Evolved Cobalamin-Independent Methionine Synthase (MetE) Improves the Acetate
and Thermal Tolerance of *Escherichia coli*

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ABSTRACT

Acetate-mediated growth inhibition of *Escherichia coli* has been found to be a consequence of the accumulation of homocysteine, the substrate of the cobalamin-independent methionine synthase (MetE) that catalyzes the final step of methionine biosynthesis. To improve the acetate resistance of *E. coli*, we randomly mutagenized the MetE enzyme and isolated a mutant enzyme, designated MetE-214 (V39A, R46C, T106I and K713E), that conferred accelerated growth in the *E. coli* K-12 WE strain in the presence of acetate. Additionally, substitution of cysteine 645, which is a unique site of oxidation in the MetE protein, with alanine improved acetate tolerance, and introduction of the C645A mutation into the MetE-214 mutant enzyme resulted in the highest growth rate in acetate-treated *E. coli* cells among three mutant MetE proteins. *E. coli* WE strains harboring acetate-tolerant MetE mutants were less inhibited by homocysteine in L-isoleucine-enriched medium. Furthermore, the acetate-tolerant MetE mutants stimulated the growth of the host strain at elevated temperatures (44 and 45°C). Unexpectedly, the mutant MetE enzymes displayed a reduced melting temperature (*T*<sub>m</sub>) but an enhanced *in vivo* stability. Thus, we demonstrate improved *E. coli* growth in the presence of acetate or at elevated temperatures solely due to mutations in the MetE enzyme. Furthermore, when an *E.coli* WE strain carrying the MetE mutant was combined with a previously found MetA (homoserine o-succinyltransferase) mutant enzyme, the MetA/MetE strain was found to grow at 45°C, a non-permissive growth temperature for *E.coli* in defined medium, with a similar growth rate as if it were supplemented by L-methionine.
INTRODUCTION

Inhibition of bacterial growth and metabolism by weak organic acids is a well-known phenomenon that has been exploited for food preservation for hundreds of years (1). Undissociated weak acids can permeate the cell membrane, and once inside, they can dissociate to release anions and protons, resulting in a decrease in the intracellular pH and growth inhibition (2, 3). The anion accumulation inside the cell affects cell turgor pressure (3). Through facilitating anion accumulation, the external pH also has a strong effect on the toxicity of weak acids. Moreover, Takahashi et al. (4) demonstrated an enhanced toxic effect of acetic acid on *Escherichia coli* at a lower extracellular pH level, resulting in a decreased growth rate and biomass yield.

The mechanism underlying this weak acid toxicity has not been easy to elucidate. Formic and propionic acids were found to inhibit macromolecular synthesis, particularly DNA biosynthesis (5). Weak acids have been shown to reduce the intracellular concentration of some amino acids, including glutamate, aspartate, lysine, arginine, glutamine and methionine (3, 6). Interestingly, supplementation with exogenous methionine abrogates most of the inhibitory effects of acetate on *E. coli* growth (6, 7), and a similar effect has been observed when cultures are treated with either benzoate or propionate (6). Furthermore, an increased level of intracellular methionine in *metK* mutants almost completely protects *E. coli* cells against the inhibitory effect of acetate, suggesting a critical role for methionine in overcoming growth limitation or inhibition in acetate-treated cells (6). Roe et al. (6) demonstrated that the increased accumulation of L-homocysteine (HCY), the substrate of the MetE enzyme, in acetate-treated cells seriously inhibits *E. coli* growth and they have proposed that MetE is a key enzyme associated with acetate-induced growth inhibition.
The MetE enzyme is a cobalamin-independent methionine synthase (EC 2.1.1.14), encoded by the metE gene, that catalyzes the final step in methionine biosynthesis in *E. coli* under aerobic conditions (8). Under anaerobic conditions, this reaction is driven by the MetH enzyme, a cobalamin-dependent methionine synthase (EC 2.1.1.13) encoded by the metH gene (8). Both enzymes transfer a methyl group to HCY to form methionine (8). However, the methyl donors involved in these reactions are different: 5-methyl-tetrahydro-pteroyltri-L-glutamate is the donor for the MetE enzyme, whereas 5-methyltetrahydrofolate is the donor for the MetH enzyme (9). The MetE enzyme catalyzes the direct transfer of the methyl group to HCY in what appears to be a catalytically less ideal solution compared with the use of cobalamin as a cofactor by MetH (9), as cobalamin is one of most potent nucleophiles known (10), in contrast to the thiol of the MetE enzyme functioning as an intermediate methyl acceptor (8). The MetE enzyme is approximately 50 times less active than MetH (11), which may explain why MetE is quite abundant in *E. coli* cells growing aerobically in glucose minimal medium, where it accounts for approximately 3-5% of the total cellular protein content (12, 13).

Recent studies have shown that the MetE protein is sensitive to two types of stress conditions: elevated temperature (14) and oxidative stress (15, 16). Roe et al. (6) suggested that acetate may inhibit MetE enzyme activity and they attempted (unsuccessfully) to protect *E. coli* cells through overexpression of the metE gene. Mogk et al. (14) showed that MetE is a major aggregation-prone enzyme in *E.coli* cells at an elevated temperature (45°C). Thus, the MetE protein could limit methionine availability under stress conditions (heat, acid and oxidation), which leads to slowing down of many cellular biosynthetic processes (protein, RNA and DNA biosynthesis) and total growth arrest (17). Thermolabile MetA, another aggregation-prone protein in the methionine biosynthesis pathway (18), has been proposed as a “metabolic fuse” (19) that senses
stress conditions destabilizing cellular proteins and, consequently, blocks protein synthesis via the inherent instability of MetA. The advantage of having a metabolic fuse is to spare cell energy under non-permissive growth conditions where the cells need to spend most of their energy for maintenance and protein quality control to survive under stress conditions (17). These observations suggested that stabilization of the inherently unstable MetE protein might increase the growth temperature of E. coli and provide novel biotechnological applications for developing a microbial cell factory (20).

