Escherichia coli O157:H7 glutamate- and arginine-dependent acid-resistance systems protect against oxidative stress during extreme acid challenge

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Micro-organisms may simultaneously encounter multiple stresses in their environment. To investigate the protection that several known *Escherichia coli* O157:H7 acid-resistance systems might provide against both oxidative and acid stress, the addition of diamide, a membrane-permeable thiol-specific oxidizing agent, or hydrogen peroxide were used concurrent with acid challenge at pH 2.5 to determine bacterial survival. The addition of either diamide or hydrogen peroxide decreased bacterial survival in a dose-dependent manner for *E. coli* O157:H7 during challenge at pH 2.5 following overnight growth in LB MES pH 5.5 (acid-resistance system 1, AR1). In contrast, the presence of either glutamate or arginine during challenge provided significant protection against diamide- and hydrogen peroxide-induced oxidative stress during pH 2.5 acid challenge. Oxidative stress protection during acid challenge required *gadC* and *adiA* for the glutamate- (AR2) and arginine- (AR3) dependent acid-resistance systems, respectively. In addition, maximal protection against oxidative stress in the presence of glutamate required a low external pH (pH 2.5), since pH 5.5 did not protect. This study demonstrates that the glutamate- and arginine-dependent acid-resistance systems of *E. coli* O157:H7 can simultaneously protect against oxidative stress during extreme acid challenge.

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INTRODUCTION

Human infections with *Escherichia coli* O157:H7 often result in a bloody diarrhoea that may last 2–8 days, but more severe symptoms can occur in young children and elderly individuals (Karch *et al.*, 2005). Occasionally, a consequence of *E. coli* O157:H7 infection is haemolytic uraemic syndrome, which may result in renal failure. The infectious dose for *E. coli* O157:H7 is low and estimated to be less than 100 bacterial cells.

Whether a commensal or a pathogen, it is proposed that most enteric organisms require effective acid-defence systems to traverse the acidity of the stomach and colonize the gastrointestinal tract. Investigation of microbial stress-response systems in *E. coli* indicates that these bacteria have complex acid-defence systems that protect against acid stress at pH 2.5 for several hours (Foster, 2004). Although it has been suggested that the acid resistance of *E. coli*

O157:H7 may result in a low infectious dose, comparison of acid resistance for commensal and pathogenic strains of *E. coli* indicates that both virulent and non-virulent strains have multiple, effective acid-protection systems (Large *et al.*, 2005; Lin *et al.*, 1996).

At least three principal acid-resistance (AR) systems with some overlapping components protect *E. coli* from extreme acid stress (pH 2.5). Acid-resistance system 1 (AR1) is induced in stationary-phase cells grown in buffered LB medium (pH 5.5) and requires the alternative sigma factor, RpoS, as well as the cAMP receptor protein (CRP) (Castanie-Cornet *et al.*, 1999; Lin *et al.*, 1996). Specific AR1 gene products that protect the bacterial cell from acid stress remain largely unknown. Growth in the presence of glucose represses AR1; therefore, *E. coli* grown in LB containing glucose are acid sensitive at pH 2.5 unless either glutamate or arginine is present in the challenge medium. The glutamate (AR2) and arginine (AR3) decarboxylase AR systems afford acid-stress protection and require the decarboxylation of glutamate and arginine, respectively (Hersh *et al.*, 1996; Lin *et al.*, 1995). These amino

Abbreviations: AR, acid resistance; FRT, Flp recombination target; GABA, γ -aminobutyric acid.

acid-dependent AR systems consist of an amino acid decarboxylase and an end-product antiporter, which are necessary for acid protection. For the glutamate-dependent AR system (AR2), there are two glutamate decarboxylase isozymes (GadA and GadB), either of which can decarboxylate glutamate, consuming a cytoplasmic proton in the production of γ -aminobutyric acid (GABA). Subsequently, the GadC antiporter exports GABA from the bacterial cytoplasm in exchange for external glutamate. The arginine-dependent AR system (AR3) functions in an analogous manner to AR2, with the decarboxylation of arginine to agmatine by AdiA and the exchange of agmatine and arginine by the AdiC antiporter. In complex medium (LB), the glutamate decarboxylase AR system is induced in stationary phase and the arginine decarboxylase AR system is induced at low pH values under anaerobic conditions (Foster, 2004).

