

# Specific Growth Inhibition by Acetate of an *Escherichia coli* Strain Expressing Era-dE, a Dominant Negative Era Mutant

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## Abstract

*Escherichia coli* Era is a GTP binding protein and essential for cell growth. We have previously reported that an Era mutant, designated Era-dE, causes a dominant negative effect on the growth and the loss of the ability to utilize TCA cycle metabolites as carbon source when overproduced. To investigate the role of Era, the gene expression in the cells overproducing Era-dE was examined by DNA microarray analysis. The expression of *lipA* and *nadAB*, which are involved in lipoic acid synthesis and NAD synthesis, respectively, was found to be reduced in the cells overproducing Era-dE. Lipoic acid and NAD are essential cofactors for the activities of pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex and glycine cleavage enzyme complex. The expression of numerous genes involved in dissimilatory carbon metabolism and carbon source transport was increased. This set of genes partially overlaps with the set of genes controlled by cAMP-CAP in *E. coli*. Moreover, the growth defect of Era-dE overproduction was specifically enhanced by acetate but not by TCA cycle metabolites both in rich and synthetic media. Intracellular serine pool in Era-dE overproducing cells was found to be increased significantly compared to that of the cells overproducing wild-type Era. It was further found that even the wild-type *E. coli* cells not overproducing Era-dE became sensitive to acetate in the presence of serine in a medium. We propose that when Era-dE is overproduced, carbon fluxes to the TCA cycle and to C1 units become impaired, resulting in a higher cellular serine concentration. We demonstrated that such cells with a high serine concentration became sensitive to acetate, however the

reason for this acetate sensitivity is not known at the present.

## Introduction

Era is a 34 kDa GTP-binding protein with GTPase activity and is essential for growth in *Escherichia coli* (March *et al.*, 1988; Chen *et al.*, 1990). Era is both cytosolic and associated with membrane and auto-phosphorylated in a GTP-dependent manner (Lin *et al.*, 1994; Sood *et al.*, 1994). Era was originally found in *E. coli* (Ahnn *et al.*, 1986) and has been shown to be highly conserved not only in prokaryotes but also in eukaryotes, including humans (Britton *et al.*, 2000).

The crystal structure of *E. coli* Era has been solved (Chen *et al.*, 1999). It consists of two domains: a GTP-binding N-terminal domain whose three dimensional structure is similar to those of the GTPase domains of other G-proteins (Bourne *et al.*, 1991) and a C-terminal domain which is conserved within the Era subfamily, containing a putative KH-like RNA binding motif (Johnstone *et al.*, 1999).

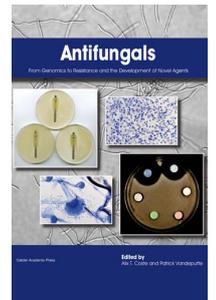
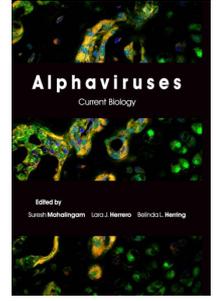
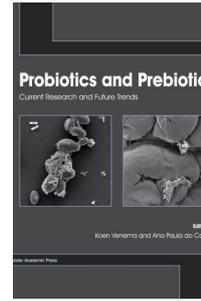
Like all GTP-binding proteins, the Era subfamily has three conserved regions (G1, G3 and G4) known to be associated with the bound guanidine nucleotide (Bourne *et al.*, 1991). However, it does not have homology with the other Ras-family proteins in the G2 region which is known as an effector region and interacts with other regulatory proteins such as GAP (Sigal *et al.*, 1986). To study the role of the putative effector region of Era, a mutant designated Era-dE (deletion of effector region), in which a 10-residue segment (from position 40 to 49) has been deleted from the effector region, has been constructed (Shimamoto and Inouye, 1996). Era-dE is not able to complement the defective chromosomal *era* gene. It shows a severe growth inhibition in a wild-type *E. coli* strain when overproduced in synthetic medium containing tricarboxylic acid (TCA) cycle intermediates such as succinate, 2-oxoglutarate, malate, and fumarate as the sole carbon sources (Pillutla *et al.*, 1996). However, the growth inhibition is less severe when glucose is used as the sole carbon source. In fact, cells overproducing Era-dE lose the ability to utilize the TCA cycle intermediates as the sole carbon source judging from ES microtiter plates (Biolog Inc.), which allow detection of the cell's ability to produce reducing agent from any of 95 different carbon sources. Overproduction of Era-dE not only causes an inhibition of cell growth, but also shows a significant decrease in cell viability (Pillutla *et al.*, 1996). Interestingly, Era depletion at 42°C increases the utilization of TCA

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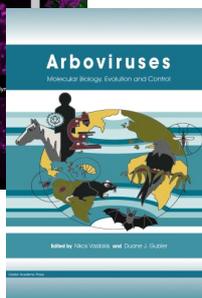
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cycle metabolites as sole carbon sources (Lerner and Inouye, 1991). It should be noted that the TCA cycle plays two essential roles in metabolism. First, the cycle is responsible for the total oxidation of acetyl coenzyme A (CoA). Second, TCA cycle intermediates are required in the biosynthesis of several amino acids (Clark and Cronan, 1996). *E. coli* cells can utilize acetate as the sole carbon source, however mutants lacking any one of the TCA cycle enzymes fail to grow with acetate as the sole carbon.

Here we report that overproduction of Era-dE reduced the expression of genes involved in lipoic acid synthesis and NAD synthesis and increased the expression of numerous genes involved in the dissimilatory carbon metabolism and carbon source transport. Moreover, the growth defect of Era-dE overproducing cells is specifically enhanced by acetate. Intracellular serine pool was found to be increased after overproducing Era-dE. In the absence of sufficient flux of carbon to the TCA cycle and to C1 units, Era-dE overproducing cells may increase the cellular concentration of serine, resulting in the severe growth defect in the presence of acetate. Although the exact reason for the toxicity is not known at present, this is the first report that acetate functions as a growth inhibitor under a certain metabolic condition at neutral pH.

## Results and Discussion

### Effect of Era-dE Overproduction on Gene Expression

To investigate the function of Era *in vivo*, we first examined the effects of Era-dE overproduction on the global gene expression by DNA microarray analysis. Total RNAs were isolated from DH5 $\alpha$  cells harboring pJR302 and pJR302-dE, grown for 2 h in LB medium in the presence of 1 mM IPTG and gene expression between wild-type Era and Era-dE overproducing cells was compared.

