping divergent promoters (35). To test whether the *chr-1* mutation might confer increased thermotolerance by altering the expression of *treR*, we constructed *treR-lacZ* fusions using DNA fragments that carried the *chr-1* or *chr⁺* alleles. The β -galactosidase assays indicated the *chr-1* mutation did not alter the transcription from this promoter (Table S2), demonstrating that the increased thermotolerance is not caused by change in the expression of *treR*. We confirmed in *S. enterica* the observation made in *E. coli* (10, 36) that loss of the RpoS protein causes a drastic reduction in thermotolerance in both exponential and stationary phase. However, the *chr-1* mutation still conferred increased thermotolerance in an *rpoS::amp* mutant background in both growth phases (Fig. S3), demonstrating that increased Mg^{2+} transport confers increased thermotolerance by a mechanism that does not depend on RpoS function.

The *chr-1* mutation did not stimulate the growth rate of the cells near the upper limit of temperature in the absence or the presence of methionine, which is required above 43 °C because of the lability of homoserine transsuccinylase (37). This result suggests that the growth cessation at nonlethal high temperatures and the loss of viability at lethal high temperatures are the consequences of different deleterious effects of thermal stress. As was the case with the ΔUTR^{e-100} mutant (19, 27, 31), expression of the *mgtA-lacZ* fusion in the *chr-1* mutant was reduced by a *phoP::Tn10* insertion to background levels (to 9 and 7 Miller units at 0.016 and 16 mM Mg^{2+} , respectively), demonstrating that transcription from the *mgtA* promoter in this mutant still depends on the PhoPQ system. The *phoP* insertions also abolished the increased thermotolerance in the *chr-1 mgtA⁺* background.

Last, increasing the Mg^{2+} concentration of the medium did not increase the thermotolerance of the WT or the *chr-1* mutant. However, in view of the relative insensitivity of the internal Mg^{2+} pool to the external concentration (16, 17), high-level expression of Mg^{2+} transport systems might be more effective in altering the control of the intracellular Mg^{2+} homeostasis than by increasing the external concentration.

Discussion

Why Does Increased Mg^{2+} Transport Confer Increased Thermotolerance? The isolation of the *chr-1* mutation provided the insight that increased expression of Mg^{2+} transport proteins dramatically increases the thermotolerance of *Salmonella*. Richmond et al. (38) reported that *corA* is induced by heat shock in *E. coli*, providing a prior line of evidence for the beneficial effects of increased Mg^{2+} uptake at high temperature. Although we cannot propose a definitive model for the connection between thermotolerance and Mg^{2+} homeostasis, we can offer a number of hypotheses.

It is generally believed that protein denaturation is the primary cause of death at high temperature, which is supported by the fact that prominent members of the heat shock regulon are chaperonins and proteases (3). Increased cytoplasmic concentrations of free Mg^{2+} might enhance thermotolerance by stabilizing proteins or protein–nucleic acid interactions. A second possibility is that membrane damage may contribute to the thermal death. There are data indicating that Mg^{2+} is needed for membrane integrity (14). High external concentrations of this cation increase the resistance of yeast to both high temperature and ethanol by counteracting increased membrane leakage (39). It is possible to isolate *E. coli* mutants that are almost completely devoid of phosphatidylethanolamine by disrupting the *pss* (phosphatidylserine synthase) gene (40). In a *pss* mutant, phosphatidylethanolamine, which is zwitterionine, is replaced by the

anionic lipids phosphatidylglycerol and cardiolipin. A *pss* null mutant is inviable in media containing normal concentrations of Mg^{2+} , but it can grow in media augmented with ≥ 10 mM Mg^{2+} , Ca^{2+} , or Sr^{2+} . High levels of these divalent cations may be able to repair the growth defect of the *pss* mutant by neutralizing the excess negative charge of the phospholipids (41). Lusk and Kennedy (42) isolated an *E. coli* mutant with an uncharacterized defect in lipid biosynthesis that was also inhibited at low Mg^{2+} , but could be rescued by high concentrations of this cation. Induction of *mgtA* by the overproduction of the Rob protein has been found to increase the resistance of *S. enterica* to cyclohexane (30), which could be a manifestation of increased membrane stability resulting from increased Mg^{2+} accumulation. If high temperature causes membrane damage, it is possible that increased cytoplasmic concentrations of Mg^{2+} resulting from higher transport activity might improve the thermotolerance of the cells by stabilizing the phospholipid bilayer from the inside.

