Control of Acid Resistance Pathways of Enterohemorrhagic *Escherichia coli* Strain EDL933 by PsrB, a Prophage-Encoded AraC-Like Regulator

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Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes bloody diarrhea and hemolytic-uremic syndrome (HUS) and is the most prevalent *E. coli* serotype associated with food-borne illness worldwide. This pathogen is transmitted via the fecal-oral route and has a low infectious dose that has been estimated to be between 10 and 100 cells. We and others have previously identified three prophage-encoded AraC-like transcriptional regulators, PatE, PsrA, and PsrB in the EHEC O157:H7 EDL933 strain. Our analysis showed that PatE plays an important role in facilitating survival of EHEC under a number of acidic conditions, but the contribution of PsrA and PsrB to acid resistance (AR) was unknown. Here, we investigated the involvement of PsrA and PsrB in the survival of *E. coli* O157:H7 in acid. Our results showed that PsrB, but not PsrA, enhanced the survival of strain EDL933 under various acidic conditions. Transcriptional analysis using promoter-*lacZ* reporters and electrophoretic mobility shift assays demonstrated that PsrB activates transcription of the *hdeA* operon, which encodes a major acid stress chaperone, by interacting with its promoter region. Furthermore, using a mouse model, we showed that expression of PsrB significantly enhanced the ability of strain EDL933 to overcome the acidic barrier of the mouse stomach. Taken together, our results indicate that EDL933 acquired enhanced acid tolerance via horizontally acquired regulatory genes encoding transcriptional regulators that activate its AR machinery.

*Escherichia coli* strains of serotype O157:H7 are associated with epidemic and sporadic infection worldwide. EHEC is a food- and waterborne pathogen that can cause hemorrhagic colitis and hemolytic-uremic syndrome (1–4). The main reservoir of EHEC is cattle and other ruminants that shed the microorganism in feces. Many outbreaks have been associated with contaminated beef products, milk, vegetables, and fruits (5, 6). The infectious dose of EHEC is extremely low (∼10 to 100 cells), which significantly increases the risk of infection (7).

EHEC expresses one or two cytotoxins, Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2), which are responsible for the life-threatening manifestations of infection (8, 9). In addition, EHEC (O157:H7) strains carry a pathogenicity island known as the locus of enterocyte effacement pathogenicity island (LEE PAI), which encodes portions of a type III secretion pathway that allows *E. coli* to enter intestinal epithelial cells and form attaching-and-effacing lesions, which cause the destruction of microvilli and the rearrangement of cytoskeletal proteins (11, 12). Expression of the five operons within the LEE PAI is tightly regulated by a number of transcriptional regulators including H-NS, Ler, GrlA, integration host factor (IHF), GdeE, and QseA, which allows the various virulence factors to be expressed when EHEC enters its preferred environmental niches in the host intestine (13–15).

To reach its site of colonization, EHEC must traverse the acidic environment of the human stomach. Although the environment within the large intestine is less acidic, EHEC also encounters volatile organic acids produced from anaerobic fermentation by the local microbiota (16). *E. coli* strains in general have evolved different acid resistance (AR) mechanisms that allow it to overcome the acidic conditions within its various hosts (17, 18). Like other *E. coli* bacteria, EHEC possesses an oxidative or amino acid-independent, acid resistance pathway (acid resistance pathway 1 [AR1]), and at least three amino acid-dependent acid resistance pathways (AR2 to AR4), which require glutamate, arginine, and lysine, respectively, to function (17, 19). Whereas the mechanism of action of AR1 remains unclear, AR2, AR3, and AR4 have been well characterized (20, 21). Each of these three systems (AR2 to AR4) consists of an inner membrane antiporter and one or two decarboxylase enzymes, which transfer intracellular protons to an amino acid (glutamate, arginine, or lysine) and expel the resulting amines into the extracellular medium in exchange for a related amino acid (21). In addition, *E. coli* strains carry an *hdeAB* operon encoding acid stress chaperones that protect periliposomal proteins from aggregation at low pH (22, 23).

