The expression of gadA and gadB, which encode two glutamate decarboxylases (GADs) of Escherichia coli, is induced by an acidic environment and participate in acid resistance. In this study, we constructed a polyamine-deficient mutant and investigated the role of polyamines in acid resistance. The expression of gadA and gadB was shown to be dependent on polyamines. For that reason, the polyamine-deficient mutant was completely devoid of GAD activity and was very susceptible to low pH if large amounts of polyamines were not provided. We also showed that the polyamine-deficient mutant contained higher cAMP levels than the isogenic polyamine-proficient wild type, and cAMP negatively regulated the expression of gadA and gadB. Therefore, introduction of the eya (encoding adenylate cyclase) mutation alone into the polyamine-deficient mutant resulted in the increment of GAD activity and thus restored the reduced acid resistance of the mutant. The positive regulators, H-NS (histone-like protein, encoded by the hns gene) and RpoS (alternative RNA polymerase subunit, encoded by rpoS gene), also significantly governed the expression of gadA and gadB, respectively. However, polyamines did not regulate either the intracellular H-NS level or rpoS expression under these culture conditions. These results strongly suggest that there are at least two different regulatory systems in acid resistance, one is positive regulation via a H-NS/RpoS system and the other is negative regulation via a polyamine/cAMP system.

Escherichia coli maintains its internal pH between 7.4 and 7.8 during aerobic growth under an external pH of 5.0–9.0 (33); however, the mechanisms of pH homeostasis remain elusive (19). The ability to survive in an acidic environment is essential to enable them to combat this acidic stress (11, 26).

The glutamate decarboxylase (GAD, EC 4.1.1.15) system has been extensively studied because of its major role in the acid resistance of enteric pathogens such as E. coli O157, Shigella flexneri, and Listeria monocytogenes (6, 7, 8, 29). E. coli contains two genes, gadA and gadB (mapped at 78 and 33 min on its chromosome), which encode two biochemically indistinguishable forms of GAD and are very similar in sequence (2, 9, 24). Sequential reaction through GAD and the putative glutamate/GABA antipporter encoded by the gadC gene allows cells to remove intracellular protons (8).

The expression of gad genes is induced at the stationary phase under normal aerobic conditions and positively responds to acidic, hyper-, and hypo-osmotic shocks (8). The extent of gadA and gadB expression can be differentiated depending on the culture conditions, and both the stationary phase and acidic pH activate distinct regulatory circuits (8). Two distinct regulatory proteins, H-NS (histone-like protein, encoded by hns) and RpoS (alternative RNA polymerase subunit, encoded by rpoS), are known to be involved in the regulation of gadA and gadB expression. The expression of gadA and gadB is negatively regulated by H-NS during the exponential growth phase, while stationary phase induction of gadA and gadB expression requires sigma factor RpoS (sigma S) (5, 6, 8). Another regulatory mechanism via the action of cAMP receptor protein (CRP) has been suggested, in which CRP represses gad expression (5).

Polyamines, the ubiquitous amine-containing molecules, have various important physiological roles (32). They participate in many cellular processes, including modulation of gene expression, signal transduction, protein synthesis, regulation of cell growth and differentiation, and an oxidative defense mechanism (13, 16, 18, 22, 25, 31). Despite their biological importance, the participation of polyamines in acid resistance has been rarely studied to date.

The present work was undertaken to identify the environmental and regulatory factors affecting the expression of gad genes in E. coli. Some evidence that polyamine is essential for the normal expression of gad genes. It is suggested that both H-NS and RpoS are “positively” involved in gad expression as transcriptional regulators. We also suggest that the polyamine/cAMP system as well as the H-NS/RpoS system plays an important part in acid resistance through the regulation of the intracellular cAMP concentration by polyamines. Finally, the possible regulatory circuits for acid resistance via a GAD system are presented.