In the present study, we employed random mutagenesis to obtain acetate-tolerant MetE mutants. The MetE-214 mutant, which exhibited faster growth in the presence of sodium acetate, contained multiple amino acid substitutions, including V39A, R46C, T106I and K713E. Alanine substitution of the surface-exposed cysteine 645, which has been identified as a unique site of oxidation in the MetE protein (15, 21), also improved acetate tolerance. However, the best acetate resistance was demonstrated by the MetE-214A mutant, in which the multiple substitutions from MetE-214 and the single-site C645A substitution were combined. We found an increased tolerance to propionate but not to benzoate in E. coli WE strains harboring the mutated MetE enzymes. The acetate-tolerant MetE mutants were found to stimulate the growth of the host strain at elevated temperatures (44 and 45°C). Unexpectedly, the mutated MetE proteins displayed reduced melting temperatures ($T_m$) but were more stable in vivo. We propose that MetE, similar to MetA (the first enzyme in methionine biosynthetic pathway), may serve as a “metabolic fuse” for the detection of unfavorable environmental conditions (19) and that MetE and MetA may be reengineered to improve E. coli growth at stressful conditions, including acetate and high temperature.
MATERIALS AND METHODS

Bacterial strains, media and culture conditions. The strains and plasmids employed in this study are listed in Table 1. E. coli strains were grown in minimal M9 medium (22) supplemented with glucose (0.2%) or in rich LB medium (Difco, San Jose, USA). Antibiotics were used at the following concentrations: ampicillin at 100 μg ml\(^{-1}\) and kanamycin at 25 μg ml\(^{-1}\). L-methionine and L-isoleucine were added to the medium to a final concentration of 50 μg ml\(^{-1}\). Bacterial cultures were grown in 25 ml of M9 glucose medium in 125 ml Erlenmeyer flasks. Seed cultures were grown overnight at 30°C and then diluted to an OD\(_{600}\) of 0.1, after which the cells were incubated at 32, 37 or 42°C with shaking. Growth was measured by monitoring the optical density at 600 nm at 1 h intervals.

To examine the effect of weak organic acids on the growth of different E. coli strains, cells were cultivated in M9 glucose medium (pH 6.0) supplemented with sodium acetate (20 mM), sodium benzoate (4 mM) or sodium propionate (10 mM) in a TVS126MB automatic growth-measuring incubator (Advantec MFS Inc., Tokyo, Japan). The specific growth rate (μ, h\(^{-1}\)) was calculated through linear regression analysis of ln(X/X\(_0\)) data using Sigma Plot software, where the initial OD\(_{600}\) (X\(_0\)) was 0.15 at the zero time point, and X represents the OD\(_{600}\) values measured every 10 min in an exponentially growing culture for 1 h. All cultures were repeated twice.

Library construction through error-prone PCR and selection of acetic acid-tolerant metE mutants. The metE gene, together with its promoter region, was amplified from genomic DNA from E. coli strain W3110 using the primers metE1 (CCGCAACGCTTGTAGCG) and metE2 (GATGGCTGGCAGCGTATG), gel-purified and employed as a template for error-prone PCR. Random mutagenesis was conducted with the GeneMorph® II Random Mutagenesis Kit (Stratagene, La Jolla, USA), using the primers metE1 and metE2 to obtain the maximal number...
of nucleotide substitutions per kb, as according to the manual. The PCR product was purified with the QIAquick PCR Purification Kit (Qiagen, Germantown, USA), amplified one additional time using the primers MutMetE1 (CCTTCCCTAAATCCATAGGATTACATATAATTAGAGGAAGAAAAATG) and MutMetE2 (GCTGGAATGGTTTAAGCAGTATGGTGGGAAGAAGTCGCTGTAATGAGAAAAGACCGGGTGTTTATTAC) and Vent polymerase, and subsequently was digested with DpnI. The amplified sequence was then transfected into freshly prepared *E. coli* WEΔmetE(pKD46) cells via electroporation, as described previously (23). The WEΔmetE strain was obtained through P1vir transduction of a kanamycin-resistance construct from the JW3805ΔmetE donor strain into the WE strain (24). The transformed cells were finally incubated at 37°C in M9 minimal medium plates supplemented with glucose to select clones containing a functional *metE* gene. The *metE* mutants were cultivated in 100 μl of M9 glucose medium adjusted to pH 6.0 in a BioScreen C incubator (Labsystems, Helsinki, Finland) in the presence of sodium acetate (20 mM) at 37°C for 49 h, with shaking at 15 min intervals. The growth curve for each mutant was compared to the control strain harboring the non-mutated *metE* gene to select acetate-tolerant clones. The candidate clones were then flask-cultivated in 25 ml of M9 glucose medium (pH 6.0) supplemented with 10 mM sodium acetate at 37°C to determine the fastest-growing clones. Finally, the *metE* gene was amplified from the genomic DNA of the acetate-tolerant mutant and sequenced.

**Construction of the single-site MetE mutants.** The V39A, R46C, T106I, C645A, K713E single-site mutants were constructed through overlap extension PCR using the QuikChange II-E
Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) with the primers MetEA1-forward
(GAAGAACTGCTGGCGGCGAGGGCGTGAGTTGCGTG); MetEC1 – forward
(CGTGAATTCGTGCTGTACTGGGATCAACAAAG); MetEI1 – forward
(CGTGGAGCTGCGGCCATTGGCGAATCTGCGG); MetEC2-forward
(CACACTCACATGTGTTATGCGGAGTTCAACGACATC); MetEE1-forward
(CTGAAGAAAGCGGCAGAACGCATTCCGGCAGAGCG); and the reverse primers,
completing the forward primers, respectively. The changes in the sequence are underlined. The
mutated metE sequences were introduced into the E. coli WEΔmetE chromosome using the λ
Red recombination system (23).