Recently, we and others showed that the prototypical EHEC O157:H7 strain, EDL933, carries three prophage-encoded loci, *patE*, *psrA*, and *psrB*, which encode AraC-like transcriptional regulators that downregulate the expression of the LEE-en-
TABLE 1 Bacterial strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics</th>
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<td><strong>E. coli strains</strong></td>
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<td>EDL933(Δstx1-2)</td>
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a Ap′, ampicillin resistance; Kan′, kanamycin resistance; Strr, streptomycin resistance; Tpr, trimethoprim resistance.

coded type III secretion pathway (24, 25). Our previous work also demonstrated that PatE is involved in transcriptional activation of AR pathways of strain EDL933 (24). The aim of the current study was to determine whether PsrA and PsrB proteins play a role in control of the AR pathways. Our findings indicated that PsrB, but not PsrA, functions as a positive regulator which enhances the ability of EDL933 to survive in different acidic environments.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and primers. The bacterial strains and plasmids used in this study are listed in Table 1. Unless stated otherwise, bacteria were grown at 37°C in Luria-Bertani broth (LB) or on Luria-Bertani agar (LA) plates supplemented with the appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; trimethoprim, 40 μg/ml. The PCR primers used in this study are listed in Table 2.

Molecular biological techniques. Plasmids were isolated using a QIAprep miniprep kit (Qiagen) or Wizard plus SV miniprep kit (Promega). The yield and purity of genetic products were determined by using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Restriction enzyme digestions were performed using enzymes and buffers from New England BioLabs (NEB) according to the manufacturer’s instructions. DNA sequencing was performed using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) in accordance with the manufacturer’s instructions. Sequencing reactions were completed in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems). Analysis of sequencing results was achieved using the Sequencer (Gene Codes) and DNA Strider (http://cellbiol.com/soft.htm) programs.

Construction of trans-complementing plasmids, pY1 and pY2. For complementation of mutants, 0.8-kb DNA fragments containing psrA or psrB were amplified from EHEC EDL933 genomic DNA by using primer pairs, psrAB-Fw and psrAB-Rv (Table 2). These fragments were cloned into pCR2.1-TOPO. The inserts of the two plasmids were confirmed by sequencing and cloned into the BamHI and Sall sites of plasmid pACYC184 to yield pY1 and pY2.

Acid tolerance assays. Growth phase acid tolerance assays were conducted as described previously (24). Briefly, bacterial cells from an over-
night culture were diluted 1 in 1,000 in HEPES-buffered LB (pH 7.0) and grown aerobically at 37°C under constant agitation (200 rpm). Aliquots were withdrawn over a 20-h period and diluted 1 in 100 in either fresh LB or a challenge medium of acidified LB adjusted with HCl to pH 2.5, 3.0, or 3.5. Bacterial numbers before challenge were determined by viable counts of the initial inoculum on LA. The challenged cells were incubated aerobi-

cally for 2 h at 37°C and 200 rpm and then immediately diluted in LB for enumeration as described above. Survival of cells was determined by com-

paring the viable counts of challenged cells to those in the original inocu-

ulum.

Glutamate-dependent acid resistance, AR2, was assayed as described by Vogel and Bonner (26). Briefly, cells were grown overnight (20 h) in HEPES-buffered LB (pH 7.0). Cultures were then diluted 1 in 1,000 in HCl-acidified EG medium (0.8 mM MgSO4, 10 mM citric acid monohy-

drinate, 73 mM K2HPO4, 17 mM NaNH4PO4, 0.4% glucose) supple-

mented with 1.5 mM glutamate (the pH of the medium was 3.0). The cells were then incubated for 2 h at 37°C and 200 rpm, after which survival was assessed as described above.

Resistance to volatile organic acids was assessed in a similar manner to AR2 except that the challenge medium was HCl-acidified LB supple-

mented with 40 mM sodium acetate (NaOAc). For all acid tolerance ass-

ays, the pH of the challenge medium was reconfirmed after incubation to

ensure consistency of challenge.