RpoS is a master regulator of the RpoS stress response that is activated by several different stress signals, including reduction or cessation of bacterial growth (Dempfle, 1991; Hengge-Aronis, 2002; Storz & Imlay, 1999). Induction of the RpoS stress response contributes to protection against multiple environmental stresses including starvation, thermal tolerance, acidic pH, osmotic and oxidative stresses. Therefore, the RpoS stress response may be activated due to a particular signal but the response can cross-protect against other types of environmental stress. The requirement for RpoS in AR1 protection against extreme acid stress suggests that AR1 is a member of the RpoS stress response (Lin *et al.*, 1996). However, as mentioned earlier, AR1 is only induced in buffered LB medium (pH 5.5) since an undefined inhibitor is produced in LB at pH 8 (Castanie-Cornet *et al.*, 1999). Due to the apparent induction of AR1 in buffered LB medium at pH 5.5, these bacterial cultures are considered to be acid adapted, whereas cells grown in LB glucose medium are considered unadapted since expression of *rpoS* is repressed.

During aerobic growth of *E. coli*, reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical ($\cdot\text{OH}$) are continuously produced by the bacterial cell (Gonzalez-Flecha & Dempfle, 1995; Imlay & Fridovich, 1991). Oxidative stress occurs when an imbalance arises between the production and removal of these reactive oxygen species. An accumulation of $\cdot\text{OH}$ radicals can cause DNA and protein damage, lipid peroxidation and disulfide bond formation (Cabiscol *et al.*, 2000; Storz & Imlay, 1999). In response to oxidative stress, bacteria rapidly react to repair the damage incurred, using various inducible genetic systems including the *oxyR* regulon (Aslund & Beckwith, 1999; Dempfle, 1991). Dempfle & Halbrook (1983) demonstrated an adaptive response for *E. coli* K-12 to oxidative stress by exposing *E. coli* to a low concentration (30 μM) of hydrogen peroxide for 30 min followed by challenge with a high concentration (5 mM) of hydrogen peroxide for up to 60 min. The survival of bacterial cells that were previously adapted to the oxidative stress was enhanced compared to cells that were directly

challenged with hydrogen peroxide without prior adaptation. Jenkins *et al.* (1988) demonstrated that glucose starvation (4 h) can induce cross-protection against 15 mM hydrogen peroxide challenge for at least 1 h (Jenkins *et al.*, 1988). Interestingly, glucose starvation induced greater protection against oxidative stress (15 mM hydrogen peroxide) challenge than adaptation with 60 μM hydrogen peroxide for 1 h. It was further demonstrated that RpoS (KatF) was required for starvation-induced resistance to oxidative stress (McCann *et al.*, 1991).

Induction of oxidative stress genes and proteins in *E. coli* K-12 during exposure to acid stress suggests that protection against oxidative stress during acid challenge may be important for bacterial survival (Blankenhorn *et al.*, 1999; Maurer *et al.*, 2005; Stancik *et al.*, 2002). Two-dimensional gel analysis demonstrated that alkyl hydroperoxide reductase (AhpC) and iron superoxide dismutase (SodB), components of the oxidative stress response, are acid-induced proteins (Blankenhorn *et al.*, 1999; Stancik *et al.*, 2002). In addition, DNA microarray analysis demonstrated that *grxA* (glutaredoxin 1), *gshA* (γ -glutamyl-cysteine synthetase) and *gshB* (glutathione synthetase) were acid induced at pH 5.0 compared to pH 7.0 and 8.7 (Maurer *et al.*, 2005). The gene products of *grxA*, *gshA* and *gshB* are components of the glutaredoxin system that catalyses the reduction of disulfides via reduced glutathione (Aslund & Beckwith, 1999; Carmel-Harel & Storz, 2000). Furthermore, it has been demonstrated that another member of the glutaredoxin system, *gor* (glutathione reductase), is regulated by RpoS (Becker-Hapak & Eisenstark, 1995).