Cloning, expression and protein purification. The wild-type and mutated metE sequences
were cloned into the NdeI/HindIII restriction sites of the pET22b plasmid in frame with a C-
terminal 6x-histidine tag using the primers MetE3 (CGCCTCCATATG
CCGATTCGTGTGCCG) and MetE4 (CGCCTCAAGCTT
CCCCCGACGCAAGTTCTG). The plasmid DNA was purified
from ampicillin-resistant clones and sequenced to verify that the correct genes had been cloned.
Competent E. coli BL21(DE3) cells were then transformed with the constructed plasmids. A
single colony of the E. coli BL21(DE3) strain harboring each plasmid was cultivated overnight at
30°C in 75 ml of LB medium with ampicillin. Two liters of 2xYT medium containing ampicillin
and 0.5 mM of zinc sulfate (25) was inoculated with an overnight culture, incubated at 30°C to
an OD_{600} of 0.6, induced with IPTG (1 mM final concentration) and cooled to 18°C. Following
overnight induction, the cells were harvested via centrifugation, and the pellets were resuspended
in ice-cold buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM MgCl₂, 5 mM imidazole, pH 7.5) at
a ratio of 3 ml of buffer g⁻¹ of wet cells. The cells were lysed by incubation with 1 mg ml⁻¹
lysozyme, Halt™ protease inhibitor cocktail (Pierce, Rockford, USA) and DNase I at 4°C for 30
min with stirring, followed by sonication for 10 x 1 min at 30 s intervals using a Branson Sonifier (model 450). The cell debris was removed via centrifugation at 13,000 x g for 30 min. The proteins were purified from the supernatants using Ni-nitrilotriacetic acid agarose (Qiagen, Germantown, USA). Six milliliters of agarose slurry was incubated with 20 ml of supernatant overnight at 4°C with rocking. The unbound proteins were removed through gravity filtration, and the agarose was washed with 12 ml of buffer (50 mM Tris-HCl, 300 mM NaCl, 100 mM imidazole, pH 7.5). The proteins were then released from the agarose via elution with 20 ml of buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 7.5), and the eluate was dialyzed against two changes of dialysis buffer (50 mM K-phosphate buffer, 150 mM NaCl, pH 7.6), and then concentrated with an Amicon Ultra-15 centrifugal device over a 30,000 NMWL membrane (Millipore, Billerica, USA). The presence of pure protein in all samples was confirmed via SDS-PAGE.

**Differential scanning calorimetry.** The thermal stabilities of the MetE proteins were measured calorimetrically over a temperature interval of 15-90°C at a scan rate of 90°C/h. A VP-DSC calorimeter (MicroCal, LLC, Northampton, USA) was employed for these measurements, using 20 µM protein in 50 mM K-phosphate buffer (pH 7.5). Three scans were obtained from independent protein preparations.

**In vivo MetE stability analysis.** The WE, WE-214, WE-CA and WE-214A strains were grown in M9 glucose medium with pH values of 6.0 and 7.0 at 37°C to exponential phase (OD600 of 0.3), and then were treated with 200 µg chloramphenicol ml⁻¹. One half of the pH 7.0 culture was shifted to 45°C. Samples (2 ml) were collected before and after the addition of chloramphenicol every 30 min for 2 h, centrifuged at 14,000 rpm for 5 min at 4°C and resuspended in 50 µl of
distilled water, after which 25 μl aliquots were mixed with 25 μl of 2 x sample buffer, and the remaining 25 μl was used for the quantification of total protein with a Bio-Rad protein assay kit. A 3 μg sample of total protein was loaded into a 4-15% Criterion™ TGX™ Pre-cast Gel (Bio-Rad, Hercules, USA), followed by Western blotting. A rabbit anti-MetE antibody (a generous gift from Dr. Axel Mogk) was employed as the primary antibody, and horseradish peroxidase-conjugated anti-rabbit IgG (Pierce, Rockford, USA) was employed as the secondary antibody. The immunoblots were developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, USA), scanned with a Fujifilm LAS-3000 Image Reader and analyzed with WCIF ImageJ software.

Tryptic digestion of the MetE mutants. Samples the mutated MetE enzymes (1 nmol) were digested with 0.01% trypsin (w/v) at 37°C in a total volume of 200 μl of 20 mM potassium phosphate buffer (pH 7.2) (25). Samples were collected at the indicated time points and analyzed via SDS-PAGE. Band intensities were quantified using WCIF Image J software.

RESULTS

Directed evolution of MetE increased the acetate tolerance of *E. coli* cells. It has been shown that the slowed growth of *E. coli* observed in the presence of acetate is mainly due to a malfunction of the MetE enzyme, resulting in accumulation of HCY, a substrate of MetE that is toxic to cells at high concentrations (6). However, overexpression of the *metE* gene does not protect cells against growth inhibition by acetate, revealing that the observed growth inhibition is not due to insufficient MetE enzyme activity but rather is due to a malfunction of MetE (6). To improve the acetate tolerance of *E. coli* cells, we performed random mutagenesis of the *metE* gene, followed by insertion of the mutant *metE* into the chromosome of the *E. coli*
WEΔmetE::kan strain, as described in the Materials and Methods. A mutant library consisting of 870 clones was constructed and tested for growth in the presence of sodium acetate (20 mM) using a BioScreen C incubator. A 20 mM concentration of sodium acetate normally inhibits the growth of the E. coli WE strain in a BioScreen C incubator. One clone grew faster than the other mutants in acetate-enriched medium and was designated as the WE-214 strain (Fig. 1). Sequencing analysis revealed the presence of four amino acid substitutions in the metE-214 mutant: V39A, R46C, T106I and K713E.