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1 The abbreviations used are: GAD, glutamate decarboxylase; CRP, cAMP receptor protein; GABA, γ-aminobutyrate; H-NS, histone-like protein; PUT, putrescine; RpoS, RNA polymerase σ subunit; SPD, spermidine; wt, wild type; PBS, phosphate-buffered saline.
Growth in a minimal M9 medium containing 10 mM glycolic acid as a sole carbon source was determined (Table II). A total of 41 of 172 transductants did not produce the lacZ gene in QC2461 with selection of the linked lacZ(o) gene. To construct a Tc-s derivative designated as JIL585, to construct a Tc-s derivative designated as JIL585, to construct a Tc-s derivative designated as JIL585, to construct a Tc-s derivative designated as JIL585. 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detected at an additional concentration of 25 µM for the growth of strains containing the gad::lacZ fusion. Intracellular polyamine concentrations were determined as described under “Experimental Procedures.” Data from two independent experiments are shown as mean ± S.D.

* ND, not detectable (<1 µM).
ylation, and the mixture was extracted with 2 volumes of diethyl ether. After separation by centrifugation, the upper layer was transferred into a new tube and dried in a stream of nitrogen. For analysis of polyamines, a Waters liquid chromatograph (Waters 2690, Waters Co.) equipped with a photodiode array detector (Waters 996, Waters Co.) set at 225 nm was applied. A symmetry C18 column (3.9 × 150 mm, 5 μm, Waters Co.) was used for the separation; the injection volume was 20 μl, and the gradient program proceeded from 95% to 30% methanol. The flow rate was 0.9 ml/min, and spermine was used as an internal standard.

\[ \text{cAMP Determination—Intracellular cAMP concentrations were determined using a CAMP Enzyme Immunoassay Kit (Assay Design Inc.).} \]

Cells grown overnight in LB were washed with a minimal M9 medium and subcultured to an initial OD_{600} of 0.01 in a minimal M9 medium containing 0.4% glucose and cultivated for 24 h. Cells were then harvested, washed, and resuspended to equal cell densities (1 × 10^8) in 0.9% NaCl. cAMP concentrations of cells boiled for 10 min at 100 °C were determined following the manufacturer’s recommendation. The cAMP concentrations were calculated from the typical standard curves, and the detectable range was 0.2–19.8 pmol/ml.

\[ \text{β-Galactosidase Activity Assay—At the end of the growth period, an aliquot of each culture was transferred to new tubes containing 30 μg/ml chloramphenicol (final concentrations) and prechilled on ice.} \]

β-Galactosidase activity was measured by monitoring the hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) as described by Miller and expressed as Miller units (21). Results were confirmed by at least two or three independent experiments.

\[ \text{GAD Activity Assay—Cells grown for 24 h in a minimal M9 medium containing 0.4% glucose were harvested, washed with 0.9% NaCl, and resuspended to equal cell densities (5 × 10^8 cells/ml) in the same solution.} \]

A GAD activity assay was carried out using GAD reagent with some modifications (23). The GAD reagent consisted of 1 g of L-glutamic acid, 0.05 g of brom cresol green (colorimetric indicator), 90 g of NaCl, and 3 ml of Triton X-100 per liter. An aliquot of cells (5 × 10^8 cells) was transferred to a test tube, and 1 ml of GAD reagent was added, mixed immediately, and allowed to vortex vigorously for 30 s. The tubes were then incubated in a 35 °C water bath, and the change in pH was measured after 1 h.

\[ \text{Western Blot Analysis—Strains were grown in a minimal M9 medium containing glucose. At the stationary phase, cells were collected by centrifugation and washed twice with phosphate-buffered saline (pH 7.4). Protein extract was separated on a 12.5% polyacrylamide gel. After transferring the proteins onto nitrocellulose membranes, the proteins were revealed using primary antibody (rabbit anti-H-NS, kindly supplied by Dr. E. Bremer; see Ref. 10), secondary antibodies (anti-rabbit antibody), and an ECL detection kit (Amersham Biosciences). Protein concentrations were determined by the Bradford method (4).} \]

