TITLE- Polyamines are critical for the induction of the glutamate decarboxylase-dependent acid resistance system in *Escherichia coli*

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** I dedicate this manuscript to the memory of Dr. Celia White Tabor, my collaborator in the polyamine field for over 50 years.

RUNNING TITLE- Polyamines and acid resistance in *E. coli*

ABBREVIATIONS- AR- acid response; cAMP- cyclic adenosine monophosphate; CAD-cadaverine; GAD- glutamic acid decarboxylase; GDAR-glutamate dependent acid resistance; PUT-putrescine; SPD-spermidine.

KEY WORDS- acid-stress; cAMP; gene expression; glutamic acid; microarray; putrescine; spermidine

Background: Polyamines are present in all organisms.

Results: Polyamines induce various components of the glutamate-dependent acid resistance pathway (GDAR) in *Escherichia coli* and are important for protection against acid-stress.

Conclusion: A unique function of polyamines is the induction of the GDAR system.

Significance: Polyamines are important for the survival of *Escherichia coli* when passing through the acid-environment of the stomach.

ABSTRACT

As part of our studies on the biological functions of polyamines we have used a mutant of *Escherichia coli* that lacks all the genes for polyamine biosynthesis for a global transcription analysis on the effect of added polyamines. The most striking early response to the polyamine addition is the increased expression of the genes for the glutamate dependent acid resistance system (GDAR) that is important for the survival of the bacteria when passing through the acid environment of the stomach. Not only were the two genes for glutamate decarboxylases (*gadA* and *gadB*) and the gene for glutamate -γ-aminobutyrate antiporter (*gadC*) induced by the polyamine addition, but also the various genes involved in the regulation of this system were induced. We confirmed the importance of polyamines for the induction of the GDAR system by direct measurement of glutamate decarboxylase activity and acid-survival. The effect of deletions of the regulatory genes on the GDAR system and the effects of overproductions of two of these genes were also studied. Strikingly, overproductions of the alternate sigma factor *rpoS* and of the regulatory gene *gadE* resulted in very high levels of glutamate decarboxylase and almost complete protection against acid stress even in the absence of any polyamines. Thus, these data show that a major function of polyamines in *E. coli* is protection against acid stress by increasing the synthesis of glutamate decarboxylase, presumably by increasing the levels of the *rpoS* and *gadE* regulators.

Polyamines (such as putrescine, spermidine, spermine) are abundantly present in essentially all organisms, ranging from bacteria to humans and have been associated with such biological processes as nucleic acid and protein biosynthesis and structure, and cell growth and differentiation (1-6). As part of our effort to study the biological functions of
the polyamines, we have carried out a microarray analysis to find which genes are induced by the addition of polyamines to a polyamine deficient mutant of *E. coli*. Most strikingly we found that five of the seven genes that showed the largest induction soon after polyamine addition are involved in the *E. coli* acid response system; three are specifically involved in the glutamate decarboxylase dependent acid-resistance (GDAR) system. The induction of the GDAR system is of particular importance since it is necessary for the survival of various pathogenic and non-pathogenetic bacteria (such as *Escherichia coli*, *Shigella flexneri*, *Listeria monocytogenes* and *Lactococcus lactis*), when exposed to the acids present in the stomach (7-11).

Three clearly elucidated acid resistance systems are involved in the survival of *E. coli* from acid stress; namely, oxidative (AR1), glutamate-dependent (AR2, GDAR) and arginine-dependent (AR3) systems (12-14). However the glutamate-dependent acid resistance system is the most effective in protecting cells against an acid environment and has been extensively studied, especially by Foster and his associates (12, 13, 15, 16). The mechanism of the acid resistance involves the decarboxylation of glutamate by two glutamic acid decarboxylases (encoded by *gadA* and *gadB*) and export of the resultant γ-aminobutyric acid by the antiporter *gadC*, which causes the loss of intracellular protons (7, 9, 17). Several regulators of the GDAR system have been described such as *gadE* (18-20), *gadX*, *gadW* (21, 22) and *gadY* a small regulatory RNA (23). Several other factors are also involved in the transcriptional control of GDAR during different growth conditions including *rpoS* (the stationary phase specific alternate RNA polymerase subunit σ^35^) (21, 24), the global transcription regulator H-NS (8, 25, 26) and cAMP systems (27).

Stimulated by our microarray data on the effect of polyamines on the transcription of the genes in the GDAR pathway we proceeded to study the involvement of polyamines on the various components of this pathway. For these studies we used our strain that contained deletion mutations in all nine of the genes involved in polyamine biosynthesis^1^ and that contains no polyamines when grown on a purified medium (28). We feel that the use of this strain with multiple mutations is important since we have found that strains (often used in the literature) that only have a few of these mutations still contain small amounts of polyamines. Surprisingly, despite the lack of any intracellular polyamines this strain can still grow indefinitely in a purified medium, albeit at 30-40% of the normal growth rate. We have made an important additional mutation in this strain since we found that our strain had an amber mutation in the *rpoS* gene; this amber mutation in codon 33 has been reported as being present in high frequency in many *E. coli* strains (29), (30). Since RpoS is known to be important in various stress response systems, we have converted the *rpoS* amber mutation in our strain to *rpoS* (wild type) for use in the current experiments.

**EXPERIMENTAL PROCEDURE**

**Bacterial strains, medium and growth**-The *E. coli* strains and plasmids and their source are listed in Table 1. Strain HT776 was modified to convert the amber mutation (TAG) that we found in position 33 of the *rpoS* gene of HT776 to the wild type CAG sequence. To effect this modification, we converted *rpoS^amb* to *rpoS^WT* by P1 transduction with a strain (ΔcycC/ JW2720-1) that contains *rpoS^WT* and the nearby ΔcysC, selecting for a transductant (HT838-11) requiring cysteine and containing the *rpoS^WT* sequence (CAG). The ΔcysC was then removed by another P1 transduction with a strain containing the wild type cysC gene to make strain HT839 used in this paper. In this

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1 *speA*- arginine decarboxylase; *speB*- agmatine ureohydrolase; *speC*- ornithine decarboxylase; *speD*- S-adenosylmethionine decarboxylase; *speE*- spermidine synthase; *speF*- inducible ornithine decarboxylase; *cada*- inducible lysine decarboxylase; *ldcc*- lysine decarboxylase; *adiA*- inducible arginine decarboxylase.
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strain the sequence of the *rpoS* wild type gene was determined by amplification of *rpoS* from genomic DNA followed by DNA sequencing.