A third conceivable reason for the thermoprotective effect of Mg^{2+} could be that it might mitigate oxidative stress at high temperature. The production and the reactivity of oxygen radicals increases with increasing temperature, suggesting that oxidative damage could contribute to the lethal effects of thermal stress (43). Limitation for Mg^{2+} increases the accumulation of Fe^{2+} (44, 45), and thereby, renders the cells hypersensitive to oxidative damage. Increased accumulation of free Mg^{2+} may result in improved thermotolerance by lessening oxidative damage preventing the excessive uptake of Fe^{2+} , or competing with Fe^{2+} for binding to macromolecules.

Last, it is possible that Mg^{2+} might be a metabolic signal that up-regulates the thermotolerance response. Mg^{2+} has been proposed to be an important regulatory signal for the activation of mitosis in animal cells (46), and it might have a regulatory role in inducing or activating some thermoprotective functions in bacteria. If Mg^{2+} functions as a regulatory signal, it might exert this role as the free cation or in a complex with some other metabolite, such as ATP or ADP (46). Because the binding of proteins to nucleic acids is exquisitely sensitive to the ion concentration (47), small changes in the free Mg^{2+} concentration could have profound effects on gene expression. It will be of interest to carry out transcriptomic or proteomic studies to determine the effects of increased Mg^{2+} accumulation on global gene expression.

Why Does the *chr-1* Mutation Increase the Expression of the *mgtA* Gene? According to the model proposed by Cromie et al. (31), the *mgtA* riboswitch RNA can shift between two different conformations in regulating transcription termination upstream of the *mgtA* gene in response to the cytoplasmic Mg^{2+} concentration. However, surprisingly, the nucleotide at position +98 that was changed by the *chr-1* mutation is in a single-stranded region that would not be predicted to be crucial for the formation of stem structures of the riboswitch (Fig. 2). This result suggests that the regulatory mechanism of the riboswitch may be more intricate than proposed by Cromie et al. (31). It is possible that regulation by the riboswitch might involve a protein that recognizes the base altered by the *chr-1* mutation or it may be mediated by some higher-order structure in the RNA, such as a pseudoknot, that is affected by the mutation. Another interesting possibility could be that the *chr-1* mutation affects a nucleotide that is part of the Mg^{2+} “binding site” of the riboswitch.

Does Mg^{2+} Transport Have a Role in the Regulation of Thermotolerance in Other Organisms? Our finding that overproduction of the *B. subtilis* MgtE protein resulted in increased thermotolerance of *Salmonella* (Fig. 4) suggests that Mg^{2+} transport may be involved in the regulation of thermotolerance in other bacteria. Using the database SEED (www.theseed.org/wiki/Main_Page), we found that in *Yersinia*, the *mgtE* ortholog is 136 nt upstream of and in the same orientation as the Hsp70 family heat shock gene *yegD*,

and in *Vibrio cholerae*, *mgtE* is 186 nt upstream of and in the same orientation as the Hsp60 chaperonin genes *groES* and *groEL*. Because in prokaryotes genes that have related functions or share regulatory signals are often located in close proximity to each other (48, 49), the clustering of an *mgtE* ortholog with heat shock genes raises the possibility that this Mg^{2+} transport gene may be part of the heat shock regulon in these bacteria. Although at present this is only speculative, if *mgtE* were induced by heat shock in these organisms, this result would provide additional support for the conclusion that increased Mg^{2+} uptake is generally beneficial for high temperature tolerance.

Materials and Methods

Media, Growth Conditions, and Bacterial Strains. All bacterial strains used in this work were derived from strain TL1, which is our laboratory's line of WT *S. enterica* serovar Typhimurium LT2; the construction of the strains is summarized in Table S3. The experiments were carried with strains grown aerobically at 37 °C in minimal medium M63 (50), which contains 0.16 mM Mg^{2+} .

Heat Killing Experiments. Thermotolerance measurements were carried out at 53 °C with early exponential- or stationary-phase cultures grown in M63,

as described in SI Materials and Methods. The data shown are typical of results obtained in at least five independent measurements.

Isolation of the Thermotolerant Mutants. The isolation of the heat resistant mutants is described in detail in SI Materials and Methods, but briefly, the *chr-1* and *chr-2* mutants were obtained from two independent cultures of strain TL1 that were mutagenized with ethylmethane sulfonate and subjected to three cycles of heat treatment at 53 °C for 4 min M63 + glucose + 0.3 M NaCl + glycine betaine, each followed by outgrowth at 30 °C in the same medium. Because the isolation of the mutants involved three cycles of amplification of the survivors of the 53 °C treatment, we cannot estimate of the frequency of the heat resistance mutants in the mutagenized population.

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