The acid-resistance pathways of *Shigella flexneri* 2457T

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The stationary-phase acid-resistance pathways of *Shigella flexneri* 2457T have not previously been studied. The two acid-resistance systems, the glutamate-dependent acid-resistance (GDAR) and the oxidative pathways, reported elsewhere for *Escherichia coli* and *S. flexneri* 3136, were both detected in *S. flexneri* 2457T. However, *S. flexneri* 2457T cells grown overnight under fermentative conditions and acid-shocked in minimal media in the absence of glutamate, an acid test often described as a negative control for both pathways, were capable of surviving acid challenge. It is possible that this resistance is due to the oxidative pathway operating in a non-glucose-repressible manner, or to a novel pathway present in *S. flexneri* 2457T. The construction of *gadB* and *gadC* mutants ruled out any contribution by the GDAR pathway, whilst further characterizing the GDAR properties of *S. flexneri* 2457T. Interestingly, study of the role of *rpoS* in the oxidative pathway and the unusual acid-resistance phenotype revealed that the frameshift present in the 2457T *rpoS* gene results in expression of a truncated RpoS protein, which may be reduced in activity and is not essential for the acid-resistance phenotype of *S. flexneri* 2457T.

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INTRODUCTION

Shigella flexneri causes the most communicable of bacterial diarrhoeas, shigellosis. The pathogenesis of shigellosis is dependent on the bacterium's ability to invade and colonize the gastrointestinal tract. However, in order to do so, *S. flexneri* must pass through the acid environment of the human stomach, which can be at pH < 3 (Giannella *et al.*, 1972). Additionally, enteric bacteria must survive low-pH stress external to the host, such as that encountered during passage through the environment, in acidic foods and in fermenting acidified faecal material (Bhagwat & Bhagwat, 2004; Lin *et al.*, 1995). The ability of *S. flexneri* to survive these low pH levels appears to be an important pathogenic characteristic, and may be a contributing factor to the low infectious dose of 10–100 organisms required for shigellosis to occur (Gorden & Small, 1993).

The *in vitro* test of bacterial acid resistance measures bacterial survival after 2 h of acid exposure at pH 2.5, where an inoculum survival $\geq 10\%$ is considered to represent resistance (Gorden & Small, 1993). It is believed that these conditions mimic the pH and gastric-emptying time of a normal fasting stomach (Gorden & Small, 1993). Bacteria able to survive this length of acid exposure are theoretically capable of breaching the gastric acid barrier to reach the more favourable environment of the intestine.

Acid resistance has been extensively studied in *Escherichia coli*, whilst the acid-resistance phenotype of *S. flexneri* has been described in fewer instances. Studies have shown that *S. flexneri* possesses at least two acid-resistance pathways that seem to be similar to two of the three pathways identified in *E. coli*. Acid-resistance pathway 1 (AR1) is a stationary-phase, acid-induced, glucose-repressed oxidative pathway, while acid-resistance pathway 2 (AR2) is a stationary-phase, glutamate-dependent acid-resistance (GDAR) pathway (Lin *et al.*, 1995).

The GDAR pathway, which is induced by growth in mildly acidic conditions (pH 5) and in fermentatively grown cells, is a glutamate decarboxylase system consisting of two homologous decarboxylase enzymes, *gadA* and *gadB*, and an antiporter, *gadC* (Hersh *et al.*, 1996; Lin *et al.*, 1995; Waterman & Small, 1996). This system appears to act by mopping up protons leaking into the bacterial cytosol through the decarboxylation of glutamate to gamma-aminobutyric acid (GABA). GABA is then exchanged for external glutamate by the antiporter GadC, thereby maintaining the pH homeostasis of the cytoplasm, reversing the cell membrane potential to create an internal positive charge, and gradually alkalizing the extracellular medium (Castanie-Cornet & Foster, 2001; Richard & Foster, 2004). Thus, this pathway is dependent on glutamate being present in the acid-shock media (Lin *et al.*, 1995). A number of genes involved in regulating this pathway have been identified in *E. coli*, revealing a complex

Abbreviations: AR4, acid-resistance pathway 4; GABA, gamma-aminobutyric acid; GDAR, glutamate-dependent acid resistance.

network of regulation (Hommais *et al.*, 2004; Masuda & Church, 2003).