Since the RpoS stress-response system cross-protects against multiple environmental stresses, AR1 might protect against combined stresses, such as oxidative stress during acid challenge. The objective of the current study was to determine whether AR1, the glutamate (AR2) or arginine (AR3) decarboxylase AR systems could simultaneously protect *E. coli* O157:H7 from oxidative stress during acid challenge (pH 2.5). The data indicate that the glutamate (AR2) and arginine (AR3) decarboxylase AR systems, but not AR1, provide concurrent protection against oxidative stress during acid challenge at pH 2.5.

METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. Bacteria were grown in either Luria-Bertani (LB) broth or minimal E medium containing 0.4 % glucose (EG medium) (Vogel & Bonner, 1956) at 37 °C. Buffered LB broth contained either 100 mM MES for pH 5.5 medium or 100 mM MOPS for pH 8.0 medium. Antibiotics were used at the concentrations of 100 $\mu\text{g ml}^{-1}$ for ampicillin and 50 $\mu\text{g ml}^{-1}$ for kanamycin. For diamide and hydrogen peroxide experiments, various volumes of either 100 mM diamide [azodicarboxylic acid bis(dimethylamide)] or 100 mM hydrogen peroxide were added to the challenge medium to obtain the desired concentrations. Unless otherwise indicated, all chemicals were purchased from Sigma. Assays for *E. coli* AR systems 1, 2 and 3 were performed as previously described (Castanie-Cornet *et al.*,

Table 1. Bacterial strains

Strain	Genotype or relevant information	Reference
EXB43	<i>E. coli</i> O157:H7 86-24 (NADC 5570)	P. Tarr (Griffin <i>et al.</i> , 1988)
EBB36	EXB43/pKD46	This study
EBB288	EXB43 <i>gadC</i> :: <i>neo</i>	This study
EBB291	EXB43 <i>adiA</i> :: <i>neo</i>	This study
EBB292	EXB43 Δ <i>gadC</i>	This study
EBB295	EXB43 Δ <i>adiA</i>	This study
EBB300	EXB43 Δ <i>gadC</i> /pBADoBBI215/216 (<i>gadBC</i> ⁺)	This study
EBB301	EXB43 Δ <i>adiA</i> /pBADoBBI217/218 (<i>adiA</i> ⁺)	This study

1999). Briefly, following overnight growth in LB MOPS pH 8.0 (control), LB MES pH 5.5 (AR1) or LB glucose (AR2 and AR3), cultures were acid challenged in EG pH 2.5 (AR1), EG 2 mM glutamate pH 2.5 (AR2) or EG 0.6 mM arginine pH 2.5 (AR3) for the indicated periods of time. Percentage survival was determined by dividing the number of c.f.u. ml⁻¹ after challenge by the initial number of c.f.u. ml⁻¹ at *t*₀ and multiplying by 100. For all acid assays, the data represent the means $\pm$ SD of experiments performed a minimum of three times. Statistical analysis was performed on AR assays using SAS Analyst using the two-sample *t*-test for the means. Results were considered significant when the *P*-value was *P* $\leq$ 0.01.