Previously, Hondorp and Matthews (15, 21) showed that substitution of MetE cysteine 645 with alanine completely eliminated methionine auxotrophy in oxidatively stressed cells. We hypothesized that cysteine 645 might represent a site of weak organic acid sensitivity and that substitution of this residue with alanine would further enhance the acetate tolerance of the MetE protein. Thus, we constructed the metE-CA (C645A) and metE-214A (V39A, R46C, T106I, C645A, K713E) mutant genes and inserted them into the chromosome of the WEΔmetE::kan strain. The WE-214, WE-CA and WE-214A strains and the control WE strain harboring wild-type metE were cultured in M9 glucose medium (pH 6.0) supplemented with sodium acetate (20 mM), sodium benzoate (4 mM) or sodium propionate (10 mM) in an automatic growth-measuring incubator at 37°C. As shown in Fig. 2 and Table 2, the mutants grew faster than the control strain in the presence of sodium acetate or sodium propionate. The WE-214A mutant that harbored all of the amino acid substitutions present in MetE-214 as well as C645A demonstrated the highest resistance among the three tested mutants (Fig. 2; Table 2). Supplementation of the culture medium with sodium benzoate did not reveal any differences between the control and mutant strains (Table 2). Apparently, this compound has another target in the methionine biosynthesis pathway, as the addition of methionine has been found to relieve the inhibitory
The effect of benzoate on *E. coli* growth (6). Price-Carter et al. (19) have proposed that, in *Salmonella enterica*, benzoate inhibits the first enzyme in the methionine biosynthesis pathway (MetA).

Acetate-resistant MetE enzymes are more tolerant of homocysteine. Roe et al. (6) showed that supplementation of minimal medium with sodium acetate leads to intracellular accumulation of HCY, which is a substrate for the MetE enzyme, and excess HCY has been shown to inhibit *E. coli* growth (6, 26, 27). We hypothesized that the acetate-resistant MetE mutants may be tolerant of an increased concentration of HCY. Thus, the MetE mutants and the control strain were cultivated in minimal medium supplemented with 8 mM DL-HCY (Table 3). Under these conditions, the growth of all strains was inhibited approximately 50% in comparison with their growth in HCY-free medium, and we did not detect any differences in growth rates between the mutant and control strains (Table 3). Supplementation of the HCY-enriched medium with L-methionine slightly relieved the growth inhibition of all the tested strains (Table 3). Previously, Tuite et al. (26) demonstrated that the *E. coli* growth defect observed in the presence of HCY is caused by the inhibition of threonine deaminase, the first enzyme in the isoleucine biosynthesis pathway, and this inhibition leads to isoleucine auxotrophy. To exclude the toxic effect of HCY on threonine deaminase, we added L-isoleucine in the HCY-enriched medium. L-isoleucine relieves the *E. coli* growth defect caused by HCY to a greater extent than L-methionine (Fig. 3, Table 3), which is consistent with the previous findings of Sikora and Jakubowski (27). Mutants expressing the acetate-resistant MetEs grew approximately 10% faster than the control strain in the presence of L-isoleucine in the HCY-enriched medium (Fig. 3, Table 3). Thus, we have demonstrated that the stabilized MetE mutants conferring an increased acetate tolerance in *E. coli* also become more resistant to HCY. Simultaneous supplementation of the culture medium with
L-methionine and L-isoleucine resulted in a higher growth rate but did not completely restore *E. coli* growth in the presence of HCY (Table 3), which suggests a more complex effect of HCY on *E. coli* cell physiology. Sikora and Jakubowski (27) have shown that the HCY-induced inhibition of *E. coli* growth is accompanied by a significantly increased accumulation of HCY-thiolactone. The conversion of HCY to HCY-thiolactone occurs through an ATP-consuming reaction that unproductively reduces cellular energy (27). In addition, HCY-thiolactone modifies *E. coli* proteins (N-homocysteinylilation) in a way that alters or impairs their functions (27). Both of these negative consequences of increased HCY-thiolactone accumulation, reduced cellular energy and impaired protein function, could inhibit *E. coli* growth.

**Acetate-tolerant MetE enzymes displayed reduced thermal transition midpoint values but higher in vivo stability.** To determine whether the accelerated growth of the metE mutants in the presence of acetate correlated with an increase in MetE thermal stability (unfolding free energy), the melting temperature (*T*<sub>m</sub>) of the proteins was measured through differential scanning calorimetry (DSC). The wild-type and mutant MetE enzymes, containing a C-terminal six-histidine tag, were purified as described in Materials and Methods. The *T*<sub>m</sub> of the native MetE enzyme was 54.7±0.25°C (Table 4), and the single C645A substitution slightly increased the *T*<sub>m</sub> to 55.9±0.08, which contrasted with the multiple mutants MetE-214 and MetE-214A, whose *T*<sub>m</sub> values were 51.3±0.1 and 48.5±0.4, respectively (Table 4).