The GDAR pathway appears to be highly effective at acid protection in both *E. coli* and *S. flexneri* (Castanie-Cornet *et al.*, 1999; Waterman & Small, 1996). However, it is still unclear how important the oxidative system is to the *S. flexneri* acid-resistance phenotype, as so little is understood about the pathway. Besides some regulatory proteins, the mechanism and major components of the *Shigella* oxidative system are not yet known. It has been shown in *E. coli* that the alternative sigma factor RpoS and the cyclic AMP receptor protein (CRP) play a role in control of this pathway (Castanie-Cornet *et al.*, 1999; Waterman & Small, 1996). The *Shigella* oxidative system, characterized in strain 3136, displays similar properties to those of the oxidative pathway in *E. coli*, including a requirement for complex media, oxidative growth, acid induction and repression of the pathway by glucose (Lin *et al.*, 1995). Recently, it has been shown that a *fur* mutant in the *S. flexneri* serotype 2a strain, SA100, is defective in the oxidative system. This defect appears to be due to the constitutive expression of a small regulatory RNA molecule, RyhB, in the absence of Fur regulation, repressing transcription of YdeP, an oxidoreductase that reduces acidic metabolic products in the cell (Oglesby *et al.*, 2005).

In this study, the acid-resistance pathways of the *S. flexneri* serotype 2a strain 2457T were studied. This strain is a highly researched *Shigella* strain, which has been widely used in virulence studies (Wei *et al.*, 2003). It is not known what contribution the acid-resistance pathways of 2457T make to the virulence of this strain, as they have not been previously characterized. Earlier acid-resistance studies have used *S. flexneri* 3136, SA100 or M25-8A (Oglesby *et al.*, 2005; Small *et al.*, 1994; Waterman & Small, 2003). The known GDAR gene components are present in the 2457T sequence (Wei *et al.*, 2003). However, there is a key difference between 3136, the most studied *S. flexneri* strain for acid resistance, and the sequenced 2457T strain; it has been shown by Small *et al.* (1994) that 3136 possesses an

intact *rpoS* gene, producing a 330 aa protein. Instead, the 2457T sequence has a frameshift, 675 bp into the *rpoS* sequence, creating a truncated protein of 255 aa (Wei *et al.*, 2003). It is unclear whether this truncated RpoS retains its function as a major regulator of stationary-phase growth genes and whether this has implications for the regulation of the acid-resistance pathways of *S. flexneri* 2457T.

The goal of this study was to characterize the acid-resistance phenotype of *Shigella flexneri* 2457T.

METHODS

Bacterial strains and culture conditions. The strains of *S. flexneri* and *E. coli* used are listed in Table 1. Minimal E salts glucose (EG) medium was prepared as described by Vogel & Bonner (1956). The complex medium used was Luria-Bertani (LB), supplemented with 100 mM MOPS (pH 8), MES (pH 5) or 0.4 % glucose. Nicotinic acid (2 µg ml⁻¹), methionine (2 µg ml⁻¹) and tryptophan (2 µg ml⁻¹) were added to minimal media cultures of *S. flexneri*. Glutamate was added to EG to a final concentration of 0.012 %. Bacterial strains were routinely grown at 37 °C in an orbital shaker or incubator. The media were supplemented with antibiotics as necessary. Antibiotics were used at the following concentrations: ampicillin, 10 µg ml⁻¹ and 100 µg ml⁻¹; chloramphenicol, 30 µg ml⁻¹.

Genetic and molecular techniques. Molecular cloning, alkaline-lysis preparation of plasmid DNA and agarose gel electrophoresis were performed according to the protocols of Sambrook & Russell (2001). DNA was purified from agarose gels using the QIAquick kit (Qiagen). Primers used for cloning complementation vectors were obtained from Sigma Proligo. PCR was performed with *Pfu* polymerase, as specified by the manufacturer (Promega). DNA sequencing was performed at the Biomolecular Resources Facility, John Curtin School of Medical Research (Australian National University), with the ABI 3730 capillary sequence analyser using the Big Dye version 3.1 (Applied Biosystems) sequencing protocol. The molecular weight marker SPP-1/*EcoRI* was used to determine DNA fragment sizes. Chromosomal DNA was prepared using the procedure described by Huan *et al.* (1995).