Construction of *E. coli* O157:H7 mutants. The primers used in this study are listed in Table 2. Recombineering (Datsenko & Wanner, 2000) was performed to construct strains containing *gadC* or *adiA* knockouts by transformation of a linear PCR product containing the *neo* gene encoding kanamycin resistance. The oBBI92 and oBBI93 primer pair containing FRT sites (underlined in Table 2) were used to amplify the *neo* gene from pCRII (Invitrogen). The 5' ends of both oBBI92 and oBBI93 have stop codons in all three reading frames to truncate protein translation. Following gel purification, the oBBI92/93 *neo* PCR product was used as a PCR template to amplify the *gadC* and *adiA* knockout fragments using primers oBBI219/207b and

oBBI208b/209b, respectively. Primers oBBI219 (upstream) and oBBI207b (downstream) contain 47 and 46 bases of *gadC* (Z2216) nucleotide sequence (bold in Table 2), respectively. Primers oBBI208b (upstream) and oBBI209b (downstream) contain 45 and 44 bases of *adiA* (Z5719) nucleotide sequence (bold in Table 2), respectively. Following PCR purification of oBBI219/207b *neo* and oBBI208b/209b *neo*, the fragments were transformed into arabinose-induced EBB36 containing pKD46 (Datsenko & Wanner, 2000). Transformants were plated to LB kanamycin to select for the *gadC* and *adiA* knockout strains. Deletion of the *neo* gene, between the FRT sites, was performed by transformation of pCP20 containing the FLP recombinase into EBB288 and EBB291 and screening for loss of kanamycin resistance (Cherepanov & Wackernagel, 1995). The *gadC* (EBB292) and *adiA* (EBB295) deletion mutants were confirmed by PCR amplification of the *gadBC* and *adiA* regions using primer pairs oBBI215/216 and oBBI217/218, respectively. Plasmids to complement the *gadC* and *adiA* deletion mutants were constructed by PCR amplification of the *gadBC* and *adiA* regions from EXB43 genomic DNA using primer pairs oBBI215/216 and oBBI217/218, respectively. PCR fragments were TA cloned into the pBAD-Topo vector (Invitrogen) and transformed into chemically competent *E. coli* TOP10 (Invitrogen). Following plasmid extraction from TOP10, plasmids were transformed into the corresponding *E. coli* deletion mutants.

Table 2. Oligonucleotide sequences

FRT sites are indicated by the underlined nucleotide sequence. Homology to the *gadC* or *adiA* region of the *E. coli* O157:H7 genome is indicated by the bold nucleotide sequence. 'P' at the 5' end of oBBI207b, 208b, 209b and 219 represents phosphorylation.

Oligo	Sequence (5'–3')
Universal FRT primers	
oBBI92	cgatagctgaatgagtgacgtgctgaagtcctatactttctagagaataggaacttcaagcgaaccggaattgccag
oBBI93	gcatagagcagtgacgtagtcgcgaagtcctattctctagaaagtataggaacttcattcagaagaactcgtcaagaagg
<i>gadC</i> knockout primers	
oBBI207b	Pgtagtggtttctgtcattcatcacaatatagtggtggaacgtgcatagagcagtgacgtagtcgc
oBBI219	Pgctaagcagctcacattactcggctttttgccataacggcatcgaatgtagtgacgtgc
<i>adiA</i> knockout primers	
oBBI208b	Pcataacccggtaagcaatgatgaaagtattattgttgaagcgaatgtagtgacgtgc
oBBI209b	Pcagtttcgtgttcaaatccagggaatgatggtccaggattgcatagagcagtgacgtagtcgc
<i>gadBC</i> primers	
oBBI215	cggtagagaatgtaacactg
oBBI216	gtagtggtttctgtcattcatc
<i>adiA</i> primers	
oBBI217	ctcgatattgccctgacgg
oBBI218	gttacgctttcacacataac

RESULTS

Oxidative stress imposed during acid challenge decreases survival of *E. coli* O157:H7

Multiple studies have demonstrated that adapting *E. coli* to a particular environmental stress can cross-protect against another stress (Hengge-Aronis *et al.*, 1993; Jenkins *et al.*, 1988; Lin *et al.*, 1996). For example, Hengge-Aronis *et al.* (1993) demonstrated that osmotic induction (0.3 M NaCl) can enhance survival against oxidative stress (15 mM hydrogen peroxide). Most cross-protection studies have utilized only a single environmental stress during challenge. However, in the environment as well as *in vivo*, bacteria may simultaneously encounter multiple stress conditions. As a result, protection from one microbial stress response may concurrently protect the micro-organism from another stress. On the other hand, compounding stress experiences may be detrimental to the pathogen. To investigate the simultaneous exposure of *E. coli* O157:H7 to oxidative and acid stress, diamide (a membrane-permeable thiol-specific oxidizing agent) and hydrogen peroxide were used to determine if oxidative stress imposed during extreme acid challenge would decrease the survival of the pathogen. Diamide presumably promotes the formation of disulfide bonds in the cytoplasm, a normally reduced environment (Kosower &