\[ \text{Measurement of Acid Survival Rate—Strains were grown in a minimal M9 medium containing glucose and without polyamines (1 mM) until they entered the stationary phase. After 24 h of cultivation, the cells were harvested and resuspended in a PBS buffer (pH 7.0). Two aliquots of cells (each 1 × 10^8 cells/ml) were transferred to new tubes and adjusted to a 1 ml final volume using PBS buffer (pH 2.5 and 7.0, respectively). The mixtures were incubated for 1 h in a 37 °C water bath. Cells were serially diluted with PBS (pH 7.0), and viable cells were counted on a LB agar plate.} \]

\[ \text{RESULTS} \]

\[ \text{Deficiency of GAD Activity Caused by the Genetic Inactivation of Both gadA and gadB Genes Causes Elevated Sensitivity under Low pH—Despite the importance of GAD under acidic conditions, no gadA/gadB double mutant phenotypes have been reported to date. We constructed a gadA/gadB double mutant in the polyamine-synthesizing wild-type background to investigate the role of GAD on normal cell growth. While each single mutant showed a normal growth pattern, the gadA/gadB double mutant showed a decreased growth rate (−70% of the level of the wild type) in a medium containing glucose as the carbon source (Fig. 1A).} \]

\[ \text{We also assayed GAD activity of the wild type and gadA and/or gadB mutant by investigating the pH variation. Each single mutant containing either gadA or gadB mutation represented approximately half the activity of total GAD, and the gadA/gadB double mutant was completely devoid of GAD activity, indicating that GadA and GadB comprise the whole GAD activity (Fig. 1, B and C). When decarboxylation occurs,} \]

\[ \text{the pH of the GAD reagent progressively increases, causing the indicator color to change from yellow to green (23). The wild type containing normal gadA and gadB genes showed a green color with time, but the gadA/gadB double mutant sustained its yellow color. As might be expected, the single mutant in either gadA or gadB gene, showed mixed colors of green and yellow.} \]

\[ \text{It is well known that GAD is needed for survival under low pH (6, 14). We compared acid resistance under an extreme acidic environment (pH 2.5) in the wild-type and gadA and/or gadB mutants. The wild type showed higher levels of acid resistance than the gadA or gadB single mutant and the gadA/gadB double mutant. Complete deficiency of GAD activity in the gadA/gadB double mutant showed a great sensitivity to low pH (Fig. 1D).} \]

\[ \text{Polyamines Are Necessary to Keep Normal GAD Activity and Acid Resistance through the Induction of gadA and gadB Expression under Normal Aerobic Conditions—To investigate the expression patterns of gadA and gadB with growth time in the presence or absence of polyamines, we measured the β-galactosidase activities of both genes under normal aerobic conditions in a minimal M9 medium containing glucose as the carbon source. We identified that a polyamine-deficient mutant showed growth reduction if polyamines were not provided (Fig. 2A). While the gadA expression was not significantly induced in the absence of polyamines, exogenous supplementation of polyamines restored gadA expression to the wild-type level (Fig. 2B). As well as gadA, the gadB expression was induced at an extremely low level in the absence of polyamines and greatly induced by the supplementation of polyamines (Fig. 2C). The above results indicate that polyamines are definitely required for the induction of both genes (gadA and gadB) in a minimal M9 medium containing glucose as the sole carbon source, even under normal aerobic conditions.} \]
Polyamines and Acid Resistance

Regulation of *gadA* or *gadB* expression, modulation of GAD activity, and restoration of the deleterious effect of acid shock by polyamines. A, growth profiles of the wild type (QC2461, ○) and the polyamine-deficient mutant (JIL601) in the absence (○) or presence of polyamines (▼, putrescine; ▲, spermidine). Polyamines were added to each culture medium at 1 mM final concentrations when needed. B, *gadA-lacZ* expression of the wild type (JIL571, ●) and the polyamine-deficient mutant (JIL650) in the absence (○) or presence of putrescine (▼) or spermidine (▲). C, *gadB-lacZ* expression. Symbols are identical to B. D, measurement of GAD activity in the wild type (wt, QC2461) and the mutant (JIL601). Cells were grown as described in the legend to Fig. 1B. Data from three independent experiments are expressed as mean ± S.D. PUT, putrescine; SPD, spermidine. E, relative GAD activity was visualized by taking photographs of the test tubes containing the reaction mixtures of D. F, acid survivability of the wild type (wt, QC2461) and the polyamine-deficient mutant (JIL601) at pH 2.5. Data from two independent experiments are expressed as mean ± S.D.