Construction of insertion mutants. Two different knockout approaches were used to produce chromosomal insertions. For construction of *rpoS* and *gadC* insertion mutants, a suicide plasmid

Table 1. Bacterial strains used in this study

Strain	Characteristics	Reference or source
<i>S. flexneri</i>		
SFL1001	<i>S. flexneri</i> wild-type 2457T, serotype 2a	Walter Reed Army Institute of Research
SFL1629	SFL1001 <i>rpoS</i> ::pNV1234	This work
SFL1641	SFL1001 <i>gadC</i> ::pNV1206	This work
SFL1647	SFL1001 <i>rpoS</i> ::pNV1234/pNV1314	This work
SFL1648	SFL1001 <i>rpoS</i> ::pNV1234/pNV1315	This work
SFL1650	SFL1001 <i>gadB</i> ::Cm	This work
SFL1723	SFL1001 <i>gadB</i> ::Cm/pNV1389	This work
SFL1729	SFL1001 <i>gadC</i> ::pNV1206/pNV1397	This work
<i>E. coli</i>		
B1401	MG1655 K-12 wild-type	P. R. Reeves, University of Sydney

approach was taken. Internal fragments of 281 and 395 bp for *rpoS* and *gadC*, respectively, were amplified by PCR using gadCSaxF (5'-CGAGCTCTTACCGTTCTGATGTCCC-3'), gadCXbaR (5'-CG-TCTAGATTTACCCCTTTACCACC-3'), rpoSF294Sac (5'-AGAGC-TCCCTGCGTCTGGTGTTAAA-3') and rpoSR575Xba (5'-CATC-TAGATCTCTCCGCACTTGGTT-3'). Gene fragments were cloned into the *SacI/XbaI* sites of pGP704 to create pNV1234 for *rpoS* and pNV1206 for *gadC*. The resulting plasmids can only replicate in hosts expressing the π (*pir*) protein. Thus, when introduced into *S. flexneri* 2457T, which does not contain the *pir* protein, the only way to maintain the plasmid is for it to integrate into the chromosome via homologous integration. Ampicillin-resistant ($10 \mu\text{g ml}^{-1}$) colonies were screened for correct integration by PCR, where integration generates a larger band. Correct chromosomal location of the integration of pNV1234 into *rpoS* in SFL1629 was confirmed by Western blotting, and integration of pNV1206 into *gadC* in SFL1641 was confirmed by Southern blotting (data not shown). The *rpoS* gene was amplified from *S. flexneri* 2457T with primers 2457TRpoSSacF (5'-TGAGCTCGGCGGAACCAGGCTTTTG-3') and RpoSXbaR (5'-CATCTAGACCTGAATCTGGCGAACAC-3'), and amplified from *E. coli* MG1655 with MG1655SacF (5'-TGA-GCTCCAAGGGATCACGGGTAGG-3') and MG1655XbaR (5'-GTT-CTAGAGTTGCGTATGGGCGGTAA-3'). *S. flexneri* 2457T and *E. coli* PCR-amplified *rpoS* genes were cloned into *SacI/XbaI* sites of pBC SK+ (Stratagene) to create pNV1314 and pNV1315, respectively. *gadC* was amplified from *S. flexneri* 2457T using gadCSac (5'-TCGAGCTCTCACTGGCATTAGCAACG-3') and gadCXba(5'-GT-CTAGACGCTGGTCTCTTAATCGT-3'), and cloned into *SacI/XbaI* sites of pBC SK+ to create pNV1397.