Kosower, 1995). Investigators have previously demonstrated that the addition of diamide to growing cultures temporarily inhibits bacterial growth until the cells can effectively reduce the cytoplasmic disulfides (Prinz *et al.*, 1997; Ritz *et al.*, 2000). Similar affects of diamide on bacterial growth were found with an overnight culture of *E. coli* O157:H7 that was grown in LB MES pH 5.5 and challenged in EG medium in the presence of increasing concentrations of diamide. As shown in Fig. 1(a) (hatched and horizontal-lined bars), bacterial growth was inhibited in a concentration-dependent manner at pH 7.0 and 5.5 during the 4 h assay. Bacterial growth increased (>100%) in the presence of lower concentrations of diamide (0, 62.5 and 125 μ M) but viability remained at ~100% (growth inhibition) for cells treated with 250 and 500 μ M diamide. This indicates that at pH 7.0 and 5.5, the higher concentrations of diamide inhibit growth but do not result in loss of cell viability. However, as shown in Fig. 1(a) (solid bars), increasing the concentration of diamide (62.5–500 μ M) significantly decreased the survival of *E. coli* O157:H7 during the 4 h acid challenge at pH 2.5 ($P < 0.01$ at 62.5–500 μ M compared to the control without diamide). These results demonstrate that the addition of diamide, which is known to decrease the cellular thiol:disulfide ratio, resulted in a dose-dependent decrease in survival of *E. coli* O157:H7 during acid challenge at pH 2.5. While *E. coli* O157:H7 cell growth (but not

Fig. 1. Concentration-dependent sensitivity of *E. coli* O157:H7 to diamide and hydrogen peroxide at pH 2.5. Overnight cultures of EXB43 were grown in LB MES pH 5.5 and challenged in EG at pH 7.0 (horizontal-lined bar), pH 5.5 (hatched bars) and pH 2.5 (solid bars) with the indicated concentrations of diamide (a) or hydrogen peroxide (b) for 4 h. Standard deviation is shown.

survival) was inhibited at pH 7.0 and 5.5 in the presence of 250 or 500 μM diamide, these higher diamide concentrations were lethal to the pathogen during acid challenge.

To determine whether the decrease in bacterial viability was specific to diamide during acid stress, *E. coli* O157:H7 was acid challenged in the presence of hydrogen peroxide. As shown in Fig. 1(b), the addition of increasing concentrations of hydrogen peroxide significantly decreased *E. coli* O157:H7 survival at pH 2.5 ($P < 0.01$ for 62.5–1000 μM compared to no hydrogen peroxide, solid bars). At a hydrogen peroxide concentration of 1 mM, the survival of *E. coli* O157:H7 decreased 330- and 100-fold following 4 h of challenge at pH 7.0 and 5.5, respectively. Only slight effects on cell viability were seen in the presence of 250 and 500 μM hydrogen peroxide at pH 5.5 and pH 7.0 with two- to sixfold and 1.4- to twofold reductions, respectively. Similar to the diamide challenge at pH 7.0 and 5.5, bacterial growth was noted in the cultures challenged at pH 7.0 and 5.5 in the absence of hydrogen peroxide and in the presence of hydrogen peroxide concentrations up to 125 μM , resulting in survival greater than 100 %. These results indicate that, similar to diamide, oxidative stress imposed by hydrogen peroxide during acid challenge at pH 2.5 decreases the viability of *E. coli* O157:H7 grown in LB MES pH 5.5.