P1 transductions were carried out essentially as described by Miller (33). The kanamycin marker from the transduced strains was excised by Flp recombinase as described in references (31) and (34).

Strains were grown in amine free M9 medium (33) or Vogel Bonner medium (35) containing proline (10 µg/ml), threonine (10 µg/ml) and 0.4% glucose and supplemented with different concentrations of amines as indicated. Incubations were at 37°C in air. For strains containing plasmids the appropriate antibiotic was added; 20 µg/ml chloramphenicol for strains containing the pACYC or pArpoS plasmids or 100 µg/ml of carbenicillin for the strain containing the pQE gadE plasmid.

RNA isolation, rRNA removal and microarray- *E. coli* polyamine mutant (HT839) cultures were inoculated from an LB plate in triplicate in amine-free M9 medium. The cultures were deprived of amines by growing them overnight in amine-free medium. Then the cultures were diluted to OD\textsubscript{600} of 0.2 and grown until the OD\textsubscript{600} reached 0.5-0.6. Each culture was divided into two parts. To one part a mixture of putrescine and spermidine was added to a final concentration of 100 µM; the other part was left untreated and used as a control. The amine-supplemented cultures were harvested 20 min, 40 min and 60 min after amine addition. Total RNA from each sample was isolated as described previously (36). Briefly, for each RNA isolation 10\textsuperscript{9}-10\textsuperscript{10} cells were resuspended in Tris-EDTA buffer (100 mM Tris, 10 mM EDTA, pH 8.0) containing 2 mg/ml lysozyme (Sigma, St. Louis, MO) and RNA was isolated according to the protocol described in the RNeasy mini kit (Qiagen, Germantown, MD). The mRNAs were enriched from total RNA by removing the 16S and 23S ribosomal RNAs using the MICROB*Express* method and kit (Ambion/Life Technologies, Grand Island, NY, part # AM1905). The quantity and quality of RNA were evaluated by OD\textsubscript{260}/OD\textsubscript{280} assays and by RNA capillary electrophoresis (Agilent Technologies, Santa Clara, CA) and used for microarray analysis in the NIDDK genomic core facility by Dr. Weiping Chen and Dr. Keembiyechetty Chithra\textsuperscript{2}. Enriched mRNA (40 ng/reaction) was reverse transcribed and amplified using an Ovation WTA pico-V2 Kit (Part# 3302, NuGEN, San Carlos, CA) according to the manufacturer’s protocol. 2.5 µg of cDNA was fragmented and biotinylated and then hybridized for 18 h to *E. coli* GeneChip arrays (Genome 2.0 array; Affymetrix Santa Clara, CA; n= 3 each for each sample). After washing and staining with an Affymetrix kit (#FS450; HWS Kit), the GeneChips were scanned using an Affymetrix 3000G scanner and GCOS software. ANOVA (analysis of variance) was performed and p-values were calculated using Partek Pro-software (Partek, St. Louis, MO) and plotted in negative log scale on the y-axis against the Affimetrix signal ratios for each probe set on the x-axis. Up- and down-regulated genes were selected based on p-values of <0.05 and fold change >+2 or -2.

Acid stress and cell survival assay- *E. coli* cultures were inoculated from LB plates into M9 medium without amines and grown overnight to deplete intracellular amines. The cultures were further diluted in M9 medium with and without different supplements as indicated and grown for 20-24 h to full growth. The cultures were harvested and re-suspended in Vogel Bonner medium (pH 7.0 with 0.4% glucose) at a cell density of 1X10\textsuperscript{10} cells/ml and used for acid survival assays as described by Castanie-Cornet et al (7) after some modifications. For assay of cell survival in acid each culture was diluted 1:100 in Vogel Bonner medium (pre-warmed at 37°C) with 0.4% glucose adjusted to pH 2.5 with the same supplements. At the indicated times an aliquot was taken using a caliper.

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of each culture was diluted in phosphate buffer saline, spread on LB plates and incubated at 37°C overnight to determine cell survival.

**Glutamate decarboxylase assay** - Glutamate decarboxylase was measured by direct measurement of decarboxylation of L-glutamic acid. Cell extracts were prepared by suspending the harvested cells in a buffer containing 20 mM potassium phosphate, 2X protease inhibitor cocktail (Pierce, Rockford, IL), 100 mM NaCl, 100 µM pyridoxal-phosphate, 100 µg/ml lysozyme and 20 µg/ml DNase1 (Sigma), pH7.4, incubating the cell suspension at room temperature for 10 min, and subjecting the suspension to one cycle of freezing and thawing. The suspension was then centrifuged (13,000 g) and the protein content of the supernatant was determined by Bradford assay (37) using Biorad reagent (Biorad, Hercules, CA). Enzyme assay buffer contained 50 mM sodium acetate, 100 mM NaCl, 50 µM pyridoxal-phosphate, pH 4.6.

For each assay 4-10 µg of protein was mixed with 100 µl of assay buffer and the reaction was initiated by addition of 10 µl of 10 mM L-glutamic acid containing 10 µCi/ml of L-[14C(U)]-glutamic acid (260 mCi/mmol, #NEC 290, Perkin Elmer, Waltham, MA) in a total volume of 125 µl in a 1.5 ml locked microfuge tube. Liberated CO₂ after the decarboxylation reaction was trapped in a small filter paper containing a saturated solution of Ba(OH)₂ that had been placed on the cap of the microfuge tube. The reaction was incubated at 37°C for 20 min with intermittent shaking, and terminated by addition of 125 µl of 6N HCl to inactivate the enzyme and incubated further for 30 min to release dissolved CO₂. Radioactivity was measured in a liquid scintillation counter (LS6500, Beckman, Indianapolis, IN), and enzyme activity was expressed in µmoles of CO₂ released/mg protein/h.

**cAMP measurement** - Polyamine mutants were inoculated from LB plates into M9 medium plus 0.4% glucose with proline and threonine as above and grown overnight without amines to deplete intracellular amines. The cultures were then diluted in the absence or presence of different amines (100 µM each) and grown for 24 h. Cells were harvested, washed and resuspended in 0.9% NaCl to a density of 1X10⁹ cells/ml. Intracellular cAMP was measured (10 µl of the cell suspension/assay) using the direct cyclic AMP Enzyme Linked Immunosorbent Assay (ELISA) following the manufacture’s protocol (Cat #: ADI-900-066, Enzo Life Sciences, Farmingdale, NY).