The *gadB* mutant was constructed using the PCR λ red integration approach (Datsenko & Wanner, 2000). Primers gadBKOF (5'-GTCGCATTTTCAGATTATCAATGATGAATTATATCTTGAGT-GTAGGCTGGAGCTGCTTC-3') and gadBKOR (5'-GTTTCGGGTG-ATCGCTGAGATATTTACGGGAGGCTTTGTACATATGAATATC-CTCCTTAG-3') were designed, carrying 40 nt of sequence from the 121–161 and 1330–1360 bp regions of the *gadB* gene. These primers also carry the P1 and P2 sequences, designed to amplify the *cat* gene from pKD3; the P2 sequence contains a ribosomal binding site to create non-polar knockouts. The resulting PCR product of the *cat* gene, containing flanking regions with *gadB* homology, was electroporated into *S. flexneri* 2457T carrying the temperature-sensitive pKD46 helper plasmid. Chloramphenicol-resistant colonies were screened for integration by PCR, using the c1 primer specific to the *cat* gene with a *gadB*-specific primer (Datsenko & Wanner, 2000). Correct insertion of the *cat* PCR fragment in SFL1650 was confirmed

by PCR and sequencing (data not shown). The loss of pKD46 was tested for by antibiotic plating. The *S. flexneri* 2457T *gadB* gene was amplified by the primers gadBcompF (5'-CTCTAGACACTT-GCTTACTTTATCG-3') and gadBcompR (5'-TAGAGCTCCGTTG-CTAATGCCAGTGAAC-3') and cloned into the *XbaI/SacI* sites of pBS KS+ to create pNV1389. Table 2 shows the plasmids used in this study.

Western blotting. Strains were grown at 37 °C overnight to stationary phase in LB containing the required antibiotics. The optical densities of cultures were determined and the cell numbers were calculated from growth curves. Equal numbers of cells were collected by centrifugation and resuspended in 100 μl 1 $\times$ loading buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol (v/v), 2.5% β -mercaptoethanol, 0.1% bromophenol blue] and left at 100 °C for 5 min. Protein samples were run on a 15% SDS-polyacrylamide gel. After protein transfer by wet blotting onto PVDF membranes, RpoS was detected using rabbit anti-RpoS polyclonal sera (Ruiz & Silhavy, 2003) and sheep anti-rabbit IgG horseradish peroxidase (ICN). The signal was developed with Supersignal West Pico chemiluminescent substrate (Pierce) and captured on Hyperfilm (Amersham).

Catalase assay. The qualitative catalase activity was estimated by dropping 10 μl 30% H_2O_2 onto individual colonies grown overnight on LB plates at 37 °C (Subbarayan & Sarkar, 2004). The rapidity and degree of bubbling were measured for each strain. Five individual colonies were tested for each strain.

Acid-resistance assays. Acid-resistance assays were carried out as described by Lin *et al.* (1995) and Castanie-Cornet *et al.* (1999). Cells were grown overnight in one of several media, including LBG (LB plus 0.4% glucose), buffered LB [100 mM MOPS (pH 8) or 100 mM MES (pH 5)] and EG. To examine the GDAR pathway, cells were grown overnight to stationary phase at pH 5 or 8, in the presence or absence of oxygen, and acid-shocked at pH 2.5 in EG containing 0.012% glutamate. The pH 8 cultures did not contain glucose, as the consequent fermentative growth lowers the pH of the growth medium to pH 5 overnight. Cultures grown for 24 h were diluted 1:1000 into 5 ml warmed pH 2.5 EG, supplemented where indicated with 0.012% glutamate, and incubated for 2 h at 37 °C, shaken at 140 r.p.m. Cultures grown without oxygenation were grown in 5 ml Bijou bottles with tightly closed lids with shaking at 20 r.p.m. Viable cell counts were determined at 0 and 2 h post acid challenge by serial dilution and plating on LB agar plates. At least three repetitions were performed for each experiment.