Glutamate- and arginine-dependent AR systems protect *E. coli* O157:H7 from oxidative stress during acid challenge

As shown in Fig. 1, the addition of either 500 μM diamide or 250 μM hydrogen peroxide decreased survival by 1000-fold for wild-type *E. coli* O157:H7 grown in LB MES (AR1) and challenged at pH 2.5 in EG medium. In contrast, bacterial cells grown in LB glucose and treated with 250 μM hydrogen peroxide in the presence of 2 mM glutamate (AR2) or 0.6 mM arginine (AR3) during acid challenge were acid resistant (Fig. 2). For *E. coli* O157:H7 grown in LB glucose, glutamate and arginine significantly enhanced survival at pH 2.5 in the presence of 250 μM hydrogen peroxide >5000- and 900-fold compared to cells challenged only at pH 2.5 in the absence of amino acid supplementation, respectively. Similarly, the presence of glutamate and arginine protected *E. coli* O157:H7 (LB glucose grown) during acid challenge in the presence of 500 μM diamide (data not shown). The data indicate that in the presence of either glutamate (AR2) or arginine (AR3), *E. coli* O157:H7 is protected against oxidative stress during acid challenge, whereas AR1 is unable to protect, resulting in significantly decreased survival. Other investigators have previously demonstrated that deletion of *gadC* and *adiA* eliminates the acid protection provided during acid challenge in the presence of glutamate and arginine, respectively (Hersh *et al.*, 1996; Lin *et al.*, 1995). Oxidative stress protection provided by these AR systems toward diamide (data not shown) and hydrogen peroxide was also eliminated by deletion of *gadC* and *adiA* (Fig. 3).

Fig. 2. Effect of hydrogen peroxide on AR1, AR2 (glutamate) and AR3 (arginine) during challenge at pH 2.5. Overnight (O/N) cultures of EXB43 were grown in LB MOPS pH 8.0 (MOPS), LB MES pH 5.5 (MES, AR1) or LB glucose (LBG, AR2 and AR3) and challenged in EG at pH 2.5 for 4 h. Where indicated, the final concentration of hydrogen peroxide was 250 μM . The presence and absence of 2 mM glutamate or 0.6 mM arginine during challenge is indicated. Standard deviation is shown.

Furthermore, both acid and oxidative protection was restored by complementation of the *gadC* and *adiA*

Fig. 3. Requirement for glutamate and *gadC* or arginine and *adiA* for protection against hydrogen peroxide at pH 2.5. Overnight (O/N) cultures of the wild-type strain EXB43 and mutants EBB292, EBB300, EBB295 and EBB301 were grown in LB glucose (LBG) and challenged in EG containing 250 μM hydrogen peroxide at pH 2.5 for 4 h. The presence and absence of 2 mM glutamate or 0.6 mM arginine during challenge is indicated. Standard deviation is shown.

mutants with plasmids containing the *gadBC* and *adiA* genes, respectively. Thus, unlike AR1, the protection afforded by the glutamate- and arginine-dependent AR systems in *E. coli* O157:H7 can simultaneously protect against oxidative stress during extreme low pH challenge.

Maximal protection against oxidative stress requires a low external pH for optimal glutamate decarboxylase activity