**Western blot analysis** - Cells were grown as above and cultures were harvested at the indicated times for protein extraction. The same samples used for in vitro glutamic acid decarboxylase assays were used for western blotting and immunodetection using the ECL Prime western blotting kit (GE Health Care/Life Sciences, Piscataway, NJ). Anti-glutamate decarboxylase (GadA) primary antibody was raised commercially against GadA-peptides (peptide #1 INDELYLDGNARQNLAT; peptide #2 CLKYLSDHPKLQGIAQQNSFKHT; 21st Century Biochemicals, Marlboro, MA), and confirmed by comparing cell extracts from gadA⁻·gadB⁺ and ΔgadA·ΔgadB strains by western blotting. Although the antibody was raised against GadA peptides, because of very high sequence identity in the two decarboxylases (more than 95%), the primary antibody recognizes both GadA and GadB proteins. The high-titer GadA/B antibody was affinity purified and used in western blot analysis (1:25,000 dilution) containing 1 µg protein extract/lane. Horseradish-peroxidase conjugated rabbit secondary antibody (GE Health Care) was used at 1:20,000 dilutions. For immunodetection of RpoS, cell extract containing 5 µg protein/lane was used. Anti-RpoS mouse monoclonal antibody (Abcam # ab81737, Cambridge, MA) was used after 1:10,000 dilutions and secondary antibody (anti-mouse, Horseradish-peroxidase conjugated, GE Health Care) was used at 1:20,000 dilutions.
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**RESULTS**

Global gene expression in response to polyamine supplementation to a polyamine mutant of *E. coli*: Polyamines induce genes involved in the acid response pathway - For these studies we used the *E. coli* mutant that we described previously (28) that has deletions in all nine of the genes involved in polyamine biosynthesis, modified as described in the Materials and Methods section to have a wild type sequence in the rpoS gene. This polyamine mutant does not contain any detectable amines when grown in purified medium (minimum detection limit less than 0.02 nmol/mg cells). In the absence of amines this polyamine mutant grows at 30-40% of the growth rate obtained with an amine supplemented culture. We have used this strain for global transcriptome analysis to identify genes responsive to polyamine addition at very early time points (20 min, 40 min, 60 min after polyamine addition); at these times there is no increase in the overall growth rate, as measured by optical density, due to the polyamine addition. As shown in the volcano plot (Fig. 1), within 60 minutes after polyamine addition 54 genes were up-regulated more than 2-fold and 15 genes were up-regulated more than 3-fold; 94 genes were down-regulated more than 2-fold. The entire data set for the microarray study on the effect of polyamine addition has been deposited with a GEO accession number GSE49918.

Most striking was the finding that of the seven genes most induced (6.9-3.5 fold) after polyamine addition, five were genes known to be involved in the GDAR and other acid-resistance pathways (above the heavy bar in Table 2). In Table 2 we have also listed other genes that are known to be involved in the acid response pathway in *E. coli*; all of these were also induced by the polyamine addition but to a lesser degree. We therefore next examined the effect of polyamines on the survival of our mutant strain in acid, and on inducing the synthesis of glutamate decarboxylase as well as on other components of the GDAR system.

Both polyamines and glutamic acid are required for the protection of *E. coli* against acid-stress. As shown in Figure 2A survival at pH 2.5 requires both polyamines and exogenous glutamic acid. In the absence of either polyamines or glutamic acid the cells are highly sensitive and die within 15 minutes, whereas addition of 100 µM putrescine plus 100 µM spermidine together with 300 µM glutamic acid affords very good protection (>50% in 1 hour). The relative effect of the different amines is presented in Fig. 2B, and shows that spermidine is much more effective than putrescine or cadaverine. The experiment presented in Figure 2C shows that the protective effect of polyamines and glutamic acid against pH 2.5 depends on the decarboxylation of the glutamic acid since there was no protection if the bacteria also contained deletions in the genes for both glutamate decarboxylases (*gadA* and *gadB*).

**Induction of glutamate decarboxylase requires polyamines** - Fig. 3A shows that polyamine-deficient cells have little or no glutamate decarboxylase activity. Addition of either 0.1 mM putrescine or 0.01 mM spermidine to the polyamine-deficient cells caused a 10-40-fold increase in glutamate decarboxylase activity. 1 mM cadaverine supplementation had little stimulatory effect (3-4-fold). A dose dependent study (5 µM to 5 mM putrescine and/ or spermidine) showed that maximum induction of glutamate decarboxylase was obtained by addition of 100 µM putrescine and spermidine to the culture. Above this concentration the enzyme activity did not increase further (data not shown). Thus, in all further experiments a combination of 100 µM putrescine and spermidine was used. The large stimulatory effect of these concentrations of putrescine or spermidine was not due to stimulation of the growth rate of the polyamine mutant since we found that there was very little stimulatory effect of 10 µM putrescine or 1 µM spermidine on the formation of glutamate decarboxylase even though normal growth rate was attained with these concentrations of the amines.
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These assays were carried out on cells in stationary phase, since it is well known that the GDAR pathway is induced in overgrown cultures (19). In the next experiments we also determined the effect of polyamine addition on the induction of glutamic acid decarboxylase at different stages of growth. As shown in Figure 3B, and as expected from the literature (19), the activity of glutamate decarboxylase was much lower in the logarithmically growing cells than in the overgrown culture, but these cells still showed the requirement for polyamines for induction of glutamate decarboxylase. We confirmed by western blots the absence of glutamate decarboxylase protein in the polyamine-deficient cells and the presence of the enzyme in the cultures containing added polyamines, especially in overgrown cultures (Fig. 3B).

As shown in Fig. 3C, polyamine induced activation of the GDAR system is transcriptionally controlled and requires new RNA synthesis. Thus, when polyamines were added to polyamine deficient cultures pretreated with or without 10 µg/ml concentration of rifampicin (a bacterial DNA-dependent RNA polymerase inhibitor), induction of glutamate decarboxylase by polyamines was almost completely inhibited in the rifampicin pre-treated cells.