Noticeably, the gene expression of *lipA*, *nadA* and *nadB* was severely repressed in the Era-dE overproducing cells (Table 1). The gene products of *lipA* and *nadAB* are involved in lipoic acid and NAD biosynthesis respectively, which are indispensable cofactors for pyruvate dehydrogenase complex (pyruvate to acetyl-CoA) (Guest, 1978), 2-oxoglutarate dehydrogenase complex (2-oxoglutarate to succinyl-CoA) (Pettit and Reed, 1967) and glycine cleavage enzyme complex (glycine to CO<sub>2</sub>, NH<sub>3</sub> and C1 unit) (Steiert *et al.*, 1990). These complexes share a common component, the *lpd*-encoded lipoamide dehydrogenase which covalently binds lipoic acid. A mutation in the *lpd* gene results in a nutritional requirement for both acetate and succinate (Steiert *et al.*, 1990), suggesting that this component is essential for activation of the TCA cycle metabolism. NAD is essential for all living cells. It is also an important cofactor in several oxidation-reduction reactions (Penfound and Foster, 1996). The *nadAB* deficient mutant shows auxotrophy for niacin, nicotinamide, nicotinamido mononucleotide or quinolinic

acid (Penfound and Foster, 1996). The reduced *nadAB* expression will affect not only the TCA cycle metabolism but also other metabolisms, which require NAD as a cofactor. It can be speculated that the loss of ability to utilize TCA cycle metabolites as the carbon source in Era-dE overproducing cells may be due to the reduced level of indispensable cofactors, lipoic acid and NAD. Since pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase require both lipoic acid and NAD, the reduced expression of *lipA* and *nadAB* in Era-dE overproducing cells may have a more severe effect on the reactions involved in these dehydrogenases than on other reactions which require only NAD but not lipoic acid.

Bacteria can utilize a wide range of carbon sources and have a variety of specific transport systems for these sources (Gunnewijk *et al.*, 2001; Tchiew *et al.*, 2001). *E. coli* can grow on a variety of compounds such as hexitols and hexonic and hexuronic acids (Lin, 1996). As shown in Table 1, expression of genes related to dissimilatory pathways for sugar, polyols, and carboxylates was increased significantly in Era-dE overproducing cells. These include catabolic pathways for (i) hexuronates (*kgdT* and *uxaC*), (ii) hexitol (*srlR* and *srlA*), (iii) D-galactose and D-galactoside (*mgIAB* and *melAB*), (iv) maltodextrines and maltose (*malFGKMS* and *lamB*), (v) methylpentose (*fucK* and *fucI*), (vi) pentose (*rbsC* and *araG*), (vii) hexitol (*gatY*), (viii) D-glucoside (*treC*), metabolic pathway of glycerol (*glpF*) and periplasmic acid glucose-1 phosphatase (G-1-P) (*agp*). Interestingly, some of the gene expressions are controlled by cAMP-CAP, such as *malFG*, *malKM*, *lamB*, *melAB* (through MelR) and *glpF*, suggesting that Era-dE overproduction affects the carbon source metabolism. Inability to utilize TCA cycle metabolites as carbon source in Era-dE overproducing cells may induce these carbon source metabolism to replenish energy source.

### Effect of Metabolites on the Growth of Era-dE Overproducing Cells

We reported that the utilization of TCA cycle metabolites as sole carbon sources is increased in Era-depletion cells at 42°C (Lerner and Inouye, 1991). On the other hand, its ability is decreased in Era-dE overproducing cells (Pillutla *et al.*, 1996). To examine whether cellular metabolites will affect the Era-dE dominant negative phenotype, DH5 $\alpha$  cells harboring pJR302 and pJR302-dE were grown in LB medium in the presence of different cellular metabolites. The growth of DH5 $\alpha$  cells harboring pJR302-dE was significantly slowed down by the addition of 1 mM IPTG, confirming that Era-dE has a dominant negative phenotype as previously observed (Pillutla *et al.*, 1996). When various metabolites were added to the medium, growth of the cells overproducing the Era-dE was severely inhibited only in the presence of acetate (10 mM) out of 11 metabolites examined (citrate, isocitrate, succinate, fumarate, malate, 2-oxoglutarate, glycerol, pyruvate, glyoxylate, glucose, and acetate). The acetate effect was not observed in cells overproducing wild-type Era and could not be suppressed by adding TCA cycle metabolites in the

Table 1. Genes showing significant expression ratios.