Infection of mice. E. coli EDL933(Δstx1-2, Δ3) containing either pY2 (PsrB\(^+\)) or pACYC184 (control) was grown for 20 h in LB. A total of 10\(^8\) CFU of each strain were administered by gavage to nine c57BL/6 mice using a 20-gauge needle (Cole-Palmer). After 40 min, mice were killed by CO2, inhala-

tion, and the stomach and entire intestine were removed. Samp-

les were homogenized in phosphate-buffered saline (PBS) with a Poly-

tron homogenizer (Kinematica) and diluted in PBS, and the bacteria were enumerated on selective agar. The percentage survival of bacteria was calculated from the ratio of the number of bacteria isolated from each mouse to that in the inoculum. All work with animals was approved from The University of Melbourne Animal Ethics Committee and conducted in accordance with the guidelines for animal experimentation of the Austra-

lian National Health and Medical Research Council.

β-Galactosidase assay. Bacteria were grown to mid-log phase (optical density at 600 nm [OD\(_{600}\)] of ~1.0). β-Galactosidase activity was assayed by the method of Miller (27) and expressed in Miller units.

Expression and purification of maltose-binding protein-PsrB fu-

sion protein (MBP::PsrB). The coding sequence of psrB was amplified from EDL933 genomic DNA by using primer pair MBP::PsrB-BamHI-

Fw/MBP::PsrB-HindIII-Rv, and the resulting amplicon was cloned into pJY1 and pJY2, which express PsrA and PsrB, respectively, as well as the control vector, pACYC184, into the HEC strain EDL933(Δstx1-2, Δ3), in which the genes encoding the two Shiga toxins and the three AraC-like regulatory proteins (PatE, PsrA, and PsrB) are inactive (24).

Bacterial numbers before challenge were determined by viable counts on LA. The challenged cells were incubated aerobically at 37°C under constant agitation (200 rpm). Aliquots of each strain were withdrawn at both the early (6 h) and late (20 h) stationary phase, and the bacteria were assessed for their ability to survive for 2 h in LB at pH 2.5, 3.0, and 3.5, conditions consistent with those in the human stomach (29, 30).

Early stationary-phase cultures of the control strains, EDL933 (Δstx1-2, Δ3) strain carrying pACYC184 [EDL933(Δstx1-2, Δ3)/pACYC184] and PsrA\(^+\) strain [EDL933(Δstx1-2, Δ3)/pY1], failed to survive the 2-h acid treatment at pH 2.5 and pH 3.0 (Fig. 1B). In contrast, approximately 4.2% and 11.8% of the PsrB protein.

Statistical analysis. Quantitative data were confirmed to be normally distributed and then analyzed by using Student’s t test. A two-tailed P value of <0.05 was taken to indicate statistical significance.

RESULTS

Induction of acid tolerance in EHEC strain EDL933 by PsrB. We previously showed that overexpression of the PatE regulatory protein in EHEC EDL933 enhances its AR capacity in stationary phase (24). To determine whether the two homologs of the PatE protein, PsrA and PsrB, are also involved in AR, we did acid tolerance assays. To do this, we first introduced the pACYC184 derivatives, pY1 and pY2, which express PsrA and PsrB, respectively, as well as the control vector, pACYC184, into the EHEC strain EDL933(Δstx1-2, Δ3), in which the genes encoding the two Shiga toxins and the three AraC-like regulatory proteins (PatE, PsrA, and PsrB) are inactive (24).

Early stationary-phase cultures of the control strains (pACYC184) or pACYC184 (control) survived at pH 2.5 and 3.0, respectively (Fig. 1B). At pH 3.5, ~6.4% and ~9.5% of the control and PsrA\(^+\) strains survived, respectively, compared to ~30.6% of the PsrB\(^+\) strain (Fig. 1B).

Late cultures of the control and PsrA\(^+\) strains also did not survive at pH 2.5 and pH 3.0, compared with survival rates of 8.9% and 32%, for the PsR\(^+\) strain at pH 2.5 and 3.0, respectively (Fig. 1C). Furthermore, after the 2-h acid treatment at pH 3.5, the survival rates of the control, PsrA\(^+\), and PsrB\(^+\) strains increased to ~10.7%, ~14.2%, and ~54.5%, respectively (Fig. 1C).