These results confirm and extend the findings of Jung and Kim (45) with a simpler polyamine-deficient mutant that only had deletions in genes for *speA* (arginine decarboxylase), *speB* (agmatine ureohydrolase) and *speC* (ornithine decarboxylase). With this mutant they showed that polyamines were required for the induction of glutamate decarboxylase and that polyamines were needed for protection of their mutant against acid stress. However, as described in the next section, our results differ completely from their report that changes in the cAMP level are responsible for the effect of polyamines in inducing glutamate decarboxylase activity or protection from acid stress.

The polyamine-dependent induction of glutamate decarboxylase and of acid survival is independent of changes in cAMP levels- Previous work by Castanie-Cornet and Foster had shown that glutamate decarboxylase activity is much higher in wild type strains that lack the gene for cAMP synthesis, indicating a repressor function for cAMP on glutamate decarboxylase induction (27). Jung and Kim (45) claimed that the addition of polyamines caused a decrease in the cAMP level and that this decrease accounts for the effect of polyamines in stimulating the induction of glutamate decarboxylase. Our results, however, do not support this postulated mechanism since we found that the polyamine deficient cells have a low cAMP level, and that the cAMP level is increased 3-5-fold by the addition of polyamines (Fig. 4A).

We also showed that the polyamine effect in inducing glutamate decarboxylase still occurred in a polyamine deficient strain that also contained a deletion in *cyA* (adenylate cyclase) or in *crp* (cAMP receptor protein). As presented in Figure 4B, we found that these cells still required polyamines for full glutamate decarboxylase activity; addition of polyamines resulted in a marked increase in enzyme activity. Western blot analysis with anti-GadA/B antibody confirmed these results; i.e. the glutamate decarboxylase protein was present in a very high level in the plus amine cultures as compared to the minus amine cultures, both in the presence or absence of *cyA* or *crp* (Fig. 4C). These data are consistent with the acid stability assays presented in Fig. 4D, showing that the Δ*cyA* polyamine mutant (HT840) required polyamines for proper protection during acid stress.

*rpoS* is required for the induction of glutamate decarboxylase activity- Although the sigma factor *rpoS* has been shown to be essential for the induction of glutamate decarboxylase activity in cells grown to stationary phase in rich medium, there has been some disagreement on whether this is true for cells grown in minimal medium (19, 45). In Fig. 5A we show that *rpoS* is required for the induction of glutamate
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decarboxylase synthesis and activity even when the cells were grown to stationary phase in minimal medium containing polyamines, confirming the findings of Jung and Kim (45). The deletion of rpoS also resulted in a large decrease in cell viability in acidic pH even in the presence of glutamic acid and polyamines (data not shown), which is consistent with the lack of glutamate decarboxylase activity in the absence of rpoS shown in Fig. 5A.

To determine if the absence of glutamate decarboxylase activity observed in polyamine deficient cells (Fig. 3A, B) was due to a lower rpoS level, we measured the level of RpoS protein by western blot analysis in the presence and absence of polyamines. Fig. 5B shows that polyamine deficient cells contain a reduced level of RpoS protein both in late log phase and overgrown cultures. This is consistent with the 2-fold increase in tpoS gene expression seen within 20 min after polyamine addition in the microarray experiments (Table 2). These data are consistent with the postulated hypothesis that the RpoS level does affect the induction of the GDAR pathway either directly or indirectly (45).

The fact that there was still a substantial amount of RpoS protein present in the absence of polyamines suggests that this level of RpoS protein is apparently not enough to stimulate glutamate decarboxylase induction. Some support for this hypothesis is provided by the experiments presented in Fig. 5C where we measured the effect of increasing the rpoS level by induction of an rpoS-containing plasmid. These data show that increase in the rpoS level resulted in some increase in the glutamate decarboxylase activity even in the absence of polyamines, and a rather large additional increase in the cultures grown with polyamines.

Role of GDAR regulators, gadX, gadY, gadE, ydeO and rcsB in polyamine requirement for induction of glutamate decarboxylase -
To determine which genes are directly or indirectly involved in polyamine induction, we deleted the regulatory genes in the GDAR system. Three genes involved in the regulation of glutamic acid decarboxylase activity that were found to be induced by polyamines in our microarrays (Table 2) were first targeted for this study. Deletion of gadX (a transcriptional activator) or gadY (a regulatory small RNA) resulted in slightly reduced glutamate decarboxylase activity as compared to gadX+ or gadY+ cells even in the presence of polyamines in the medium (Fig. 6). However, in the absence of polyamines there was a slightly higher glutamate decarboxylase activity in both ΔgadX and ΔgadY in comparison to their wild type counterpart (Fig. 6A), but both of these mutants needed polyamine for increased glutamate decarboxylase activity. Similar results were obtained by western blot analysis of protein extracts prepared from these mutants grown with or without polyamines (Fig. 6A).

In contrast, deletion of gadE abolished glutamate decarboxylase activity or expression of glutamate decarboxylase protein when the cultures were grown in M9 glucose containing medium both in the presence or absence of polyamines (Fig. 6B). Survival of cells in acid stress (pH 2.5 medium) was also compromised in ΔgadE cells even in the presence of polyamines and glutamic acid (data not shown). Deletion of ydeO (a regulatory gene of gadE) resulted in slightly reduced induction of glutamate decarboxylase activity in the presence of polyamines (Fig. 6C). In contrast, deletion of rcsB (a response regulator known to bind gadE) resulted in complete loss of glutamate decarboxylase activity even in the presence of added polyamines (Fig. 6C). These data confirm earlier findings in wild type E. coli cells that rcsB is required for activation of glutamate decarboxylase (46).

Polyamine requirement for glutamate decarboxylase induction and acid survival of polyamine mutants can be overcome by overexpression of gadE or gadE and rpoS -
The above results showed that functional rpoS and gadE are essential for polyamine induction of the GDAR system and acid protection (Fig. 5 and 6). We then tested the effects of overexpression of these two regulators on the polyamine requirement for
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GDAR activation and acid survival. Overexpression of gadE from a plasmid resulted in nearly 40-fold higher expression of glutamate decarboxylase activity in the absence of polyamines (Fig. 7A). gadE overexpression also resulted in 1.7-fold increased glutamate decarboxylase activity in cultures grown with polyamines. When the polyamine deficient mutant contained plasmids that overproduced both gadE and rpoS, the level of induction was even higher and there was virtually no difference in glutamate decarboxylase activity between plus or minus amine grown cultures (Fig. 7A). These results were further confirmed by western blot analyses using the same extracts (lower panel, Fig. 7A). Cell viability assays in acidic pH also showed that even in the absence of polyamines overexpression of gadE and rpoS rescued the cells and provided near complete protection for 60 min at pH 2.5 (Fig. 7B).