| Functional Group           | Gene                  | Gene Product   | Ratio (Era-dE/wild-type Era)    |         |
|----------------------------|-----------------------|--|---------------------------------|---------|
|                            |                       |  | None                            | Acetate |
| Acetate metabolism         | <i>aceA</i>           | Isocitrate lyase   | N.S.                            | 4.39    |
|                            | <i>aceB</i>           | Malate synthase  | N.S.                            | 5.33    |
|                            | <i>aceK</i>           | Isocitrate dehydrogenase (IDH) kinase/phosphatase            | N.S.                            | 5.59    |
|                            | <i>mdh</i>            | Malate dehydrogenase   | N.S.                            | 3.49    |
| Methylpentose pathway      | <i>fucI</i>           | Fucose isomerase   | 4.58                            | 11.95   |
|                            | <i>fucK</i>           | Fucokinase   | 3.07                            | 2.35    |
|                            | <i>fucO</i>           | Lactaldehyde reductase                                       | N.S.                            | 3.48    |
| Maltodextrine pathway      | <i>malF</i>           | Inner membrane protein                                       | 4.10                            | 17.86   |
|                            | <i>malG</i>           | Maltose transport protein                                    | 2.74                            | 8.96    |
|                            | <i>malK</i>           | Maltose/maltodextrin transport ATP-binding protein           | 4.62                            | 14.50   |
|                            | <i>malM</i>           | Maltose operon periplasmic protein                           | 2.15                            | 8.49    |
|                            | <i>malS</i>           | alpha-amylase, periplasmic                                   | 3.14                            | 4.41    |
|                            | <i>malT</i>           | MalT regulatory protein                                      | N.S.                            | 2.85    |
|                            | <i>lamB</i>           | Maltoporin (lambda receptor protein)                         | 3.44                            | 7.33    |
|                            | D-galactoside pathway | <i>melA</i>  | alpha-galactosidase (melibiase) | 6.27    |
| <i>melB</i>                |                       | Melibiose carrier protein (melibiose permease)               | 3.50                            | 2.80    |
| <i>melR</i>                |                       | Melibiose operon regulatory protein                          | N.S.                            | 2.10    |
| D-galactose pathway        | <i>mgIA</i>           | Galactoside transport ATP-binding protein                    | 3.23                            | 34.30   |
|                            | <i>mgIB</i>           | D-galactose binding protein                                  | 2.46                            | 14.13   |
| Pentose pathway            | <i>rbsC</i>           | Ribose transport system permease                             | 3.46                            | 4.05    |
|                            | <i>rbsK</i>           | Ribokinase   | 2.96                            | 5.31    |
|                            | <i>araG</i>           | L-arabinose transport ATP binding protein                    | 2.35                            | 2.49    |
| Hexitol pathway            | <i>srlA</i>           | PTS system, glucitol/sorbitol specific IIBC component        | 3.43                            | 3.96    |
|                            | <i>srlR</i>           | Glucitol operon repressor                                    | 2.25                            | 3.23    |
|                            | <i>gatY</i>           | Tagatose bisphosphate aldolase                               | 3.16                            | 4.30    |
|                            | <i>gatZ</i>           | Putative tagatose 6-phosphate kinase 1                       |                                 |         |
|                            | <i>agaZ</i>           | Putative tagatose 6-phosphate kinase 2                       | 2.18                            | 2.76    |
|                            | <i>ptkB</i>           | PTS enzyme II, galactitol specific, protein B                | N.S.                            | 3.34    |
| D-glucoside pathway        | <i>treC</i>           | Trehalose 6-phosphate hydrolase                              | 8.85                            | 8.13    |
| Hexuronate pathway         | <i>uxaC</i>           | Uronate isomerase (glucuronate isomerase) (uronic isomerase) | 5.64                            | 3.45    |
|                            | <i>uxuA</i>           | Mannonate dehydratase  | N.S.                            | 3.14    |
|                            | <i>kdgT</i>           | Probable 2-keto-3-deoxygluconate transport protein           | 2.06                            | 5.70    |
|                            | <i>lacA</i>           | Galactoside acetyltransferase                                | 4.62                            | 5.94    |
| Glycerol pathway           | <i>glpF</i>           | Glycerol diffusion facilitator                               | 3.05                            | 5.67    |
|                            | <i>glpX</i>           | GlpX protein, may be involved in glycerol metabolism         | 2.07                            | 4.59    |
| Acid glucose-1-phosphatase | <i>agp</i>            | Glucose-1-phosphatase  | 4.84                            | 9.48    |
| Serine metabolism          | <i>sdaB</i>           | L-serine deaminase   | N.S.                            | 3.34    |
|                            | <i>sdaC</i>           | Probable serine transport                                    | N.S.                            | 3.21    |
| Tryptophan metabolism      | <i>tnaA</i>           | Tryptophanase  | 2.06                            | 135.89  |
|                            | <i>tnaB</i>           | Low affinity tryptophan permease                             | 5.58                            | 53.18   |
| Unknown function           | <i>yjcU</i>           | Hypothetical protein   | 0.11                            | 30.82   |
|                            | <i>yjcX</i>           | Hypothetical ABC transporter periplasmic binding protein     | 0.15                            | 39.96   |
|                            | <i>yjcW</i>           | Hypothetical ABC transporter ATP-binding protein             | 0.19                            | 46.54   |
|                            | <i>yjcV</i>           | Hypothetical transcriptional regulator                       | 0.18                            | 64.09   |

Table 1. Continued

|                          |             |  |      |      |
|--------------------------|-------------|--|------|------|
| Lipoic acid biosynthesis | <i>lipA</i> | Lipoic acid synthetase                         | 0.42 | 0.26 |
| NAD biosynthesis         | <i>nadA</i> | Quinolinate synthetase A                       | 0.34 | 0.11 |
|                          | <i>nadB</i> | L-aspartate oxidase (quinolinate synthetase B) | 0.18 | 0.10 |

Genes that were induced by more than 2.0 fold or decreased by more than 50% are shown.  
N.S.; Not significant difference.

medium, suggesting that the acetate effect is not simply due to absence of TCA cycle metabolites in the cells.

### Growth Defect of Era-dE Overproducing Cells in the Presence of Acetate

To define more precisely the acetate effect on Era-dE overproducing cells, DH5 $\alpha$  harboring pJR302 or pJR302-dE was grown in M9 (0.4% glucose and 0.2% Casamino acids) medium to log phase and then 50 mM acetate and/or 1 mM IPTG were added to the medium. DH5 $\alpha$  cells harboring pJR302 grew well under all conditions tested (Figure 1A). DH5 $\alpha$  cells harboring pJR302-dE grew well with or without acetate. When Era-dE production was induced with IPTG, the cell growth was stopped within 3.5 h in the absence of acetate (Figure 1B). However, after 35 h incubation, the cell turbidity reached almost the same as that of the cells without IPTG. On the other hand, in the presence of both IPTG and acetate, the cell growth was completely inhibited within 2.5 h of induction and the cell turbidity did not increase at all even after 35 h incubation.

Table 2 shows that acetate toxicity was also observed on solid media. Colony forming units (CFU) of DH5 $\alpha$  cells harboring pJR302-dE on LB plates containing either IPTG or acetate was decreased to 60% as compared to control plates without IPTG and acetate. On the other hand, CFU on the plates containing both IPTG and acetate was dramatically decreased by a factor of  $10^4$  as compared to the control plates. Under the same condition, no significant growth defect was seen in cells overproducing wild-type Era.

It has been shown that cell viability dropped significantly when Era-dE was overproduced (Pillutla

*et al.*, 1996). To examine whether the cell viability of Era-dE overproducing cells drops more severely in the presence of acetate, DH5 $\alpha$  cells harboring pJR302-dE grown to log phase were incubated in M9 (0.4% glucose and 0.2% Casamino acid) medium with 1 mM IPTG or 1 mM IPTG plus 50 mM acetate for 0, 1, 2, 4 and 6 h, followed by plating on LB plates. The rates of the viability reduction of the Era-dE overproducing cells with or without acetate were almost identical (Figure 2). These results indicate that the acetate effect on Era-dE overproducing cells is on the rate of cell growth but not on the cell viability, indicating that the Era-dE dominant negative phenotype is not directly enhanced by acetate. The acetate effect on cell growth observed is thus likely caused at the level of a key function(s) required for cell growth such as energy metabolism.