These results clearly demonstrated that PsrB, but not PsrA, plays a positive role in acid tolerance. Consequently, our subsequent investigations of acid tolerance in strain EDL933 focused on the PsrB protein.
Activation of Acid Resistance by PsrB

FIG 1 Determination of the effects of PsrA and PsrB on growth of EHEC EDL933Δstx1-2, Δ3) (A) and its survival under acidic conditions (B and C). Overnight cultures of the control strain EDL933Δstx1-2, Δ3)/pACYC184 (Δ3 + pACYC184) and two test strains, EDL933Δstx1-2, Δ3)/pJY1 (PsrA+) and EDL933Δstx1-2, Δ3)/pJY2 (PsrB+), were diluted 1 in 1,000 in HEPES-buffered LB (pH 7.0). (A) Growth of the three strains was monitored for 20 h using a Klett-Summerson photoelectric colorimeter. (B and C) Aliquots were withdrawn at two time points, 6 h (B) and 20 h (C), and assessed for their ability to survive in LB, pH 2.5, 3.0, and 3.5, for 2 h. Percentage survival was determined by comparison of viable counts of the untreated sample to that after 2 h in the challenge medium. Results are the means ± standard deviations (SDs) (error bars) from three individual experiments. Asterisks indicate significant differences between the values for the control and test strains (P < 0.01 by two-tailed Student’s t test).

Contribution of the chromosomally encoded psrB gene to the protection of strain EDL933 in various low-pH environments. We next examined the effects of the chromosomal copy of the psrB gene on the acid tolerance of strain EDL933. This was done by comparing the survivability of the triple-knockout strain of EDL933 [EDL933Δstx1-2, Δ3)] and double-knockout strain of EDL933 [EDL933Δstx1-2, Δ2)] under various acidic conditions.

Following the growth of the bacterial cells in LB (pH 7.0) for 6 or 20 h, an acid tolerance assay was carried out. As shown in Fig. 2A and B, the EDL933Δstx1-2, Δ3) (PsrB+) strain grown for either 6 or 20 h did not survive at pH 2.5 or 3.0. Conversely, a significant proportion of the EDL933Δstx1-2, Δ2) (PsrB+) strain (~2.5% and ~4.9% from the 6-h time point and ~3.5% and ~8.6% from the 20-h time point) survived an acid challenge at pH 2.5 and 3.0, respectively. At pH 3.5, ~5.8% and ~12.6% of the EDL933Δstx1-2, Δ3) strain from early and late stationary-phase cultures, respectively, survived the acid challenge (Fig. 2A and B), compared with ~21% and ~39%, respectively, for the EDL933Δstx1-2, Δ2) strain (Fig. 2A and B). These results indicate that the chromosomally encoded PsrB protein contributes significantly to the acid tolerance of strain EDL933.

Psrb enhances AR2-mediated acid tolerance and protects strain EDL933 against volatile organic acids. To test whether expression of PsrB in strain EDL933 affected AR2-mediated acid tolerance, we compared the survival of the control strain EDL933Δstx1-2, Δ3)/pACYC184 (Δ3 + pACYC184) and the PsrB+ strain EDL933Δstx1-2, Δ3)/pJY2 (Δ3 + pJY2) to tolerate acidified minimal EG medium (pH 3.0) supplemented with 1.5 mM glutamate for 2 h (Fig. 3). The glutamate-supplemented EG medium was used to avoid providing amino acids that are employed by AR2, AR3, and AR4. Moreover, the glucose in the me-

FIG 2 Effect of a single copy of psrB on the survival of EHEC EDL933 at low pH. The PsrB+ test strain Δ2 [EDL933Δstx1-2, Δ3)] and the PsrB- control strain Δ3 [EDL933Δstx1-2, Δ3)] were each grown in HEPES-buffered LB (pH 7.0) at 37°C for 6 h (A) or 20 h (B). The cultures were then diluted 1 in 1,000 in LB at pH 2.5, 3.0, or 3.5 and incubated at 37°C with shaking (200 rpm) for 2 h. Viable counts were obtained by plating on LA. Percentage survival was determined by comparison of the viable count of the untreated sample to that of the acid-challenged sample. Results are the means ± SDs from three independent experiments. Asterisks indicate significant differences between the values for the PsrB+ strain and control strains (P < 0.001 by two-tailed Student’s t test).