Based upon the above results, we directly measured GAD activities of the wild-type and the polyamine-deficient mutant type in the presence or absence of polyamines. While GAD activity was normal in the wild type, the polyamine-deficient mutant failed to show normal activity (Fig. 2D). GAD activity in the mutant was dramatically increased by exogenous supplementation of putrescine or spermidine. The polyamine-synthesizing wild type showed a green color with time, but the polyamine-deficient mutant grown in the absence of polyamines sustained its yellow color. The color changed dramatically from yellow to green with the addition of polyamines to the polyamine-deficient mutant strain, indicating that polycations are essential for the maintenance of GAD activity (Fig. 2F).

Because a polyamine-deficient mutant is devoid of GAD activity as described above, we anticipated that a low environmental pH could lead to a severe deleterious effect on the polyamine-deficient mutant. Based upon the induction pattern of *gad* expression under normal aerobic conditions, we prepared stationary phase cells of the polyamine-deficient mutant and its isogenic polyamine-synthesizing wild type. Compared with the wild type, the mutant is at least 4,000 times more susceptible to an environmental pH of 2.5. However, exogenous supplementation of polyamines dramatically relieved the increased cytotoxicity by the acid-induced stress in the mutant (Fig. 2F).

Regulation of *gad* expression by Polyamine/cAMP System—A regulatory mechanism via the action of the cAMP receptor protein (CRP) has been suggested, in which CRP acts as a negative regulator for *gad* expression (5). We tried to suggest a characteristic polyamine-induced regulatory circuit in acid resistance, investigating the role of cAMP in the induction of *gad* expression and the modulation of the cAMP level by polyamines. A cya mutant showed increased *gadA* and *gadB* expression, indicating that cAMP acts as a negative regulator for *gad* induction (Fig. 3A). GAD activity in the cya mutant was has been induced at the stationary phase in previous studies (8), and we confirmed those results.

Fig. 3. cAMP effect on acid resistance. A, cAMP effect on the expression of *gadA* or *gadB* genes. β-Galactosidase activities were measured from the wild type (wt, JIL733, and JIL734, containing either *gadA-lacZ* or *gadB-lacZ* fusion) and the cya mutant (JIL790 and JIL791, containing either *gadA-lacZ* or *gadB-lacZ* fusion). B, visualization of relative GAD activity of the wild-type QC2461 and its cya mutant JIL582. C, modulation of the intracellular cAMP level by polyamines in the wild type (wt, QC2461) and the polyamine-deficient mutant (JIL601). JIL582 (QC2461, cya”) and JIL612 (JIL601, cya”) were used as controls. Data from two independent experiments are presented as pmol/mg of protein and shown as mean ± S.D. D, effects of the cya mutant allele on *gadA* or *gadB* gene expression and acid resistance. Left panel, β-galactosidase activities of the polyamine-deficient mutants JIL650 and JIL669 (cya”) containing either *gadA* or *gadB-lacZ* fusion, and their cya mutants (cya”) JIL790 and JIL791 containing either *gadA* or *gadB-lacZ* fusion; Right panel, acid resistance of the polyamine-deficient mutant JIL601 and its cya mutant JIL612.
putrescine (PUT) or spermidine (SPD) concentrations were determined as described under "Experimental Procedures." Data from two independent experiments are shown as mean ± S.D.