Roe *et al.* recently showed that at pH 6.0 acetate accumulates inside cells to severely inhibit cell growth (Roe *et al.*, 1998). To examine whether the growth defect was due to pH changes of the medium, DH5 $\alpha$  cells harboring pJR302 or pJR302-dE were grown to log phase. IPTG was then added in the presence of acetate and pH changes of the medium were monitored. The pH of the medium did not change with the cells overproducing either wild-type Era or Era-dE (not shown), suggesting that the growth defect due to Era-dE overproduction in the presence of acetate was not caused by the pH effect.

We also excluded the possibility that acetate might cause higher gene expression of Era-dE. The total amount of Era-dE produced with acetate was identical to that without acetate as determined by quantitative western blot analysis (not shown).

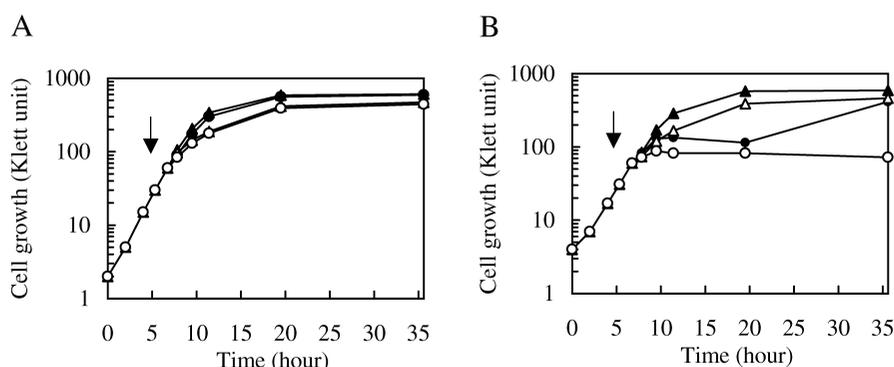


Figure 1. Growth of DH5 $\alpha$  expressing either (A) wild-type Era or (B) Era-dE mutant with or without acetate. DH5 $\alpha$  harboring pJR302 and pJR302-dE in M9 medium to log phase were incubated with ( $\blacktriangle$ ) none, ( $\triangle$ ) 50 mM acetate, ( $\bullet$ ) 1 mM IPTG, and ( $\circ$ ) 50 mM acetate plus 1 mM IPTG. The arrows indicate the time point at which acetate and/or IPTG were added in the cell culture.

Table 2. Effect of acetate on the growth of Era-dE overproducing cells.

|                | No. of colony on LB plate ( $10^5$ ) |                         |
|----------------|--------------------------------------|-------------------------|
|                | DH5 $\alpha$ /pJR302                 | DH5 $\alpha$ /pJR302-dE |
| KCl            | 1.3                                  | 1.0                     |
| KCl + IPTG     | 1.5                                  | 0.6                     |
| Acetate        | 1.5                                  | 0.6                     |
| Acetate + IPTG | 1.6                                  | 0.0002                  |

Cells were grown on LB plates in the presence of 50 mM potassium acetate and 1 mM IPTG at 37°C.

### Genes Affected Specifically in the Presence of Acetate

The gene expression in Era-dE overproducing cells was also examined in the presence of acetate using the DNA microarray analysis (Table 1). Gene expression of *lipA*, *nadA*, and *nadB*, which was reduced in the absence of acetate was also found to be reduced (Table 1). Genes involved in dissimilatory carbon metabolism and carbon source transport were also induced. On the other hand, the expression of genes for acetate utilization (*aceBAK* and *mdh*) was induced specifically in the presence of acetate when Era-dE was overproduced. Expression of *aceBAK* is known to be induced in the presence of acetate or fatty acids as sole carbon source (Cronan and LaPorte, 1996). This induction, however, has been shown to be prevented by the presence of preferred carbon sources such as glucose, glycerol or pyruvate. Notably, the expression of *aceBAK* in Era-dE overproducing cells was induced even when grown in a rich medium. Era-dE overproduction may lead to the induction of the *aceBAK* expression to reduce the cellular concentration of acetate. The gene expression of the

*yjcUVWX* operon was highly induced in Era-dE overproducing cells specifically in the presence of acetate but highly reduced in the absence of acetate. The *yjcW* gene product is a putative ATP-binding component of a transport system and similar to the galactoside transport ATP-binding protein, MglA. The *yjcX* gene product is a *lacI*-type transcriptional regulator. This operon may be involved in sugar transport system and may be involved in the acetate effect of Era-dE overproduction.

### Measurement of Amino Acid Pools in Era-dE Overproducing Cells

To examine the mechanism of the acetate effect on Era-dE overproducing cells, we measured free amino acid pools in Era-dE overproducing cells in the presence of acetate since some amino acids, such as serine and valine are known to be toxic for cell growth (Hama *et al.*, 1990). The free amino acid pools were measured in the presence of acetate 2 h after inducing either wild-type Era or Era-dE. Table 3 demonstrates that among 16 amino acids examined, the cellular concentration of serine increased three times upon induction of Era-dE in the presence of acetate. Other amino acid pools, including valine, were not significantly affected except the arginine pool, which was significantly decreased in the cells overproducing wild-type Era. Acetate did not affect the serine concentration in the cells expressing wild-type Era.

The free amino acid pools in cells overproducing Era-dE in the presence of acetate were further examined at different incubation time points to determine whether the serine accumulation correlates with the growth defect of Era-dE overproduction. Figure 3 shows that the cellular concentration of serine increased at 2 h incubation when the cell growth had not yet been inhibited. After 3.5 h incubation the cell growth became severely affected, while the intracellular serine pool was further increased 9-fold compared to that of cells before adding IPTG. After 5 h incubation the cell growth completely stopped and the cell viability significantly decreased. At this time point all amino acid pools were decreased compared to those of earlier time points. However, the serine pool was still retained significantly higher than the others. These data suggest that the accumulation of intracellular serine pool may lead to the growth defect when Era-dE is overproduced. However, serine accumulation itself is unlikely to be the primary effect for the growth defect since the observed growth defect caused by acetate

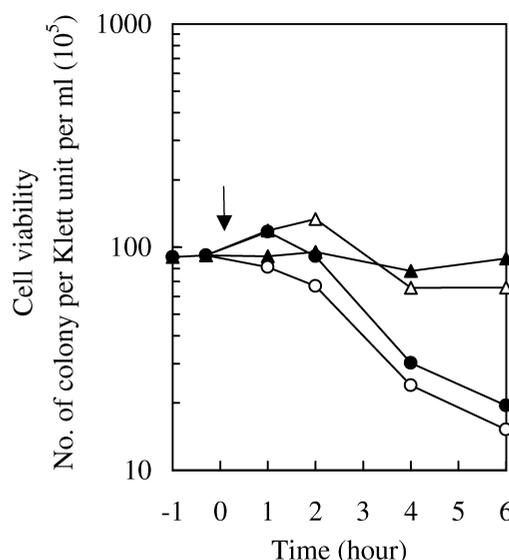


Figure 2. Viability of DH5 $\alpha$  expressing Era-dE mutant with or without acetate. DH5 $\alpha$  harboring pJR302-dE grown in M9 medium with (▲) 50 mM KCl, (△) 50 mM acetate, (●) 50 mM KCl plus 1 mM IPTG, and (○) 50 mM acetate plus 1 mM IPTG were plated on LB medium and incubated at 37°C overnight. The arrow indicates the time point at which KCl, acetate and/or IPTG were added in the cell culture.