FIG 3 Effect of PsrB on the survival of EHEC EDL933Δstx1-2, Δ3) strains in acidified EG medium supplemented with glutamate and acidified LB containing sodium acetate (NaOAc). The control strain EDL933Δstx1-2, Δ3)/pACYC184 (PsrB+) and test strain EDL933Δstx1-2, Δ3)/pJY2 (PsrB+) were grown for 20 h as described in the legend to Fig. 2. Cultures were then diluted 1 in 1,000 in EG medium supplemented with 1.5 mM glutamate (medium pH 3.0) or in LB broth supplemented with 40 mM NaOAc (broth pH 3.0). Cells were incubated for 2 h (37°C and 200 rpm), and viable counts were obtained. Survival is expressed as a percentage of the untreated sample. Results are means ± SDs from three independent experiments. Asterisks indicate significant differences between the values for the PsrB+ and control strains (P < 0.005 by two-tailed Student’s t test).
Effect of PsrB on the survival of EHEC EDL933 (Δstx1-2, Δ3) in mice. The PsrB− control strain, EDL933 (Δstx1-2, Δ3)/pACYC184, and a PsrB+ test strain, EDL933 (Δstx1-2, Δ3)/pJY2, were each grown for 20 h in LB buffered at pH 7.0. A total of 10^6 CFU of each strain were then administered perorally to C57BL/6 mice. Mice were killed 40 min later, and the stomach, small intestine, and large intestine were excised. Samples were homogenized in PBS and spread on selective agar plates for the enumeration of viable bacteria. Each symbol represents the value for an individual mouse, and the mean (black line) ± SDs (error bars) of the percent survival for the group of mice is shown. Percent bacterial survival was calculated as follows: ratio of the number of bacteria isolated from each mouse to the number of bacteria in the inoculum × 100. The mean values for the two groups were significantly different (P < 0.001 by two-tailed Student’s t test) as indicated by the asterisk.

Effect of psrB-mediated AR in vivo. We have described a mouse model to quantify the effectiveness of gastric acid in mediating resistance to infection with ingested bacteria (32). For the present study, we used this model to examine the effect of expression of the psrB gene on the AR of EHEC in vivo. The EDL933 (Δstx1-2, Δ3) strain carrying either the control plasmid, pACYC184, or the PsrB-expressing plasmid, pJY2, were each administered perorally to C57BL/6 mice. After 40 min, the mice were killed by CO2 inhalation, and the stomach, small intestine, and large intestine were excised. Samples were homogenized in PBS and spread on selective agar plates for the enumeration of viable bacteria. Each symbol represents the value for an individual mouse, and the mean (black line) ± SDs (error bars) of the percent survival for the group of mice is shown. Percent bacterial survival was calculated as follows: ratio of the number of bacteria isolated from each mouse to the number of bacteria in the inoculum × 100. The mean values for the two groups were significantly different (P < 0.001 by two-tailed Student’s t test).