Because the acetate-tolerant MetE mutants demonstrated reduced *T*<sub>m</sub> values, we analyzed the stability of the engineered and native MetE enzymes *in vivo* after protein synthesis had been blocked by the addition of chloramphenicol. Residual MetE proteins were quantified via Western blotting of cells grown in M9 glucose medium with a pH value of 6.0 or 7.0, and at 45°C under
neutral conditions as described in Materials and Methods. As shown in Fig. 4, the mutants demonstrated an increased in vivo stability under both weakly acidic and neutral conditions, displaying half-lives ($t_{1/2}$) of 84 min (MetE-214) and more than 120 min (MetE-CA and MetE-214A) at pH 6.0, and 73, >120 and 100 min at pH 7.0 (for MetE-214, MetE-CA and MetE-214A, respectively). The half-lives of the native MetE protein were 48 min at pH 6.0 and 49 min at pH 7.0. Interestingly, the acetate-tolerant MetE mutants were more stable at a lower pH (Fig. 4A and B). At an elevated temperature (45°C), the MetE mutants were also degraded more slowly than the native enzyme, with half-lives of 70, 103 and 99 min (for MetE-214, MetE-CA and MetE-214A, respectively), versus 55 min for the wild-type MetE (Fig. 4C). The half-lives of the native and mutated MetE enzymes at 45°C were similar to those half-lives found at 37°C, most likely because their $T_m$ values were higher than 45°C.

The acetate-tolerant MetE proteins conferred thermal stability in E. coli. Previously, we found that stabilized mutants of the MetA protein demonstrated an increased tolerance to acetic acid (24). Here, we hypothesized that the acetate-tolerant MetE mutants might improve the thermal stability of E. coli cells. The MetE mutants and the control strain were cultivated at 44°C in minimal M9 glucose medium (Fig. 5A). All of the mutants grew slightly more than four-times faster than the control WE strain. The specific growth rate ($\mu$) of the WE strain was 0.17 h$^{-1}$, while those of the WE-214, WE-CA and WE-214A mutant strains were 0.69, 0.7 and 0.72 h$^{-1}$, respectively. The enhanced thermal stability of the acetate-tolerant MetE mutants was confirmed through incubation of serially diluted cultures on solid M9 glucose plates at 44°C (Fig. 5B). The viability of the mutant strains was increased by at least two- to three-orders of magnitude compared with the wild-type strain (Fig. 5B, left panel), although the same maximal viability was not observed in the presence of methionine (Fig. 5B, right panel). This result can be
explained by the presence of another thermolabile protein, MetA, in the methionine biosynthetic pathway. Supplementation of the culture medium with L-methionine stimulated the growth of the wild-type and mutant strains at 44°C to the same extent, thus abolishing the differences observed among these strains (Fig. 5B, right panel).

To determine the individual contribution of each amino acid residue to improved acetate tolerance and thermal stability, single amino acid substitutions corresponding to those found in the MetE-214 were introduced into the wild-type MetE protein by site-directed mutagenesis. The single-site metE mutants were inserted into the WEA::kan chromosome to yield strains A39, C46, I106, and E713, which were then tested for growth in M9 glucose medium (pH 6.0) supplemented with sodium acetate (20 mM) at 37°C or in M9 glucose medium (pH 7.0) at 44°C (Fig. 6). Two amino acid substitutions, R46C and T106I, conferred the highest growth rates to the WE strain in the presence of acetate (specific growth rates of 0.178 and 0.185 h⁻¹ for the C46 and I106, respectively, versus 0.164 h⁻¹ for the control strain WE) (Fig. 6A). The other mutations, V39A and K713E, also stimulated the A39 and E713 mutant strain growth under acidic conditions but to a lesser extent (specific growth rates of 0.174 and 0.175 h⁻¹, respectively) (Fig. 6A). All the single-mutated MetEs stimulated growth of the E.coli strains at 44°C (specific growth rates of 0.6, 0.62, 0.62 and 0.67 h⁻¹ for the A39, C46, I106 and E713 strains, respectively, versus 0.17 h⁻¹ for the control strain WE) (Fig. 6B). The E713 (K713E) was the fastest growing mutant among those tested for growth at 45°C (specific growth rate of 0.425 h⁻¹) (data not shown). Other single-site mutants, C46 and I106, also demonstrated an accelerated growth at 45°C (specific growth rates of 0.3 and 0.32 h⁻¹, respectively) (data not shown). The control strain WE did not grow at 45°C. As seen in data presented here, all of the amino acid residues
substituted in the MetE-214 mutant enzyme are involved in the MetE stability, but to variable degrees.

Stabilized MetA and MetE enzymes synergistically increase the growth rate of *E. coli* in the presence of acetate and at elevated temperature. To test the combined effect of the stabilized MetA and MetE enzymes on the growth of the WE strain in sodium acetate medium and at the higher temperature of 45°C, the *metA*-Y229 gene, encoding the thermal-tolerant Y229 MetA mutant (28), was inserted into the chromosome of the WE-214A strain instead of the wild-type *metA* gene. We have previously found that stabilized MetA mutants displayed enhanced acetate tolerance (24). The Y229-214A double mutant MetA-MetE strain and the single mutant MetA (Y229) and MetE (WE-214A) strains were cultivated in minimal M9 glucose medium in the presence of sodium acetate (20 mM) or at 45°C, and they were compared with the control strain WE harboring the wild-type *metA* and *metE* genes (Fig. 7A and B). The growth rate of the double mutant Y229-214A was higher than the growth rates of the control strain WE and the single mutants tested for growth in sodium acetate enriched medium (specific growth rates of 0.205 h⁻¹ for the Y229-214A versus 0.162, 0.181 and 0.183 h⁻¹ for the WE, WE-214A and Y229 strains, respectively) (Fig. 7A). However, the mutant Y229-214A grew more slowly than in the presence of L-methionine (specific growth rate 0.27 h⁻¹; data not shown). This result might reflect the presence of other acetate-sensitive regions in the MetA/MetE enzymes, or it might mean that another protein in the methionine biosynthetic pathway is involved in acetate tolerance. The Y229-214A double mutant grew faster than the Y229 and WE-214A single mutants at the elevated temperature of 45°C, with specific growth rates of 0.53, 0.47 and 0.31 h⁻¹, respectively (Fig. 7B). Moreover, the specific growth rate of the Y229-214A double stabilized mutant was the same as in the presence of L-methionine (0.53 and 0.55, respectively) (data not shown), and the
Y229, WE-214A and control WE strains demonstrated similar specific growth rates in L-
methionine-supplemented medium (0.56, 0.54 and 0.54, respectively) (data not shown). The
control strain WE did not grow at 45°C (Fig. 7B).