DISCUSSION

Despite the fact that polyamines have been reported to have many physiological functions (2, 3, 5, 47-50), it is noteworthy that even though the addition of polyamines to our polyamine mutant of Escherichia coli resulted in change in expression of many genes, the most striking changes in gene transcription in our microarray experiments are not related to these functions but rather to the glutamate decarboxylase dependent acid-resistance system (GDAR). In these studies we report findings on the role played by polyamines in controlling the GDAR system. Many of the components of the GDAR system are induced by polyamines (gadA, gadB, gadC, gadE, gadX, gadY, rpoS), and the overall induction of glutamate decarboxylase is transcriptionally controlled. We found that the LuxR like response regulator gadE and the alternate sigma factor rpoS are particularly important modulators of the polyamine effect.

The system involved in the acid-resistance of some bacteria has been of particular interest over many years, particularly since in food borne pathogens acid resistance is a basic prerequisite for host colonization. Both pathogenic and non-pathogenic E. coli and S. flexineri are able to survive in an extremely acidic environment (pH≤2.5) and this correlates with their low infectious dose (<10⁷ cells/ml) (51, 52). The ability of these organisms to survive extreme acid stress primarily relies on the activation of very powerful acid-resistance (AR) systems. In his pioneering work Ernest Gale showed that in bacteria the α-decarboxylases (such as lysine, ornithine, arginine, tyrosine, histidine and glutamic acid decarboxylases) are inducible by growth to stationary phase or growth in an acidic environment (53). Later three distinct systems for acid resistance were described that protect both growing and non-growing cells from acid over a wide range of environmental conditions (12). They are all induced by growth in acid medium or growth to stationary phase. One of these systems (AR3) requires extracellular arginine and the acid-inducible arginine decarboxylase (adiA) (54). A second system (AR2) requires extracellular glutamate and decarboxylation by glutamate decarboxylase and involves several regulatory genes (12, 14, 38). A third system (AR1) does not involve any amino acid decarboxylation but is repressed by glucose (7). Systems AR1 and AR3 are present in all enterobacteria, but system AR2 is only present in some bacteria, including E. coli, and is particularly important for the survival of these bacteria in acid (12). Apart from these, acid inducible ornithine decarboxylase (speF) and lysine decarboxylase (cadA) systems have been described as providing moderate protection from acid stress in E. coli (55-57). However, previous acid-survival studies at pH 2.5 with wild type E. coli strains grown in LB medium have shown little or no protection if the cells lack the gadA-gadB genes even though these cells still contain the genes for ornithine decarboxylase, and lysine decarboxylase (7). In addition our strain does not contain the genes for these enzymes or for arginine decarboxylase, and thus our studies are only concerned with the GDAR system (AR2).

In this manuscript we report studies on the effect of polyamines on various
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components of the glutamate decarboxylase system. We used a quantitative radiometric assay that measured $^{14}$CO$_2$ formed from $^{14}$C-labeled glutamic acid. In addition, where possible we measured the glutamate decarboxylase protein level by western blot. The polyamine-requiring strain that we used (HT839) is particularly suitable for the current studies since, as opposed to other studies in the literature, it contains deletions in all nine of the genes involved in polyamine biosynthesis.

Another advantage of our strain is that we converted the amber codon that is present in the *rpoS* gene in so many laboratory strains of *E. coli* to a sense codon (29). Most studies in the literature on the effects of polyamines in *E. coli* have not considered the possible involvement of an amber mutation at codon 33 of *rpoS*. We feel that this modification of *rpoS* amber mutations to *rpoS*WT is critical for studies on the effect of polyamines since it has been shown by Yoshida et al (30) that polyamines facilitate the read-through of *rpoS* amber mutations as well as other amber mutations (58), and this read-through would lead to an increase in the level of the *rpoS* transcript, and might result in many changes in gene transcription and translation not related directly to the primary effect of the polyamine addition.

It is important to note that the data obtained in our microarray experiments were measured after a short interval (20-60 minutes) after addition of the polyamines in batch cultures. During this time interval there was no increase in the overall growth rate due to the polyamine addition. This is an important consideration since so many experiments by others and ourselves on the effect of polyamine addition to polyamine deficient cells involve assays after long-term growth. Polyamine-supplemented cultures grow more rapidly, and some of the effects seen might have been related to the change in growth rate rather than to a specific effect of the polyamines added. We also performed a microarray experiment using a chemostat to control growth and found that the most pronounced effect of polyamine addition was on the genes involved in the GDAR system (data not shown); i.e. similar to those reported in this paper with batch cultures.

We confirmed the important finding of Jung and Kim (45) that polyamines are required for the induction of glutamate decarboxylase in a polyamine mutant. However, our results disagree with their proposal that the mechanism that the polyamine addition results in a decrease in the cAMP level and that this causes a derepression of the GDAR system. In contrast we found the opposite result. As shown in Fig. 4A, polyamine addition to our polyamine deficient cells resulted in 3-5-fold increased cAMP level, similar to earlier findings of Yoshida et al (59). Further evidence that changes in cAMP are not required for the polyamine induction of glutamate decarboxylase activity was shown by our experiments with polyamine mutants that contain an additional deletion in either the adenylate cyclase gene (*cyaA*) or the cAMP receptor gene (*crp*). With both of these mutants addition of polyamines resulted in a marked increase in glutamate decarboxylase activity, indicating that cAMP is not required for the induction of glutamate decarboxylase by polyamines (Fig. 4B, 4C). Moreover, a Δ*cyaA* polyamine mutant strain still required polyamines for protection from acid stress (Fig. 4D).