Transcriptional activation of the hdeA promoter by PsrB. To determine whether PsrB functions as a transcriptional activator of AR, we investigated the effect of PsrB on the expression of the hdeA gene encoding the periplasmic chaperone HdeA, a key protein for the AR of E. coli. This experiment involved the use of four hdeA promoter-lacZ transcriptional fusions in which various hdeA promoter fragments of different lengths were cloned in front of the promoter-less lacZ structural gene on a single-copy plasmid, pJP1433 (24). The four constructs were each transformed into strain EDL933 (Δstx1-2, Δ3) containing either pACYC184 (control) or pJY2 (PsrB+), and the resultant strains were assayed for β-galactosidase activity. As shown in Fig. 5, the construct hdeA-lacZ-1 (pJB29), which contained the region between positions −443 and +174 relative to the transcriptional start site of hdeA, expressed 149 Miller units of β-galactosidase by EHEC EDL933 (stx1-2, Δ3) with or without PsrB and containing one of the four hdeA-lacZ fusions shown. Numbering of the various hdeA fragments is relative to the hdeA transcriptional start site. β-Galactosidase activity is expressed as Miller units, and the values are means ± SDs from three independent assays. The fold activation (fold act.) is the ratio of β-galactosidase activity of the PsrB+ strain to that of the PsrB− strain.

β-Galactosidase activity (Miller units)

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<td>161 ± 13</td>
<td>163 ± 10</td>
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FIG 5 β-Galactosidase expression by EHEC EDL933 (Δstx1-2, Δ3) with or without PsrB and containing one of the four hdeA-lacZ fusions shown. Numbering of the various hdeA fragments is relative to the hdeA transcriptional start site. β-Galactosidase activity is expressed as Miller units, and the values are means ± SDs from three independent assays. The fold activation (fold act.) is the ratio of β-galactosidase activity of the PsrB+ strain to that of the PsrB− strain.
FIG 6 EMSA analysis of binding of the fusion protein MBP::PsrB to the hdeA promoter region. $^{32}$P-labeled PCR fragments were incubated for 30 min at 25°C with increasing amounts of MBP::PsrB (0, 37.5, 75, 150, or 300 nM MBP::PsrB in lanes 1 to 5, respectively), after which the samples were analyzed on native polyacrylamide gels. (A to C) The $^{32}$P-labeled PCR fragments were the labeled hdeA promoter fragment (hdeA-prom, comprising nucleotides −252 to +50 relative to the transcriptional start site) (A), the $^{32}$P-labeled control fragment (hdeA-control, comprising nucleotides +162 and +363 of the hdeA coding region) (B), and the labeled hdeA-prom fragment in the presence of approximately 100-fold molar excess of unlabeled hdeA-prom fragment (C). Bands of free DNA (F) and DNA-protein complex (C) are indicated to the left of the gels.

FIG 7 Amino acid alignment of PsrA and PsrB performed by using the ClustalW2 multiple-sequence alignment tool provided by the EMBL-EBI website (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The amino acids in the putative helix-turn-helix (HTH) DNA-binding regions are underlined. Variable amino acid residues are shown in boldface type on a gray background.

DISCUSSION

In this study, we investigated the involvement of the AraC-like regulatory proteins PsrA and PsrB in AR of the prototypical EHEC O157:H7 strain, EDL933, and found that the PsrB protein plays a significant role in enhancing the AR of this pathogen. Our results indicated that PsrB is involved in the induction of AR2 and the expression of glutamate and in LB medium in the presence of sodium acetate. Data from the AR assays carried out in EG medium in the presence of glutamate and in LB medium in the presence of sodium acetate indicated that PsrB is involved in the induction of AR2 and the resistance of EHEC to volatile organic acids. Prior to this study, Tree et al. (25) showed that PsrB upregulates the expression of the gadE gene by binding directly to its regulatory region. In this study, we confirmed this finding by using a gadE promoter-lacZ fusion (data not shown). As GadE is a major regulator responsible for the expression of the hdeAB operon encoding acid stress enzymes. (iv) An MBP::PsrB fusion protein bound directly to the hdeA promoter region. Collectively, these findings provide an explanation for the ability of EHEC to withstand extreme gastric acidity.