We also tested the viability of these strains at 45°C. Serially diluted cultures were incubated on
M9 glucose plates, with or without L-methionine (Fig. S1A and B). As shown in Fig. S1A, the
Y229-214A double mutant strain was more viable than the single stabilized mutants Y229 and
WE-214A by one- or two-orders of magnitude, respectively. The survival of the Y229-214
double mutant was only slightly lower than the survival observed in the presence of L-
methionine (Fig. S1B). Thus, the stabilization of two thermolabile enzymes in the methionine
biosynthesis pathway, MetA and MetE, synergistically increased the thermal tolerance of the E.
coli WE strain, bringing it close to the maximum tolerance achieved with L-methionine
supplementation. We hypothesize that this further improvement of the thermal stability of the
MetA/MetE proteins contributes to completely overcoming the methionine auxotrophy that
occurs at elevated temperatures, and thereby accelerates E. coli growth.

DISCUSSION

Earlier observations made by Han et al. (7) and Roe et al. (6) demonstrated that L-methionine
relieves the growth inhibition of E. coli cells caused by weak organic acids, including acetic acid.
Roe et al. (6) showed that there was an increased level of HCY, which is a substrate for the MetE
protein, in acetate-treated cells and that E. coli growth was inhibited in HCY-enriched medium.
However, overproduction of MetE did not protect these E. coli cells against growth inhibition by
acetate (6), most likely due to a significant decline of the soluble MetE protein level in response
to acetate challenge. We found that the amount of soluble wild-type MetE protein was reduced
by 40% after 2 h of cultivation of the WE strain in minimal M9 glucose medium supplemented with 20 mM of sodium acetate (Fig. S2A). The level of the soluble native MetE protein in the overexpressed BL21(DE3) cells decreased by 25% compared to untreated cells after 2 h of acetate treatment (Fig. S2B). Another way to increase the acetate resistance of *E. coli* cells is random mutagenesis of *metE*, followed by selection of clones growing more rapidly in the presence of sodium acetate. Through this process, we obtained the MetE-214 mutant variant (with the V39A, R46C, T106I and K713E mutations) that displayed acetate tolerance. We found that all of the single-site mutations corresponding to those found in the MetE-214 mutant increased both acetic and thermal tolerance of the native MetE, but to variable degrees. A C645A substitution, which was found to confer resistance to oxidative stress in the MetE protein (15, 21), also increased the protein’s tolerance to acetate.

It is necessary to note that despite the increased acetate tolerance of these MetE mutants, we did not achieve the same extent of growth inhibition relief as observed in the presence of L-methionine (Table 2), which may be explained by two factors. First, the substitution of other amino acid residues can also increase the acetate tolerance of the MetE enzyme. Second, different proteins in the methionine biosynthesis pathway (e.g., the MetA enzyme) appear to be involved in acetate resistance. Arnold *et al.* (29) found that the expression of the *metA* gene was increased three-four fold in acetate-treated *E. coli* cells, and we previously observed an increased tolerance to acetic acid in stabilized MetA mutants (24).

Roe *et al.* (6) hypothesized that the hampered *E. coli* growth observed in the presence of acetate is a consequence of a drastically increased pool of HCY due to inhibition of the biosynthetic step downstream of the HCY metabolic intermediate, corresponding to the MetE enzyme. In the
present study, we demonstrated that the acetate-tolerant MetE enzymes conferred greater 
resistance to HCY in *E. coli* cells, but only in the presence of L-isoleucine. This finding is 
consistent with the previous observation made by Tuite *et al.* (26) that the primary target of HCY 
is the first enzyme in the isoleucine biosynthetic pathway, i.e., threonine deaminase. We may 
therefore assume that when branched-chain amino acid biosynthesis is protected by L-isoleucine 
addition, the stabilized MetE enzymes metabolize HCY to methionine more effectively than the 
wild-type MetE enzyme. Consequently, the *E. coli* cells can overcome two negative effects: the 
decreased intracellular levels of methionine and the accumulation of the toxic methionine 
precursor HCY.

MetE catalyzes the final step in *de novo* methionine biosynthesis in *E. coli* cells grown under 
aerobic conditions (8). The MetE protein has been found to account for approximately 3-5% of 
the total cellular protein content when *E. coli* is grown in glucose minimal medium (12, 13). This 
high abundance of MetE protein may be explained by its approximately 50-times lower activity 
compared to the MetH enzyme, which drives the same reaction, but under anaerobic conditions 
(11). A higher cellular content of the MetE protein is correlated with an increased susceptibility 
to unfavorable environments, such as heat (14), oxidative stress (15, 16, 21) and acidic 
conditions (6). Mogk *et al.* (14) determined that MetE is a major aggregation-prone protein at an 
elevated temperature (45°C) that is significantly below the melting temperature (*T*<sub>m</sub>) of the 
purified protein (55°C) (15, Table 4 of present study). It was surprising to find that the acetate- 
and temperature-tolerant MetE enzymes exhibited melting temperature below that determined 
for the wild-type protein (Table 4). The *T*<sub>m</sub> is a good indicator of thermal stability (30). 
Apparently, we are faced with another type of stabilization here, specifically kinetic stabilization, 
where specific conformational changes lead to a high unfolding barrier that ultimately results in
very slow unfolding rates (31). We previously obtained similar results for stabilized MetA enzymes (28).