As the induction of glutamate decarboxylase is transcriptionally controlled and since in our microarray experiments we found that the transcription of many of the regulatory genes in the GDAR pathway are up-regulated by polyamines, we also studied the effect of polyamines on deletion of some of these genes. A most striking effect was found with *gadE*, consistent with the studies by Richard and Foster (60) that *gadE* is the most important regulatory component of the GDAR system. Deletion of *gadE* completely abolished polyamine induced expression of glutamate decarboxylase (Fig. 6B), as expected from the experiments of Ma et al (19) with wild type strains. In *E. coli*, *gadE* regulation is extremely complex and many activators or repressors are known to control *gadE* functions in response to different conditions (12) (15, 16). To confirm the
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significance of *gadE* regulation on the polyamine induced stimulation of the GDAR system and to identify upstream regulators of *gadE*, we deleted *ydeO* and *rcsB* in our polyamine mutant strain. We find that *ydeO* gene deletion showed a partial effect on polyamine induced stimulation of glutamate decarboxylase activity. However, deletion of *rcsB* completely abolished glutamate decarboxylase activity even in the presence of polyamines (Fig. 6C). These data confirm a previous report in a wild type *E. coli* strain (containing large amount of polyamines) that *rcsB* is known to form a heterodimer with *gadE* and a functional heterodimer of *gadE/rcsB* is required for GDAR activation (46). Involvement of polyamines in the regulation of *rpoS* was also shown by the observation that the amount of RpoS protein is decreased (but not absent) in a polyamine mutant, and is increased 2-3 fold by the addition of polyamines. One of the genes that have been reported to control increased translation and maybe stability of RpoS protein is *dsrA* (61, 62), which was also induced (1.6-fold) by polyamine addition in our microarray studies (Table 2). Some evidence for the effect of polyamines in stabilizing RpoS protein has been reported by Tkachenko et al (63).

Not only does a deletion in *gadE* completely abolish the induction of glutamate decarboxylase, even in the presence of added polyamines, but also overproduction of *gadE* in a polyamine mutant overcomes the polyamine requirement for the induction of glutamate decarboxylase even in the absence of polyamines (Fig. 7A). Overexpression of both *rpoS* and *gadE* genes showed a robust increase in the glutamate decarboxylase level and near complete protection of cells from acid stress even in the absence of added amines (7A, B). This effect could be due to synergistic effects of both *rpoS* and *gadE* genes.

There are many other well-documented physiological functions attributed to polyamines in bacteria (47-50). Our current data do not exclude the validity of these findings or minimize their physiological significance. Some of the functions probably do not involve effects of polyamines on transcription or involve smaller effects on transcription than involved in the GDAR system. Smaller change in gene expression induced by polyamines or of the expressed protein might, of course, still have larger physiological effects.

Although the GDAR system is only present in some organisms, some of the effects observed in the current work might be applicable to other systems concerned with the response of bacteria to various growth conditions (as postulated by Igarashi and Kashiwagi (5)). The fact that specific systems involving polyamines (such as glutathionylspermidine, trypanothione, hypusine, and GDAR) are not present in every organism is presumably an indication that specific polyamine functions are evolutionary evolved according to the different cellular requirements of each organism.

In conclusion, the data presented in this paper demonstrate that a unique additional function of the polyamines is for the induction of the GDAR system possibly through the activation of *rpoS* and *gadE* expression. Most of the studies on GDAR regulation in the literature were performed in wild type (in terms of polyamine biosynthesis) *E. coli* cells, which contain large amount of intracellular putrescine and spermidine. Our present findings using a polyamine mutant have permitted us to study the regulatory mechanisms played by polyamines on the GDAR system.

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FOOTNOTES

1. *speA*- arginine decarboxylase; *speB*- agmatine ureohydrolase; *speC*- ornithine decarboxylase; *speD*- S-adenosylmethionine decarboxylase; *speE*- spermidine synthase; *speF*- inducible ornithine decarboxylase; *cadA*- inducible lysine decarboxylase; *ldcC*- lysine decarboxylase; *adiA*- inducible arginine decarboxylase.

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**FIGURE LEGEND**

**Figure 1.** Transcriptome analysis showing the difference in gene expression in polyamine supplemented vs polyamine deficient cultures. The volcano plot shows the significance of the treatment with polyamines on the y-axis (expressed in p-values obtained from analysis of variance (ANOVA) from assays of three biological replicates) and the fold-change in relative gene expression after polyamine addition on the x-axis. Polyamine mutant cultures were grown in M9 medium to deplete polyamines as described in the Materials and Methods section. When the cultures reached 0.6 OD$_{600}$ the cultures were divided into two parts; to one part of the culture 100 µM putrescine and 100 µM spermidine (“plus PA”; final concentrations) were added; the other part was untreated (“minus PA”). Samples were harvested after different times (20, 40 and 60 min); microarray data from the 60 min time point is shown here. Five highly induced acid response pathway genes are circled.

**Figure 2.** Glutamic acid, polyamines and *gadA-gadB* are required for acid survival of an *E. coli* polyamine mutant. (A) Survival of *E. coli* cells in pH 2.5 medium was assayed as described in the Materials and Methods section. Polyamine mutant HT839 was grown in M9 medium with or without polyamines (100 µM putrescine plus 100 µM spermidine) and with or without glutamic acid (300 µM) until stationary phase and diluted in pH 2.5 medium with the same supplements to approximately 2x10$^8$ cells/ml. At the indicated times aliquots of the cultures were assayed for cell survival on LB plates. Results are averages of three separate assays and are expressed as colony forming units/ml of culture. (B) Cells (HT839) were grown as above in the presence of glutamic acid (300 µM) with or without 100 µM of putrescine (PUT), cadaverine (CAD) or spermidine (SPD) until stationary phase and diluted into pH 2.5 medium with the same supplements and assayed for cell survival. (C) Polyamine mutants (HT839-*gadA*-*gadB*) and (HT857-*ΔgadA*-Δ*gadB*) were grown in M9 medium with glutamic acid (300 µM) and polyamine (100 µM putrescine plus 100 µM spermidine) supplements as above and cell survival was assayed at the indicated times.