**DISCUSSION**

Acid resistance is considered to be an important virulence factor of pathogenic *E. coli* strains such as O157:H7 (6). It is...
well known that GAD is needed for survival under low pH (6, 14). Cells possess specific defense mechanisms against acid environments in which the GAD system has been extensively studied because of its major role in the detoxification of acid-induced stress in *E. coli*. H-NS and RpoS are known to be common regulators that are associated with *gad* induction. This model proposes that H-NS acts as a negative regulator of *gad* induction in the logarithmic phase, and RpoS acts as a positive regulator of it in the stationary phase. In this study, we present a new model containing polyamines and cAMP as signaling molecules necessary for normal operation of the GAD system against acid-induced stress. In addition, we compare two distinct regulatory mechanisms, H-NS/RpoS and polyamine/cAMP systems, for the induction of *gad* expression, and show that polyamine/cAMP system has a major role in the defense mechanism against acid-induced stress as well as the H-NS/RpoS system.

There are many investigations demonstrating the fact that polyamines participate in various cellular processes including gene expression and protein biosynthesis, but the role of polyamines in an acid resistance has not been clarified (16). In this report, we suggested a specific defense mechanism that regulates *gad* expression via the positive function of polyamines. In addition, we reported that the two defense mechanisms, H-NS/RpoS and polyamine/cAMP system, comprise distinct regulatory circuits against acid environment. Finally, we suggested a possible model encompassing cell signaling.

**Maintenance of GAD Activity by Polyamines Is Essential for Acid Resistance**—The GAD system contains three genes. Two of the genes, *gadA* and *gadB*, each at separate locations, encode highly homologous GAD isoforms (24). A third gene, *gadC*, is functionally different from *gadA* and *gadB* and encodes a putative glutamate:GABA antiporter (6). GAD, the gene product of *gadA* and *gadB*, is a pyridoxal 5'-phosphate-dependent enzyme, catalyzing the irreversible conversion of glutamate and H⁺ to GABA and CO₂. Following decarboxylation, one intracellular proton is consumed, and GABA is exported using the putative glutamate:GABA antiporter encoded by the *gadC* gene, which is located downstream of the *gadB* gene in the *gadBC* operon (8). As the result of sequential reactions, protons are consumed during acid stress and excreted from the cell, thereby preventing the internal pH from decreasing to lethal levels.

Although GAD has major roles in the defense mechanism against acid-induced damage, no studies have been made of the phenotype of the *gadAlgadB* double mutant. Concerned about the importance of GAD, we constructed a *gadAlgadB* double mutant to investigate its phenotype. GadA and GadB are similarly involved in a large range of GAD activities, with each contributing equally. The *gadAlgadB* double mutant was absolutely devoid of GAD activity. Reduced growth rate by the *gadAlgadB* double mutant in a minimal M9 medium containing glucose may be related to acid sensitivity. This double mutant showed severe susceptibility to acid stress, suggesting that GAD is necessary for acid resistance.

Our experiments indicated that a polyamine-deficient mutant was completely devoid of GAD activity, which was also true of the *gadAlgadB* double mutant. This complete deficiency of GAD activity resulted from the repression of *gadA* and *gadB* expression caused by polyamine deficiency, because exogenous supplementation of polyamines to the mutant enabled the cell to maintain normal GAD activity through up-regulation of the *gad* genes. Therefore, in the case of the *gadAlgadB* double mutant, the polyamine-deficient mutant showed increased susceptibility against acid-induced stress. On the other hand, exogenous supplementation of polyamines dramatically restored vulnerability to acid stress to wild-type levels. This strongly suggests that the cytotoxic effect by intracellular polyamine depletion in an acidic environment must be the result of the deficient GAD activity and that polyamines participate in specific defense mechanism that causes the recovery of normal GAD activity.