None of the amino acids substituted in the acetate-tolerant MetE-214 mutant were located at the conserved active sites (11, 25, 32, 33) (Fig. S3), so we hypothesize that these mutations lead to kinetic stabilization of the MetE protein. Hondorp and Matthews noticed that cysteine 645, identified as a unique site of oxidation in the MetE protein (15, 21), is conserved only within the Enterobacteriaceae (21). The MetE enzymes from the acidophilic and thermophilic strains do not possess a homologous cysteine 645 (Fig. S3). On the other hand, the MetE enzymes from other species, which have been found to be sensitive to oxidation, do not contain a homologous cysteine 645 (34, 35). Obviously, the MetE amino acid residues involved in the response to environmental stress are quite diverse and species specific.

Hondorp and Matthews have shown that glutathione (GSSC) oxidation of MetE causes a conformational change compared with the reduced protein (15). The oxidized MetE protein was found to be more stable in response to tryptic digestion than the reduced form (15). In the present study, all of the MetE mutants were much more susceptible to tryptic digestion than the wild-type protein (Fig. 8), which suggests that the conformational changes that occur in the mutated MetE proteins prevent them from unfolding and being inactivated in a stressful environment. This assumption may explain the increased acetate tolerance of the MetE-214, MetE-CA and MetE-214A mutant enzymes that was observed when the intracellular concentration of acetate was much higher than the external concentration at moderately acidic pH values (3). In support of this notion, the mutated MetE proteins, which were more stable in vivo than the wild-type enzyme under either an acidic or a neutral pH, demonstrated a higher level of in vivo stability specifically at low pH (Fig. 4). The increased thermal tolerance of the acetate-resistant MetE
mutants may also be a consequence of the conformational changes in the thermolabile MetE protein (14).

In the present study, we demonstrated increased thermal tolerance of *E. coli* strains expressing acetate-resistant MetE enzymes. It is quite notable that *E. coli* cells acquire cross protection from acetate and heat from mutations at MetE at the single protein level. A higher heat resistance of acid-adapted *E. coli* strains has previously been shown using a complex medium (36). One of the major nutrient sources in this complex medium is the pool of amino acids, including L-methionine, peptides and proteins, derived from organic nitrogen substrates such as soybean flour, yeast extract and corn steep liquor (37). The presence of L-methionine in the culture medium represses genes involved in methionine biosynthesis, as shown by Greene (38), suggesting the existence of another mechanism of thermotolerance in the complex medium. *E. coli* cells have been found to grow significantly more slowly after a temperature upshift without L-methionine supplementation (39). This phenomenon was linked to the inherent instability of MetA, the first enzyme in the methionine biosynthetic pathway (17, 39). In heat-treated *E. coli* cells, Mogk *et al.* (14) identified MetE as another thermolabile protein in the methionine biosynthesis pathway. Here, we demonstrated that the stabilized MetE enzymes alone were responsible for the accelerated growth and higher survival observed in *E. coli* cells at elevated temperatures. The simultaneous presence of both the stabilized MetA and stabilized MetE enzymes improved the survival of heat-stressed *E. coli* cells to the levels observed under L-methionine supplementation (Fig. S1). We therefore believe that the introduction of stabilized MetA and MetE proteins is sufficient to enable the growth of *E. coli* at higher temperatures. Moreover, further stabilization of the *E. coli* MetA/MetE enzymes may completely overcome methionine auxotrophy and provide an increased growth rate and enhanced survival at higher
temperatures. This MetA/MetE reengineering may increase the temperature window for \textit{E. coli} cells, permitting them to grow and form products at higher temperatures, and thus widen the biotechnological applications for \textit{E. coli} as a cell factory.

\textbf{ACKNOWLEDGEMENTS}

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\textbf{REFERENCES}


TABLE 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant description*</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F-, supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1 deoR λ-</td>
<td>(22)</td>
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<td>JW3805</td>
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<td>ΔlacZ4787(:rmB-3),</td>
<td>Institute of Genetics, Japan)</td>
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<tr>
<td></td>
<td>Δ(rhaD-rhaB)568, hsdR514</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>Growth rate $\mu$, h$^{-1}$a</td>
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</tr>
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</table>

* Ap', ampicillin resistance; *cat*, chloramphenicol resistance gene; *kan*, kanamycin resistance gene; *tet*, tetracycline resistance gene; *metA$_{W3110}$*, *metA* gene from the *E.coli* W3110 strain.

TABLE 2. Effects of mutated MetE proteins on the growth of the *E. coli* WE strain in the presence of weak organic acids.
Strains were grown in M9 glucose medium (pH 6.0) supplemented with sodium acetate (20 mM), sodium benzoate (4 mM) or sodium propionate (10 mM) in an automatic growth-measuring incubator at 37°C for 28 h, with two repetitions. L-methionine was added to the medium to a final concentration of 50 µg ml⁻¹.

The specific growth rate (µ, h⁻¹) was calculated through linear regression analysis of \( \ln(X/X₀) \) data with Sigma Plot software, where the initial OD₆₀₀ (X₀) was 0.1-0.15 at the zero time point, and X represents the OD₆₀₀ values measured every 10 min in an exponentially growing culture over 1 h.

Symbol: ▲ – the increase in the specific growth rate is ≥10% of the rate in the control strains.