**Figure 3.** Polyamine requirement for induction of glutamate decarboxylase in *E. coli*. (A) Polyamine mutant HT839 was grown either in the absence of any polyamines (-PA) or in the presence of the indicated amounts of putrescine (PUT) and spermidine (SPD) for 24 h and glutamate decarboxylase assays were performed as described in the Materials and Methods section. Results are expressed as μmoles of $^{14}$CO$_2$ released/mg protein/h at 37°C using L-[U$^{14}$C]-glutamic acid as a substrate, and represent the average of triplicate assays (B) To study the induction of glutamate decarboxylase at different stages of growth, polyamine mutant HT839 was grown with or without a mixture of 100 µM of putrescine and 100 µM spermidine and the cultures were harvested at different optical densities for glutamate decarboxylase assays (upper panel). One µg aliquots of these of cell extracts were also used to detect the level of glutamate decarboxylase protein by western blot analysis with anti-rabbit GadA/B antibody (lower panel). (C) HT839 was grown in the absence of amines and diluted in amine free medium as above and grown to 1.2 OD$_{600}$. The culture was divided into four parts; to two parts rifampicin was added at a final concentration of 10 µg/ml concentration; two parts did not receive any rifampicin. After 30 min of further incubation at 37°C, polyamines (100 µM putrescine plus 100 µM spermidine) were added to two of the tubes as indicated on the figure and all of the tubes were incubated further. After 3h of incubation, cells were harvested (at early stationary phase) for protein extraction and glutamate decarboxylase assays.
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**Figure 4.** Change in the cAMP level is not required for polyamine induction of glutamate decarboxylase. (A) Intracellular concentrations of cAMP were measured by ELISA from cultures (HT839) grown with or without polyamines at 100 µM concentrations of either putrescine (PUT) or spermidine (SPD) or both (PA). Results are the average of three samples from each of three independent cultures. (B) Glutamate decarboxylase assays from cell extracts grown without (-PA) or with (+PA; 100 µM putrescine plus spermidine) for 24h. The strains used were HT839 (polyamine mutant, *cyA crp*), HT840 (HT839, Δ*cyA crp*), and HT841 (HT839 Δ*crp*). Results are average of three assays. (C) One microgram of protein extracts was used to detect glutamate decarboxylase (GAD) proteins by western blot assays. (D) Polyamine mutants (HT839, *cyA crp*) and (HT840, Δ*cyA crp*) were grown in M9 medium with glutamic acid (300 µM) and with or without polyamine (100 µM putrescine plus 100 µM spermidine) supplementations as above and cell survival was assayed at the indicated times.

**Fig. 5** Effect of *rpoS* levels on induction of glutamate decarboxylase. (A) We have introduced deletion of *rpoS* gene in our polyamine biosynthesis mutant strain (HT839) to make a polyamine deficient-Δ*rpoS* strain (HT843). Cultures of each strain were grown for 24 h in the presence of a 100 µM mixture of putrescine plus spermidine in M9 medium containing all the essential amino acids (present in MEM mixtures, Sigma) needed for optimum growth. Glutamate decarboxylase was measured in the cell extracts in triplicate. (B) HT839 cultures were grown in the presence or absence of polyamines, and harvested at different cell densities (late log to stationary phase). RpoS protein was detected by western blot with anti-RpoS antibody in 5 µg aliquots of protein extracts (upper panel). The blot was then scanned to quantitate the relative RpoS protein level (lower panel). (C) HT839 cells were transformed with a control plasmid (pACYC184) or with an *rpoS* overexpression plasmid (p*ArpoS*). These cells were grown with or without polyamines plus 20 µg/ml of chloramphenicol for 24 hours; glutamate decarboxylase activity was then measured in these cell extracts. Results are average of three assays.

**Fig. 6** Role of GDAR regulators (*gadX, gadY, gadE, ydeO* and *rcsB*) on the polyamine-induced activation of glutamate decarboxylase. (A) We have introduced deletions of *gadX* or *gadY* gene into the polyamine biosynthetic mutant strain (HT839) to make a polyamine mutant/Δ*gadX* strain (HT850) or a Δ*gadY* (HT851) strain. Cultures were grown with or without a 100 µM mixture of putrescine plus spermidine in M9 medium for 24 h. Glutamate decarboxylase activity (upper panel) and protein levels (lower panel) were measured in the cell extracts. (B) A strain was constructed by deleting *gadE* gene in our polyamine biosynthetic mutant strain (HT839) to make a polyamine mutant-Δ*gadE* strain (HT844). Cultures were grown with or without 100 µM mixture of putrescine plus spermidine in M9 medium for 24 h. Glutamate decarboxylase activity was measured in the cell extracts in triplicates (upper panel), and protein level was measured by western blotting using one of the triplicate extracts (lower panel). (C) Glutamate decarboxylase assays in cell extracts from cells grown without or with 100 µM putrescine plus spermidine for 24h. The strains for Fig. 6C were HT859 (HT839, Δ*ydeO*) and HT860 (HT839, Δ*rcsB*). Results are the average of three assays.

**Fig. 7** *gadE* and *rpoS* overexpression induces glutamate decarboxylase and protects cells from acid stress even in the absence of polyamines (A) HT839 cells containing no plasmids or transformed with pQE*gadE* plasmid (HT862) or both pQE*gadE* and p*ArpoS* plasmids (HT865) were grown in M9 medium (with any necessary antibiotics) with or without polyamine
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supplementation (100 μM spermidine plus 100 μM putrescine) to 0.4 OD$_{600}$. *gadE* expression was induced by adding 0.5 mM IPTG and the cultures were grown for an additional 24 h. Cell extracts were made from these cells and assayed for glutamate decarboxylase activity and western blot analysis. (B) HT865 (i.e. HT839 with both pQE*gadE* and pArpo*S*) were grown and induced by 0.5 M IPTG as described above but with the inclusion of glutamate (300 μM) in the media. At the end of the 24 h incubation, the cells were tested for acid survival at pH 2.5.
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### Table 1: Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant genotype | Source or reference |
|-------------------|-------------------|---------------------|
| **Strains**       |                   |                     |
| HT765             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC::kan thr-1 proA2 rpoS<sup>Sm</sup> | (28) |
| HT776             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Sm</sup> | (28) |
| HT838-11          | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Sm</sup> ΔcyC | HT776 X ΔacyC (JW2720-1) |
| HT839             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> | This study |
| HT840             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> ΔcyA::kan | HT839 X ΔcyA::kan (JW3778-3) |
| HT841             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> Δcrp::kan | HT839 X Δcrp::kan (JW5702-4) |
| HT843             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA ΔrpoS::kan | HT839 X ΔrpoS::kan (JW5437-1) |
| HT844             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> ΔgadX::kan | HT839 X ΔgadX::kan (JW3480-2) |
| HT850             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> ΔgadY::kan | HT839 X ΔgadY::kan (JW3484-1) |
| HT851             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> ΔgadY::kan | This study |
| HT857             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> ΔgadA ΔgadB::kan | HT389 X ΔgadA:: kan (JW3485-1)X ΔgadB::kan (JW1488-7) |
| HT859             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> ΔydeO::kan | HT839 X ΔydeO::kan (JW1494-1) |
| HT860             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> ΔrcsB::kan | HT839 X ΔrcsB::kan (JW2205-2) |
| HT862             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> pQE-gadE | This study |
| HT863             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> pACYC184/control | This study |
| HT864             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> pArpoS | This study |
| HT865             | δ(speAspE) Δ(speCglc) Δ(speDspeE) ΔcadA ΔspeF ΔdcC thr-1 proA2 ΔadiA rpoS<sup>Wt</sup> pArpoS/pQE-gadE | This study |
| **Plasmids**      |                   |                     |
| pC20              | FLP recombinase    | (31) |
| pACYC184          | Control plasmid    | (32) |
| pArpoS            | rpoS overexpression | (32) |
| pQE-gadE          | gadE overexpression | (18) |