Table 3. Intracellular amino acid pools in the cells overproducing Era-dE.

|               | Amino acid ng per mg dried cell |               |                         |               |
|---------------|---------------------------------|---------------|-------------------------|---------------|
|               | DH5 $\alpha$ /pJR302            |               | DH5 $\alpha$ /pJR302-dE |               |
|               | Before                          | After         | Before                  | After         |
| Aspartate     | 234 $\pm$ 44                    | 180 $\pm$ 57  | 228 $\pm$ 28            | 194 $\pm$ 21  |
| Threonine     | 144 $\pm$ 56                    | 186 $\pm$ 65  | 176 $\pm$ 2             | 165 $\pm$ 41  |
| Serine        | 52 $\pm$ 18                     | 75 $\pm$ 18   | 73 $\pm$ 31             | 228 $\pm$ 12  |
| Glutamate     | 631 $\pm$ 184                   | 741 $\pm$ 138 | 463 $\pm$ 103           | 319 $\pm$ 42  |
| Glycine       | 943 $\pm$ 55                    | 947 $\pm$ 110 | 963 $\pm$ 71            | 707 $\pm$ 8   |
| Alanine       | 230 $\pm$ 68                    | 203 $\pm$ 51  | 203 $\pm$ 17            | 225 $\pm$ 7   |
| Cysteine      | 168 $\pm$ 5                     | 110 $\pm$ 58  | 103 $\pm$ 7             | 84 $\pm$ 9    |
| Valine        | 351 $\pm$ 86                    | 315 $\pm$ 32  | 369 $\pm$ 19            | 262 $\pm$ 42  |
| Methionine    | 138 $\pm$ 30                    | 98 $\pm$ 12   | 136 $\pm$ 4             | 97 $\pm$ 21   |
| Isoleucine    | 171 $\pm$ 42                    | 142 $\pm$ 17  | 125 $\pm$ 5             | 99 $\pm$ 21   |
| Leucine       | 699 $\pm$ 209                   | 582 $\pm$ 8   | 582 $\pm$ 59            | 450 $\pm$ 157 |
| Tyrosine      | 208 $\pm$ 10                    | 325 $\pm$ 87  | 242 $\pm$ 75            | 170 $\pm$ 6   |
| Phenylalanine | 353 $\pm$ 93                    | 482 $\pm$ 117 | 344 $\pm$ 5             | 239 $\pm$ 54  |
| Lysine        | 1683 $\pm$ 76                   | 879 $\pm$ 121 | 1642 $\pm$ 81           | 1003 $\pm$ 41 |
| Histidine     | 198 $\pm$ 2                     | 186 $\pm$ 23  | 204 $\pm$ 20            | 165 $\pm$ 31  |
| Arginine      | 530 $\pm$ 125                   | 51 $\pm$ 9    | 611 $\pm$ 187           | 388 $\pm$ 157 |

Cells grown to early log phase in LB medium were incubated in the presence of 1 mM IPTG and 50 mM potassium acetate for 0 h (Before) and 2 h (After). Amino acids are expressed as ng per mg dry cell. Mean values standard deviations were obtained from two independent cultivations. 1 mg of dry cell weight corresponds to  $2.4 \times 10^9$  cells.

could not be suppressed by isoleucine (not shown), which is known to remove the serine toxicity (Hama *et al.*, 1990). Therefore, the acetate toxicity observed in the present paper is different from the known serine toxicity causing impairment of amino acid biosynthesis.

We also observed that the expression of *tnaA* (L-tryptophanase encoding gene) and *sdaC* (L-serine deaminase encoding gene) was induced in the Era-dE overproducing cells especially in the presence of acetate (Table 1). Serine accumulation in Era-dE overproducing cells in the presence of acetate may lead to