**TABLE 3. Effects of the mutated MetE proteins on the growth of the *E. coli* WE strain in the presence of HCY.**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth rate µ, h⁻¹&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WE</td>
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<tr>
<td>M9 glu (control)</td>
<td>0.57±0.006</td>
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<tr>
<td>+ NaAc</td>
<td>0.162±0.01</td>
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<tr>
<td>+ NaBenz</td>
<td>0.138±0.01</td>
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<tr>
<td>+ NaProp</td>
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<tr>
<td>+NaAc+L-met</td>
<td>0.27±0.004</td>
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<sup>a</sup> Values are means ± standard deviations.
Strains were grown in M9 glucose medium (pH 6.0), with or without HCY supplementation (8 mM), at 37°C in an automatic growth-measuring incubator for 24 h, with two repetitions. L-methionine and L-isoleucine were added to a final concentration 50 µg ml⁻¹.

The specific growth rate (µ, h⁻¹) was calculated through linear regression analysis of ln(X/X₀) data with Sigma Plot software, where the initial OD₆₀₀ (X₀) was 0.15 at the zero time point, and X represents the OD₆₀₀ values measured every 10 min in an exponentially growing culture during 1 h.

Symbol: ▲ - the increase in the specific growth rate is ≥10% of the rate in the control strains.

TABLE 4. Differential scanning calorimetric data for the wild-type and mutant MetE enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tₘ (°C)</th>
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<tr>
<td>MetE, wild-type</td>
<td>54.7±0.25</td>
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<tr>
<td>MetE-214</td>
<td>51.3±0.1</td>
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<tr>
<td>MetE-C645A</td>
<td>55.9±0.08</td>
</tr>
<tr>
<td>MetE-214A</td>
<td>48.5±0.4</td>
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</table>
All measurements were performed in triplicate.

FIGURE LEGENDS

Figure 1. Effect of the mutated MetE-214 protein on *E. coli* growth in the presence of sodium acetate. Strains WE and WE-214 were flask cultivated in M9 glucose medium (pH 6.0) supplemented with sodium acetate (10 mM). Symbols: WE (filled circles); WE-214 (open circles). The average of two independent experiments is presented.

Figure 2. Effects of the mutated MetE enzymes on *E. coli* growth in the presence of weak organic acids. The WE (filled circles), WE-214 (open circles), WE-CA (filled triangles) and WE-214A (open triangles) strains were incubated in M9 glucose medium (pH 6.0) supplemented with 20 mM sodium acetate (A) or 10 mM sodium propionate (B) in an automatic growth-measuring incubator at 37°C for 28 h. The average of two independent experiments is presented.

Figure 3. Acetate-tolerant MetE enzymes accelerate *E. coli* growth in the presence of HCY and L-isoleucine. The WE (filled circles), WE-214 (open circles), WE-CA (filled triangles) and WE-214A (open triangles) strains were incubated in M9 glucose medium (pH 6.0) supplemented with 8 mM HCY and L-isoleucine (50 μg ml⁻¹) in an automatic growth-measuring incubator at 37°C for 24 h. The average of two independent experiments is presented.

Figure 4. *In vivo* stability of the MetE mutants. Cells of the WE (filled circles), WE-214 (open circles), WE-CA (filled triangles) and WE-214A (open triangles) strains growing exponentially (OD₆₀₀ of 0.3) at 37°C in M9 glucose medium with pH values of 6.0 (A) and 7.0 (B) were treated with 200 μg chloramphenicol ml⁻¹, and then one half of the pH 7.0 culture was shifted to 45°C (C). Samples were collected at the indicated time points and analyzed via Western blotting, as described in the Materials and Methods. The densitometry results were normalized by setting the
Figure 5. Thermal stability of the MetE mutants. The WE (filled circles), WE-214 (open circles), WE-CA (filled triangles) and WE-214A (open triangles) strains were grown in M9 glucose medium in an automatic growth-measuring incubator at 44°C (A). The average of two independent experiments is presented.

Serial dilutions of cultures growing logarithmically at 37°C in M9 glucose medium (OD$_{600}$ of 0.5) were spotted onto M9 glucose or M9 glucose L-methionine (50 μg ml$^{-1}$) agar plates (B). The cells were then incubated for 24 h at 44°C.

Figure 6. Effects of single mutated MetE enzymes on acetate tolerance or thermal stability of the E.coli WE strain. The WE (filled circles), A39 (open circles), C46 (filled triangles), I106 (open triangles) and E713 (filled rectangles) strains were incubated in M9 glucose medium (pH 6.0) supplemented with 20 mM sodium acetate at 37°C for 28 h (A) or in M9 glucose medium (pH 7.0) at 44°C for 10 h (B) in an automatic growth-measuring incubator. The average of two independent experiments is presented.

Figure 7. Combined effects of the mutated MetA and MetE proteins on E. coli growth in the presence of acetate or at elevated temperature. The WE (filled circles), Y229 (open circles), WE-214A (filled triangles) and Y229-214A (open triangles) strains were grown in M9 glucose medium (pH 6.0) supplemented with sodium acetate (20 mM) at 37°C(A) or in M9 glucose medium at 45°C (B) in an automatic growth-measuring incubator. The average of two independent experiments is presented.

Figure 8. Tryptic digestion of the MetE proteins. The MetE enzymes (1 nmol) were digested with 0.01% trypsin (w/v) at 37°C in a total volume of 200 μl of 20 mM potassium phosphate buffer (pH 7.2). Samples were collected at indicated time points and analyzed via SDS-PAGE.
Band intensities were quantified using WCIF Image J software. Symbols: MetE, wt (filled circles), MetE-214 (open circles), MetE-CA (filled triangles) and MetE-214A (open triangles).
Fig. 1
Fig. 3
Fig. 4

Table 1

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<tr>
<th>pH 6.0, 37°C</th>
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<tbody>
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<tr>
<td>MetE-214</td>
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<td>MetE-214A</td>
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<td>MetE-214A</td>
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Fig. 5 - methionine + methionine
Fig. 6
Fig. 7
Fig. 8