**Polyamines Participate in the Detoxification of Acid-induced Stress by Reducing Intracellular cAMP Levels**—cAMP is involved in the regulation of transcription, either positively or negatively, in many *E. coli* genes (3), and is also involved in the regulation of acid resistance (5, 6). It has been shown that the polyamine supplement to the *E. coli* B strain affected the hydrolyzing activity on cAMP by increasing the phosphodiesterase activity (33). This result suggested the possibility that
polyamine could regulate intracellular cAMP levels. Based on the previous results, we hypothesized that the regulation of gad expression by polyamines during normal aerobic growth could be mediated by cAMP. To examine the possibility that the polyamine-mediated defense mechanism against acid-induced stress is mediated by cAMP, we measured the intracellular cAMP level in the polyamine-deficient mutant and its isogenic wild type. Interestingly, polyamines largely prevented cAMP accumulation. Furthermore, the introduction of a cya mutation allele into the polyamine-deficient mutant greatly induced both gadA and gadB expression, which led to increased acid resistance, even if polyamines were not provided. These results strongly suggest that the polyamine signal, acting as a positive regulator, enables effective gad induction, arising from the down-regulation of the intracellular cAMP level, acting as a negative regulator.

**Positive Regulation of gad Gene Expression via H-NS/RpoS System**—It has been suggested that H-NS protein directly or indirectly function as a transcriptional repressor (28, 30). Furthermore, H-NS has been implicated in post-transcriptional regulation of the RpoS stability (1, 30). Therefore, it could be hypothesized that nucleoid protein affected the expression of gad genes (8). Those results have been obtained using cells growing exponentially in rich media. In this study, we suggest other roles for H-NS and RpoS in cells grown in the stationary phase using M9 minimal medium.

Stationary phase-grown cells containing hns mutation failed to induce gad expression. This evidence strongly suggests that there is another function of H-NS that positively regulates gad expression in the stationary phase, in addition to H-NS acting as a negative regulator for gad expression in the logarithmic growth phase. We also confirmed that the RpoS regulator positively regulates gad expression in the stationary phase. To identify signal transduction pathways involving H-NS and RpoS, we measured rpoS expression in both hns− and hns+ backgrounds. Surprisingly, H-NS is definitely required for normal rpoS induction in the stationary phase, indicating that signal transduction from H-NS to gad is mediated by rpoS up-regulation via positive regulation of rpoS. We conclude that H-NS induces rpoS expression, thereby possibly enhancing the RpoS level, which may lead to gad induction. While H-NS is absolutely required for gad induction, RpoS is partially required for it. So, we conclude that H-NS regulates at least two different factors including RpoS. We do not know the other regulator affecting gad expression at this time, but RpoS must be the main regulator because its deficiency causes significant reduction of gad expression.

**The Relation of Signal Transduction Pathways for the Induction of gadA and gadB by H-NS, RpoS, cAMP, and Polyamines**—To investigate the relationship between H-NS/RpoS (positive regulation) and polyamine/cAMP (negative regulation) systems, we first measured the changes of intracellular H-NS level in the absence or presence of polyamines and the intracellular polyamine level in both hns− and hns+ backgrounds. We could not demonstrate significant detectable changes in either the level of H-NS or the level of polyamines, indicating that H-NS and polyamines are not directly related to each other for gad expression. We confirmed that a polyamine-deficient mutant cell failed to induce gad expression irrespective of the existence of normal H-NS. However, normal level of H-NS was essential for the induction of gad expression by polyamines because the polyamine effect on gad up-regulation disappeared in the hns mutant background. Nonetheless, we concluded that although there were two regulatory systems in acid resistance, they seem distinct.

In conclusion, we showed that multiple signals including H-NS, RpoS, cAMP, and polyamines are involved in gad induction (Fig. 5). In these investigations, we examined the sequence of signal transduction or the relationship of the signals for normal gad induction. We demonstrated two distinct regulatory circuits. H-NS positively regulates rpoS gene expression and the rpoS gene product, RpoS, induces both gadA and gadB expression. cAMP negatively regulates both gadA and gadB induction, but the negative regulation is suppressed by polyamines. The H-NS/RpoS and polyamine/cAMP systems operate independently to achieve acid resistance.

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