The PsrB protein belongs to the AraC superfamily of transcriptional regulators which control the expression of genes involved in diverse cellular functions such as carbon metabolism, stress responses, and virulence (35, 36). In enteric bacterial pathogens, a number of AraC-like activators including AggR of enteroaggregative E. coli (37), Rns of enterotoxigenic E. coli (38), PerA, RegR, and RaIR of enteropathogenic E. coli (39–41), RegA of Citrobacter rodentium (42, 43), and ToxT of Vibrio cholerae (44) play a critical role in bacterial virulence. The genes encoding these virulence activators appear to have coevolved with their principal target virulence genes, which code for secreted factors and surface proteins responsible for intestinal colonization (40, 41, 45, 46). EHEC carries three horizontally transferred genes (patE, psrA, and psrB) encoding AraC-like regulators. Although none of these proteins activates virulence gene expression directly (24, 25), our previous and current studies have shown that PatE and PsrB contribute to EHEC virulence by enhancing its ability to survive in acidic environments in the gastrointestinal tract and some foods.

Despite the high degree of similarity between PsrA and PsrB at the amino acid sequence level (94%) (Fig. 7) (24), PsrA did not significantly enhance the AR of EHEC. This inability to induce AR is not due to weak or absent expression of the PsrA protein, as we have previously shown that the psrA and psrB genes are transcribed and translated to similar extents in E. coli (24). Therefore, it is likely that the difference in these two proteins is due to variation in the amino acid residues at positions 151, 199, and 222, which are located within their helix-turn-helix DNA-binding domains. As has been demonstrated for other AraC-like regulators, such as RegA (from C. rodentium) and ToxT (from V. cholerae), single-amino-acid changes within the DNA-binding domains can lead to a major change in function (28, 47).

Results of the AR assays performed in this study demonstrated that PsrB protects strain EDL933 from acid stress as low as pH 2.5. Data from the AR assays carried out in EG medium in the presence of glutamate and in LB medium in the presence of sodium acetate indicated that PsrB is involved in the induction of AR2 and the resistance of EHEC to volatile organic acids. Prior to this study, Tree et al. (25) showed that PsrB upregulates the expression of the gadE gene by binding directly to its regulatory region. In this study, we confirmed this finding by using a gadE promoter-lacZ fusion (data not shown). As GadE is a major regulator responsible for the expression of the hdeAB operon encoding acid stress enzymes.
for expression of AR2, it is likely that the PsrB-mediated enhancement of AR2 activity is mediated via induction of the gadE gene.

Several other studies have established the role of the HdeA protein in resistance to volatile organic acids (22, 48, 49). Therefore, the PsrB-mediated increase in resistance to these acids is likely to be due to an increase in the expression of HdeA. Indeed, data from both the transcriptional analysis using promoter-lacZ fusions and the EMSA support this hypothesis by demonstrating a direct upregulation of hdeA by PsrB. Furthermore, by investigating four hdeA-lacZ fusions which contained various truncations in the promoter region, the PsrB-binding site was mapped to positions between −132 and −45 relative to the start site of transcription. This location is well situated for transcriptional activation, as binding to this region would not inhibit the binding of RNA polymerase to the promoter core element which spans the −35 and −10 region (50). Our data indicate that PsrB is a class I transcriptional activator, which characteristically binds immediately upstream of the promoter core element, facilitating the initiation of transcription through interaction with the alpha subunit of RNA polymerase (51).

Data from previous and current studies have also shown that the PatE and PsrB proteins exert parallel regulatory effects on gene expression in EHEC (24, 25). Apart from being responsible for positive control of AR pathways, PatE and PsrB negatively regulate the expression of the LEE PAI via transcriptional activation of the gadE gene (24, 25). Furthermore, all of the known PatE- and PsrB-regulated promoters (e.g., hdeA, gadE, LEE1, and LEE2 or LEE3) are controlled by multiple transcriptional regulators, including both repressors and activators.

EHEC may have acquired two regulatory genes, patE and psrB, with overlapping functions to ensure the development of an infection with effective bacterial replication and transmission to a new host. The ability of these two proteins to exert positive and negative transcriptional effects on different regulatory circuits allows EHEC to shift resources and energy from expressing LEE to produce proteins and enzymes that assist the pathogen to cross the acidic barrier at the early stage of infection. In summary, this study revealed how EHEC O157:H7 has enhanced its virulence through various regulatory pathways to facilitate bacterial survival in different environments.

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