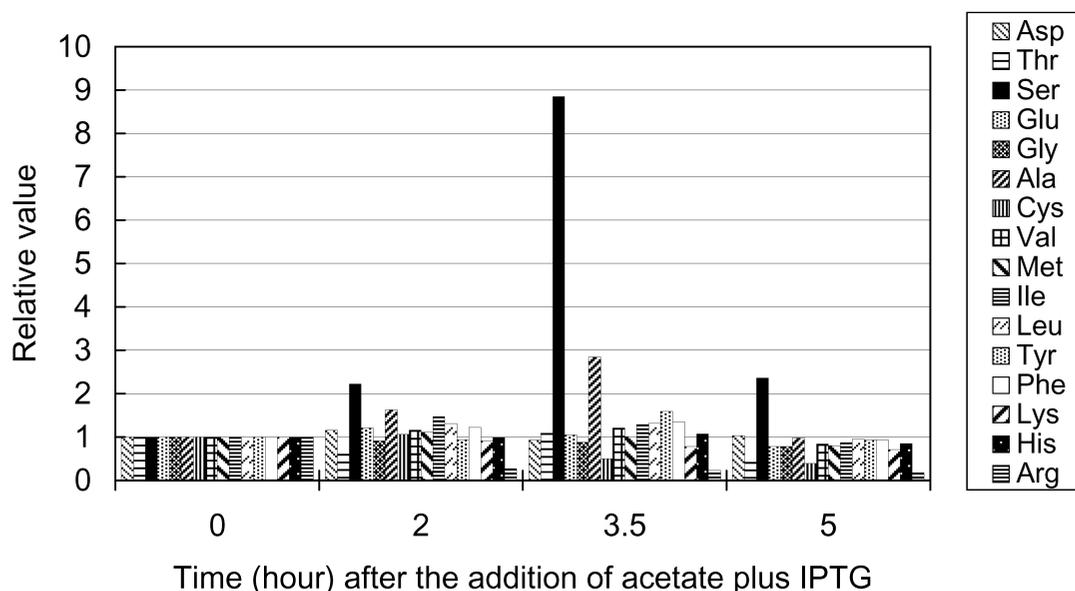


Figure 3. Intracellular amino acid pools in the cells overproducing Era-dE. DH5 $\alpha$  harboring pJR302-dE grown in LB medium to early log phase was incubated with 1 mM IPTG and 50 mM potassium acetate for 0, 2, 3.5, and 5 h. Cells were harvested by centrifugation and washed in ice-cold distilled water. Intracellular amino acids in the cell pellet were extracted in a boiling water bath for 10 min. After centrifugation (5 min at  $15,000 \times g$ ), all amino acids in the supernatant were subsequently quantitated with an amino acid analyzer. Amount of each amino acid at 0 h incubation was taken as 1, calculated from following values expressed as ng per mg dry cell; Asp, 174 ng/mg; Thr, 161 ng/mg; Ser, 46 ng/mg; Glu, 453 ng/mg; Gly, 931 ng/mg; Ala, 164 ng/mg; Cys, 186 ng/mg; Val, 291 ng/mg; Met, 130 ng/mg; Ile, 121 ng/mg; Leu, 462 ng/mg; Tyr, 274 ng/mg; Phe, 315 ng/mg; Lys, 1440 ng/mg; His, 216 ng/mg; and Arg, 583 ng/mg.

induction of these genes to reduce the amount of accumulated serine since not only L-serine deaminase but also L-tryptophanase can convert serine to pyruvate. The *sdaCB* and *tnaAB* operons require cAMP-CAP for transcription (McFall and Newman, 1996). Since Era-dE overproducing cells induce some of genes controlled by cAMP-CAP (Table 1), the expression of *sdaC* and *tnaA* may also be induced by cAMP-CAP control system. However, since the expression of the *tnaA* gene is known to be induced by tryptophan and at pH 9 (Blankenhorn *et al.*, 1999; Stewart *et al.*, 1986), we cannot rule out the possibility that *tnaA* expression in Era-dE overproducing cells was induced by accumulated tryptophan, which could not be detected with the amino acid analysis performed in the present study.

#### Growth Defect of the Wild-type Strain in the Presence of Acetate and Serine

Next we examined whether the acetate toxicity can be observed even in wild-type strains of *E. coli* without Era-dE overproduction. Cell growth of wild-type *E. coli* strains DH5 $\alpha$  and W3110 was tested on M63 agar plates in the presence and the absence of acetate plus serine. M63 plates used contained 0.4% glucose as a carbon source, 50  $\mu$ g/ml histidine, 0.2 mM isoleucine to eliminate the toxic effect of serine on the amino acid biosynthesis, and different concentrations of serine and potassium acetate as follows; 1, 50 mM acetate only; 2, 10 mM serine only; 3, 50 mM acetate and 1 mM serine and; 4, 50 mM acetate and 10 mM serine. DH5 $\alpha$  and W3110 cells were able to grow under all conditions tested except in the presence of 50 mM acetate and 10 mM serine, although the growth of DH5 $\alpha$  cells was slightly inhibited by presence of 50 mM acetate and 1 mM serine, suggesting that serine if added together with acetate is toxic in spite of the presence of isoleucine in *E. coli*. In the exponential phase, it is known that *E. coli* cells growing on a mixture of amino acids preferentially consume serine prior to utilizing other amino acids (Prüß *et al.*, 1994). In nature, it seems that *E. coli* avoids accumulation of both serine and acetate simultaneously.

On the basis of these results, we speculate that the enhancement of the Era-dE growth defect by acetate is due to the accumulation of intracellular serine pool in the absence of sufficient flux of carbon to the TCA cycle and to C1 units. This may be the reason why the acetate effect can only be seen on cell growth (Figure 1-B) but not on cell viability (Figure 2) since the acetate toxic effect is eliminated when cells are diluted for plating.

We have recently isolated multicopy suppressors for the acetate toxicity of Era-dE overproducing cells using *E. coli* genomic library. Identification and characterization of these genes may shed light into the molecular mechanism of the acetate toxicity.

In Table 4 we also listed the genes, which were significantly affected in Era-dE overproducing cells, but not discussed in the present paper in view of the Era function. However, further studies may reveal the importance of these genes in the signaling pathways associating with Era.

## Experimental Procedures

### Strains, Plasmids and Growth Conditions

Plasmid pJR302 contains the *era* gene under the control of the *lpp* promoter and *lac* promoter-operator in the high copy expression vector, pINIII (Shimamoto and Inouye, 1996). Plasmid pJR302-dE is identical to plasmid pJR302 except that the *era* gene was replaced by *era-dE* gene. *E. coli* strain DH5 $\alpha$  [ $\phi$ 80d/*lacZ* $\Delta$ M15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* ( $r_k^-$ ,  $m_k^+$ ), *supE44*, *relA*, *deoR*,  $\Delta$ (*lacZYA-argF*)U169], (Hanahan, 1983) was used as the host strain for the plasmid bearing wild-type Era or Era-dE mutant. LB, M9 and M63 media were prepared as described previously (Miller, 1972). Media were supplemented, when necessary, with 50  $\mu$ g/ml ampicillin and 1 mM isopropyl-thiogalactopyranoside (IPTG). All the reagents supplemented to medium were adjusted to pH 7.0. Unless otherwise specified, cells were grown at 37°C with constant shaking. Growth was monitored with a Klett-Summerson photoelectric colorimeter (no. 66 filter). One Klett unit corresponded to  $7 \times 10^6$  cells per ml. Viable cells were counted by the plating of diluted cultures on LB plates containing 1.5% agar, followed by overnight incubation of the plates at 37°C.

### DNA Microarray Analysis

For RNA extraction, cells grown in 20 ml LB medium supplemented with 50  $\mu$ g/ml ampicillin at 37°C to early log phase were incubated for 2 h in the presence of 1 mM IPTG with or without 50 mM potassium acetate (pH 7.0). RNA samples were prepared from a 9-ml cell culture with the hot phenol method described previously (Samientos *et al.*, 1983). Total RNA samples (200  $\mu$ g) purified further with RNeasy Mini Kit (QIAGEN) were treated with RNase free-DNase I, followed by phenol-chloroform extraction. 100 units of AMV reverse transcriptase were used to label mRNAs in 80  $\mu$ g of total RNAs with 0.1 mM Cy3-dUTP and Cy5-dUTP (Amersham), using 300 pmol pd(N)<sub>6</sub> Random Hexamer (Amersham) as primer. *E. coli* gene array, IntelliGene *E. coli* CHIP Version  $\beta$  was obtained from TAKARA shuzo, which is arrayed and immobilized with 3437 DNA fragments. This represents approximately 80% of ORF of *E. coli* K-12 W3110. Hybridizations were done as recommended by the manufacturer. Genes that were induced  $\geq 2.0$  fold or decreased by more than 50% are shown in Table 1 and Table 4.