Lin *et al.* (1996) demonstrated that RpoS is active in bacterial cells grown in LB MES pH 5.5 (AR1) but is partially repressed if glucose is present in the growth medium [LBG, conditions used herein for assays in the presence of glutamate (AR2) and arginine (AR3)]. In addition, the activity of glutamate decarboxylase has been shown by Richard & Foster (2004) to be maximal at an internal pH between 4 and 5 which corresponds to an approximate external pH of 2.5. The oxidative stress protection afforded during acid challenge at pH 2.5 in the presence of glutamate could be due to the carry-over of the RpoS stress response induced in the stationary-phase culture prior to acid challenge. If oxidative stress protection is induced in stationary phase, the bacterial cell should be protected against oxidative stress as well or better at pH 5.5 and pH 7.0 compared to pH 2.5. Conversely, oxidative stress protection during acid challenge in the presence of glutamate may require glutamate decarboxylase activity induced at low pH. Using a higher level of oxidative stress than our previous experiments, *E. coli* O157:H7 grown in either LB MES (AR1 growth medium) or LB glucose (AR2 or AR3 growth medium) were protected in the presence of glutamate against pH 2.5 and 1 mM hydrogen peroxide following 4 h of challenge (Fig. 4, second set of bars). The presence of glutamate was mandatory since a 1000-fold decrease in bacterial survival was observed when glutamate was absent during challenge at pH 2.5 with 1 mM hydrogen peroxide (Fig. 4, first set of bars). For *E. coli* O157:H7 grown in LB glucose, maximal protection against oxidative stress in the presence of glutamate requires low pH (pH 2.5) for optimal glutamate decarboxylase activity since there is a >500-fold difference in bacterial survival comparing pH 2.5 and 5.5 (Fig. 4, solid bars on second vs fourth sets of bars). Therefore, oxidative stress protection of *E. coli* O157:H7 grown in the presence of glucose requires low pH for induction of glutamate decarboxylase activity. However, at pH 5.5 and 7.0, *E. coli* O157:H7 survival in the presence of 1 mM hydrogen peroxide was 24- to 116-fold greater in cultures grown overnight in LB MES pH 5.5 (AR1, hatched bars) compared to LB glucose (AR2, solid bars). Thus, the RpoS stress response induced in *E. coli* O157:H7 grown in LB MES (but not LB glucose) can provide protection against oxidative stress at pH 7.0 and 5.5 (Fig. 4, hatched bars on the last three sets of bars). Nonetheless, the best protection for *E. coli* O157:H7 against oxidative stress (1 mM hydrogen peroxide) was observed at pH 2.5 in the presence of glutamate.

Fig. 4. Maximal protection against hydrogen peroxide challenge requires pH 2.5 for optimal glutamate decarboxylase activity. Overnight (O/N) cultures of EXB43 were grown in LB MES pH 5.5 (MES, hatched bars) or LB glucose (LBG, solid bars) and challenged in EG containing 1 mM hydrogen peroxide at pH 7.0, pH 5.5 or pH 2.5 for 4 h with (+) or without (-) 2 mM glutamate. Standard deviation is shown.

DISCUSSION

In their environment, bacteria may concurrently confront multiple stresses. Our research demonstrates that *E. coli* O157:H7 can protect itself against oxidative stress during extreme acid challenge if glutamate or arginine is present. A potential environment where the bacterium would simultaneously encounter both oxidative stress and acidic pH is in a host organism. The oral cavity serves as a first line of defence against microbial pathogens, since saliva contains several defence factors including salivary IgA, lysozyme, lactoferrin, histatins, cystatins, mucins, salivary glycoproteins, peroxidase and hydrogen peroxide (Geiszt *et al.*, 2003; Sato *et al.*, 2008). Oral neutrophils provide protection to the host by producing reactive oxygen species, nitric oxide and antimicrobial peptides. Superoxide and hydrogen peroxide are also produced by oral bacteria as potential mediators of oxidative stress (Halliwell *et al.*, 2000; Sato *et al.*, 2008). In addition, the diet may be a contributor to oxidative stress, as several common beverages contain hydrogen peroxide (Halliwell *et al.*, 2000). Therefore, the exposure of *E. coli* O157:H7 to oxidative stress in the oral cavity or oesophagus could linger during exposure to acidic pH in the stomach.