All the strains obtained in this study for relevant gene deletion were obtained from Yale *E. coli* genetic stock center, except ΔgadY::kan strain was obtained from Dr. Gisela Storz of NICHD, NIH. The pQE-gadE plasmid was obtained from Dr. John Foster of University of South Alabama (Mobile, USA) and the pArpoS and control plasmids were obtained from Dr. Udo Bläsi of University of Vienna (Vienna, Austria).
### Table 2. Induction of glutamate decarboxylase dependent acid resistance (GDAR) and other acid response genes of *E. coli* by polyamines

| Gene name/Probe set ID | Function                                      | Reference | Fold increase after 20 min | Fold increase after 40 min | Fold increase after 60 min |
|------------------------|-----------------------------------------------|-----------|---------------------------|----------------------------|----------------------------|
| *gadB* (1760545)       | Glutamate decarboxylase B                     | (38)      | 3.8                       | 5.9                        | 6.9                        |
| *gadC* (1768165)       | Glutamate-GABA antiporter                     | (17)      | 2.7                       | 3.9                        | 4.9                        |
| *slp* (1768627)        | Outer membrane lipoprotein                   | (39)      | 2.6                       | 3.3                        | 4.8                        |
| *hdeA* (1765321)       | Periplasmic chaperone of acid-denatured proteins A | (40)      | 2.9                       | 3.5                        | 3.7                        |
| *gadA* (1768498)       | Glutamate decarboxylase A                     | (38)      | 2.0                       | 2.7                        | 3.5                        |
| *ybaS* (1759740)       | Glutaminase 1                                 | (41)      | 2.0                       | 2.6                        | 3.2                        |
| *gadE* (1766528)       | Transcriptional regulator of *gadA* and *gadBC* | (19)      | 1.8                       | 1.9                        | 2.2                        |
| *rpoS* (1761030)       | Stress and stationary phase sigma S; Sigma38  | (42)      | 1.9                       | 2.0                        | 2.0                        |
| *hdeB* (1759829)       | Periplasmic chaperone of acid-denatured proteins B | (40)      | 1.6                       | 1.7                        | 1.8                        |
| *gadY* (1764675)       | sRNA regulator of *gadAB* transcriptional activator *GadX* mRNA | (23)      | 1.1                       | 1.3                        | 1.7                        |
| *gadX* (1760509)       | Transcriptional activator for *gadA* and *gadBC* | (43)      | 1.1                       | 1.4                        | 1.7                        |
| *dsrA* (1765903)       | Regulatory sRNA enhances translation of *rpoS* | (44)      | 1.2                       | 1.5                        | 1.6                        |

Data are average of three independent experiments (p<0.05); functional annotations are based on Ecogene Database ([www.ecogene.org](http://www.ecogene.org)). Probe set ID is Affymetrix probe set ID of *E. coli* microarray chip 2.0.
Fig. 1
Fig. 2

A

Cell survival (colony forming units/ml)

Minutes at pH 2.5

No polyamines/No glutamic acid

Polyamines/Glutamic acid

Glutamic acid

No polyamines/No glutamic acid

B

Cell survival (colony forming units/ml)

Minutes at pH 2.5

SPD/Glutamic acid

PUT/Glutamic acid

CAD/Glutamic acid

Glutamic acid

C

Cell survival (colony forming units/ml)

Minutes at pH 2.5

gadA⁺ gadB⁺ /

Polyamines/Glutamic acid

ΔgadA-ΔgadB

Polyamines/Glutamic acid
Fig. 3

Glutamate decarboxylase activity (μmoles of $^{14}$CO$_2$ released/mg protein/h)

- No polyamines
- +1 μM PUT
- +10 μM PUT
- +100 μM PUT
- +1 μM SPD
- +10 μM SPD
- +100 μM SPD
- PUT + SPD

A

Glutamate decarboxylase activity (μmoles of $^{14}$CO$_2$ released/mg protein/h)

- No polyamines
- No polyamines plus rifampicin
- Plus polyamines
- Plus polyamines plus rifampicin

C

Glutamate decarboxylase activity (μmoles of $^{14}$CO$_2$ released/mg protein/h)

- OD$_{600}$ 0.5, 1.0, 1.2, 1.6
- Stationary phase 0.5, 1.0, 1.2, 1.6

B

GadA/B 53 kDa

53 kDa
Fig. 4
Fig. 5

A. Glutamate decarboxylase activity (μmoles of 14CO2 released per protein/h) for strains with and without RpoS under different conditions.

- **Strain:** rpoS⁺, ΔrpoS
- **Polyamine:** +, +

B. RpoS protein (relative intensity) under varying OD₆₀₀ concentrations with and without polyamines.

- **OD₆₀₀:** 1.4, 1.8, 2.0, 2.2
- **Polyamine:** minus, plus

C. Glutamate decarboxylase activity (μmoles of 14CO2 released per protein/h) for strains with and without prpoS.

- **Strain:** rpoS⁺, ΔrpoS
- **Polyamines:** - (control), - (control), +, +
- **Plasmids:** Control, prpoS, Control, prpoS

RpoS protein at 38kDa is indicated.
Fig. 6
Fig. 7
Polyamines are critical for the induction of the glutamate decarboxylase-dependent acid resistance system in Escherichia coli
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