### Immunoblot Analysis

Cells grown in M9 medium to log phase were harvested and suspended in 50 mM Tris-HCl (pH 8.0)-1% SDS. Equivalent protein amounts were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel (10% polyacrylamide) followed by immunoblotting with anti-Era antiserum as described previously (Gallop and March, 1991). Proteins were assayed by the method of Bradford (Bradford, 1976) using a reagent purchased from BioRad with crystalline bovine serum albumin as standard.

Table 4-A. Genes showing significant expression ratios.

| Gene             | Gene Product   | Ratio (Era-dE/wild-type Era) |         |
|------------------|--|------------------------------|---------|
|                  |  | None                         | Acetate |
| <i>aldA</i>      | Aldehyde dehydrogenase   | 0.45                         | 3.28    |
| <i>aldB</i>      | Aldehyde dehydrogenase   | 4.89                         | 4.60    |
| <i>aphA/napA</i> | Acid phosphatase   | N.S.                         | 5.09    |
| <i>aspA</i>      | Aspartate ammonia lyase  | N.S.                         | 4.98    |
| <i>atpG</i>      | ATP synthase gamma chain   | N.S.                         | 2.63    |
| <i>cpdB</i>      | 2',3'-cyclic-nucleotide 2'-phosphodiesterase   | 2.02                         | 3.14    |
| <i>csiE</i>      | Stationary phase inducible protein   | 2.05                         | 2.73    |
| <i>cynX</i>      | Cyanate transport protein  | N.S.                         | 5.26    |
| <i>cysJ</i>      | Sulfite reductase beta subunit   | 9.51                         | 3.85    |
| <i>cytR</i>      | Regulatory gene for <i>deo</i> operon  | N.S.                         | 2.29    |
| <i>dctA</i>      | Transport system for dicarboxylic acid, C <sub>4</sub> amino acids                   | N.S.                         | 7.76    |
| <i>eaeH</i>      | Attaching and effacing protein homolog   | N.S.                         | 2.35    |
| <i>exo</i>       | 5'-3' exonuclease  | N.S.                         | 2.71    |
| <i>fadA</i>      | 3-ketoacyl-CoA thiolase  | N.S.                         | 3.02    |
| <i>fadH</i>      | 2,4-dienoyl-CoA reductase  | 0.39                         | 3.03    |
| <i>fdoH</i>      | Formate dehydrogenase-O iron-sulfur subunit  | N.S.                         | 2.14    |
| <i>flgB</i>      | Rod protein  | 3.15                         | 16.51   |
| <i>flgD</i>      | Basal-body rod modification protein  | 2.48                         | 5.63    |
| <i>flgE</i>      | Flagellar hook protein   | 2.33                         | 4.72    |
| <i>flhC</i>      | Flagellar transcriptional activator  | N.S.                         | 3.07    |
| <i>gadA</i>      | Glutamate decarboxylase alpha  | N.S.                         | 3.00    |
| <i>gadB</i>      | Glutamate decarboxylase beta   | N.S.                         | 2.94    |
| <i>glcF</i>      | Glycolate oxidase iron-sulfur subunit  | N.S.                         | 2.45    |
| <i>glcG</i>      | Hypothetical protein   | N.S.                         | 2.97    |
| <i>glgC</i>      | Glucose-1-phosphate adenylyltransferase  | N.S.                         | 2.48    |
| <i>gloB</i>      | Probable hydroxyacylglutathione hydrolase  | N.S.                         | 2.60    |
| <i>gltD</i>      | Hypothetical protein   | 35.50                        | 10.40   |
| <i>hdeA</i>      | Unknown function, sigmaS dependent promoter  | 5.52                         | 3.16    |
| <i>hdeB</i>      | Unknown function, sigmaS dependent promoter  | 4.79                         | 3.05    |
| <i>hyaB</i>      | Hydrogenase 1 large subunit  | N.S.                         | 4.97    |
| <i>hybC</i>      | Hydrogenase 2 large subunit, probably  | 14.96                        | 2.68    |
| <i>hypB</i>      | Hydrogenase isoenzymes formation protein   | 14.29                        | 2.47    |
| <i>lit</i>       | Locus in defective prophage e14  | N.S.                         | 2.83    |
| <i>menG</i>      | S-adenosylmethionine   | 2.16                         | 2.99    |
| <i>nrdD</i>      | Oxygen-sensitive ribonucleoside-triphosphate reductase                               | 8.81                         | 4.50    |
| <i>nrfG</i>      | Unknown function, part of formate-dependent nitrite reductase complex                | N.S.                         | 6.20    |
| <i>nuoA</i>      | NADH dehydrogenase I subunit   | N.S.                         | 2.30    |
| <i>nuoB</i>      | NADH dehydrogenase I subunit   | N.S.                         | 2.60    |
| <i>nuoH</i>      | NADH dehydrogenase I subunit   | N.S.                         | 2.36    |
| <i>paaK</i>      | Phenylacetate-CoA ligase   | N.S.                         | 3.06    |
| <i>pckA</i>      | Phosphoenolpyruvate carboxykinase  | 2.02                         | 4.28    |
| <i>pmrD</i>      | Polymyxin B resistance protein   | N.S.                         | 2.68    |
| <i>rpiR</i>      | Hypothetical protein   | 0.27                         | 22.94   |
| <i>slp</i>       | Outer membrane lipoprotein; Carbon starvation and stationary phase inducible protein | 4.95                         | 3.65    |
| <i>sodB</i>      | Superoxide dismutase, iron   | 4.61                         | 2.71    |
| <i>tsx</i>       | Nucleoside-specific channel-forming protein  | N.S.                         | 3.06    |
| <i>xasA</i>      | Extreme acid sensitivity protein   | N.S.                         | 4.31    |
| <i>yaaF</i>      | Nucleoside hydrolase   | 2.23                         | 2.43    |
| <i>yajO</i>      | Putative NAD(P)H-dependent xylose reductase  | 3.54                         | 4.10    |
| <i>ybeJ</i>      | Putative periplasmic binding transport protein                                       | 2.32                         | 2.12    |
| <i>ybeK</i>      | Pyrimidine specific nucleoside hydrolase   | 3.97                         | 9.97    |
| <i>yedE</i>      | Putative transport system permease protein   | 7.36                         | 5.20    |
| <i>yehU</i>      | Putative sensor protein  | N.S.                         | 2.10    |
| <i>yeiA</i>      | Putative oxidoreductase  | 14.30                        | 5.70    |
| <i>yfbL</i>      | Putative aminopeptidase  | N.S.                         | 2.70    |
| <i>yfcX</i>      | Putative fatty oxidation complex alpha subunit                                       | N.S.                         | 2.40    |
| <i>ygiS</i>      | Putative binding protein   | 3.60                         | 3.90    |
| <i>yhiE</i>      | Hypothetical protein   | N.S.                         | 2.80    |
| <i>yihR</i>      | Putative aldose-1-epimerase  | 2.27                         | 4.80    |
| <i>yihS</i>      | Hypothetical protein   | N.S.                         | 4.50    |
| <i>yjcG</i>      | Putative transport protein   | N.S.                         | 7.30    |
| <i>yjfO</i>      | ATP-NAD kinase   | N.S.                         | 3.10    |
| <i>yjhC</i>      | Putative dehydrogenase   | N.S.                         | 2.70    |
| <i>ymfE</i>      | Hypothetical protein   | 4.20                         | 5.80    |
| <i>yzzQ</i>      | Unknown protein  | 2.42                         | 2.40    |