E. coli is generally considered a neutrophilic organism, but both commensal and pathogenic *E. coli* are capable of surviving exposure to extreme acidic environments (pH 2.5) for several hours (Foster, 2004; Large *et al.*, 2005). Evidence in *E. coli* K-12 suggests that acid induction of oxidative stress components could assist in protecting the bacterial cell against oxidative stress during acid

challenge (Blankenhorn *et al.*, 1999; Maurer *et al.*, 2005; Stancik *et al.*, 2002). However, we demonstrated that AR1 was incapable of protecting wild-type *E. coli* O157:H7 from simultaneous challenge by both oxidative (500 μ M diamide or 250 μ M hydrogen peroxide) and acid (pH 2.5) stress. In contrast, both the glutamate- and arginine-dependent AR systems provided significant protection against the combination of oxidative and acid stress at pH 2.5. This indicates that in addition to the OxyR- and RpoS-dependent stress response systems, *E. coli* also possesses the glutamate- and arginine-dependent acid resistance systems, which can effectively manage oxidative stress during low-pH encounters.

Our data also indicate that the RpoS stress response induced in LB MES grown cultures is partially protective against oxidative stress (1 mM hydrogen peroxide) at pH 7.0 and 5.5 in the absence (or presence) of glutamate, but requires glutamate for survival at pH 2.5 (Fig. 4). Furthermore, although LB glucose grown *E. coli* O157:H7 was resistant to 1 mM hydrogen peroxide at pH 2.5 in the presence of glutamate, limited protection was observed at pH 5.5 in both the presence and absence of glutamate. This indicates that glutamate decarboxylase requires low pH for optimal induction as previously described by Richard & Foster (2004). Thus, *E. coli* O157:H7 appears to possess multiple systems (including the *rpoS* and *oxyR* regulons) that protect against oxidative stress. At least one system is induced in cells grown in buffered LB pH 5.5 and protects against oxidative stress at pH 5.5 and 7.0, whereas another system is induced in cells grown in either buffered LB pH 5.5 or LB glucose and requires glutamate during extreme acid challenge (pH 2.5). Lin *et al.* (1996) demonstrated that the glutamate- (AR2) and arginine- (AR3) dependent AR systems provided protection against a volatile fatty acid cocktail (acetic, propionic and butyric acids) at pH 4.4 for 7 h when glutamate and arginine were present, respectively. Similar to our results with oxidative stress, AR1 was much less effective for protection against the volatile fatty acid cocktail. Our data indicate that the best protection against oxidative stress (1 mM hydrogen peroxide) is provided during acid challenge (pH 2.5) in the presence of glutamate. Clearly, concurrent protection against both oxidative and acid stress by these stress-response systems provides redundancy for the microorganism with an enhanced level of stress protection.

The addition of 2 mM GABA (the decarboxylation product of glutamate) was unable to protect either the wild-type *E. coli* O157:H7 or the *gadC* mutant during acid challenge at pH 2.5 (data not shown). This indicates that protection against acid stress is not provided by GABA alone but requires the activity of the glutamate decarboxylase isozymes and the GadC antiporter. How does the decarboxylation of glutamate or arginine during acid challenge protect *E. coli* O157:H7 from oxidative damage due to diamide and hydrogen peroxide? Richard & Foster (2004) have demonstrated that *E. coli* increases internal pH and reverses membrane potential from an internal negative

to an internal positive charge during acid challenge in the presence of glutamate or arginine. Thus, one potential mechanism is that the greater internal pH in the presence of either glutamate or arginine compared to acid challenge in their absence may decrease the 'level' of stress (acid and oxidative) for the bacterial cell, resulting in increased survival. Another possibility is the production of uncharacterized stress-response proteins (RpoS-dependent or -independent) that protect *E. coli* against multiple environmental stresses including oxidative stress. In this study, the RpoS stress response appears to provide protection at pH 7.0 and 5.5 against oxidative stress but insufficient protection is afforded at pH 2.5 in the absence of glutamate or arginine. These results do not preclude the involvement of the RpoS stress response in protection against oxidative stress during extreme acid challenge, but they indicate that the RpoS stress response by itself is not sufficient for protection. In conclusion, although multiple stress-response proteins are most likely involved in protection of *E. coli* O157:H7 against concurrent challenge with both oxidative and acid stress, the glutamate-dependent AR system is a cornerstone for maximal protection.

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