Genes that were induced by more than 2.0 fold are shown.

N.S.; Not significant difference.

Table 4-B. Genes showing significant expression ratios.

| Gene            | Gene Product  | Ratio (Era-dE/wild-type Era) |         |
|-----------------|---|------------------------------|---------|
|                 |   | None                         | Acetate |
| <i>aroF</i>     | Tyrosine sensitive 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase | 0.25                         | 0.20    |
| <i>atzN</i>     | Zinc transporting ATPase (Zn(II) translocating P-type ATPase)           | N.S.                         | 0.19    |
| <i>clpB</i>     | Heat shock protein  | N.S.                         | 0.46    |
| <i>corA</i>     | Magnesium transport protein   | 0.25                         | 0.40    |
| <i>ibpA</i>     | Chaperon, heat inducible protein of HSP20 family                        | N.S.                         | 0.07    |
| <i>ibpB</i>     | Chaperon, heat inducible protein of HSP20 family                        | 0.11                         | 0.07    |
| <i>ldhA</i>     | D-lactate dehydrogenase   | 0.14                         | 0.22    |
| <i>marR</i>     | Probable <i>mar</i> operator repressor                                  | N.S.                         | 0.47    |
| <i>ompA</i>     | Outer membrane protein  | N.S.                         | 0.36    |
| <i>ompX</i>     | Outer membrane protease   | N.S.                         | 0.40    |
| <i>osmB</i>     | Lipoprotein, osmotically inducible                                      | N.S.                         | 0.33    |
| <i>phoB</i>     | Phosphate regulon regulatory protein                                    | N.S.                         | 0.12    |
| <i>phoH</i>     | Phosphate starvation inducible protein                                  | 0.13                         | 0.09    |
| <i>phoR</i>     | Phosphate regulon sensor protein  | N.S.                         | 0.15    |
| <i>phoU</i>     | Phosphate transport system regulatory protein                           | N.S.                         | 0.43    |
| <i>pncB</i>     | Nicotinate phosphoribosyltransferase                                    | N.S.                         | 0.38    |
| <i>proP</i>     | Proline/betaine transport protein                                       | 0.15                         | 0.24    |
| <i>pspA</i>     | Phage shock protein A   | N.S.                         | 0.19    |
| <i>pspB</i>     | Phage shock protein B   | 0.44                         | 0.21    |
| <i>pspC</i>     | Phage shock protein C   | N.S.                         | 0.26    |
| <i>pstB</i>     | Phosphate transport ATP-binding protein                                 | N.S.                         | 0.29    |
| <i>pstC</i>     | Phosphate transport system permease protein                             | N.S.                         | 0.21    |
| <i>pstS</i>     | Phosphate-binding periplasmic protein                                   | 0.21                         | 0.08    |
| <i>smmA</i>     | Small membrane protein A  | N.S.                         | 0.33    |
| <i>tbpA</i>     | Thiamin-binding periplasmic protein                                     | N.S.                         | 0.35    |
| <i>thiC</i>     | Hydroxymethylpyrimidine synthesis                                       | 0.03                         | 0.07    |
| <i>thiD</i>     | Phosphomethylpyrimidine kinase  | N.S.                         | 0.23    |
| <i>thiG</i>     | 4-methyl-5-(beta-hydroxyethyl)thiazole monophosphate synthesis protein  | 0.26                         | 0.12    |
| <i>uhpC</i>     | Regulator of <i>uhpT</i>  | 0.17                         | 0.32    |
| <i>uhpT</i>     | Hexose phosphate transport protein                                      | 0.17                         | 0.29    |
| <i>wza</i>      | Putative polysaccharide export protein                                  | N.S.                         | 0.40    |
| <i>ydeA</i>     | Putative resistant/regulatory protein                                   | N.S.                         | 0.24    |
| <i>yoaC</i>     | Hypothetical protein  | N.S.                         | 0.50    |
| <i>yojH/mqo</i> | Membrane-associated malate:quinone-oxidoreductase                       | 0.06                         | 0.27    |

Genes that were decreased by more than 50% are shown.

N.S.; Not significant difference.

### Intracellular Contents of Amino Acids

Cells harboring pJR302 and pJR302-dE grown to log phase in 100 ml of LB medium were induced by 1 mM IPTG in the presence of 50 mM potassium acetate. Cells (90 ml) were harvested by centrifugation and washed in 90 ml of ice-cold distilled water. The cell pellet was suspended in 10 ml of distilled water. Intracellular amino acids were extracted in a boiling water bath for 10 min. After centrifugation (5 min at 15,000 × g), each supernatant was concentrated to 0.9 ml by an evaporator. All amino acids in the supernatant were subsequently quantitated with an amino acid analyzer (L-8500A, Hitachi Co., Tokyo, Japan) by Dr. H. Takagi, Fukui Prefectural University, Japan. Amino acids are expressed as ng per mg dry cell.

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