Table 2. Plasmids used in this study

Plasmid	Characteristics	Reference or source
pBS KS+	Cloning vector	Stratagene
pBC SK+	Cloning vector	Stratagene
pKD3	PCR knockout template plasmid	Datsenko & Wanner (2000)
pKD46	Red recombinase expression plasmid	Datsenko & Wanner (2000)
pGP704	Suicide vector, R6K <i>ori</i>	Miller & Mekalanos (1988)
pNV1206	pGP704 with internal fragment of <i>gadC</i>	This work
pNV1234	pGP704 with internal fragment of <i>rpoS</i>	This work
pNV1314	pBC SK+ with SFL1001 <i>rpoS</i>	This work
pNV1315	pBC SK+ with MG1655 <i>rpoS</i>	This work
pNV1389	pBS KS+ with <i>gadB</i>	This work
pNV1397	pBC SK+ with <i>gadC</i>	This work

Table 3. Characteristics of the GDAR pathway for *S. flexneri* 2457T SFL1001

Condition	Adaptation medium*	pH 2.5 challenge medium†	Percentage survival‡	SEM
1	LBG (pH 5) WO	EG + Glt	88.6	3.90
2	LBG (pH 5)	EG + Glt	37.9	8.90
3	LB (pH 8) WO	EG + Glt	3.30	0.39
4	LB (pH 8)	EG + Glt	<0.001	0
5	EG (pH 5) WO	EG + Glt	52.0	9.90
6	EG (pH 5)	EG + Glt	5.87	1.60

*Adaptation involved overnight growth in media listed. Glt, glutamate added at 0.012 %; WO, growth without oxygenation.

†Challenge was a 1:1000 dilution of overnight culture into pH 2.5 medium for 2 h.

‡Survival is stated as a percentage of the inoculum. Results are the mean $\pm$ SEM of three experiments.

RESULTS

The GDAR pathway of SFL1001

Table 3 shows the GDAR pathway characteristics for the *S. flexneri* 2457T strain SFL1001. Acid induction was displayed by the strain at pH 5, as there was very little acid survival detected for cultures grown at pH 8, whilst cultures grown overnight at pH 5 were capable of surviving acid exposure (Table 3, condition 2 vs 4). Additionally, SFL1001 exhibited increased resistance to acid exposure when grown in culture without oxygenation (Table 3, condition 1 vs 2 and condition 3 vs 4). Finally, GDAR resistance in *S. flexneri* 2457T is not dependent on overnight growth in complex media, as acid resistance was detected when the strain was grown overnight in EG (pH 5) (Table 3, conditions 5 and 6). The negative control for the GDAR pathway, which was acid shock in EG (pH 2.5) without glutamate, is included in Table 4 and will be discussed below.

The oxidative acid-resistance pathway of SFL1001

Table 4 contains the results for the glutamate-independent oxidative acid-resistance pathway of SFL1001; cells were grown overnight to stationary phase and acid-shocked at pH 2.5 in EG. Buffers were included in the overnight medium to maintain the exact pH levels. Similar to *E. coli*, the *S. flexneri* 2457T oxidative system protected cells at pH 2.5 in minimal media in the absence of glutamate (Table 4, condition 1). There was a clear requirement for oxidative growth conditions, as resistance was not present in cells grown without oxygenation (Table 4, condition 1 vs condition 2). Furthermore, as observed by Lin *et al.* (1995) for *S. flexneri* 3136, the oxidative pathway of *S. flexneri* 2457T required overnight growth in complex media and could not be detected in cells grown overnight in minimal media at pH 5 (Table 4, condition 4).

Transfer of cells into fresh media is sufficient to remove the inhibitor and restore acid resistance (Castanie-Cornet *et al.*,

Table 4. Acid resistance for the oxidative acid-resistance pathway of *S. flexneri* 2457T strain SFL1001

Condition	Adaptation medium*	pH 2.5 challenge medium†	Percentage survival‡	SEM
1	LB-MES (pH 5)	EG	54.9	5.80
2	LB-MES (pH 5) WO	EG	0.53	0.13
3	LB-MOPS (pH 8)	EG	<0.001	0
4	EG (pH 5)	EG	<0.001	0
5	LB-MES (pH 5) + Glt	EG	57.0	8.70
6	LB-MOPS (pH 8) + Glt	EG	<0.001	0
7	LB-MOPS (pH 8) + fresh EG	EG	<0.001	0
8	LBG (pH 5)	EG	59.4	10.4
9	LBG (pH 5) WO	EG	<0.01	0

*Adaptation involved overnight growth in media listed. WO, growth without oxygenation; Glt, 5.9 mM glutamate added to overnight cultures immediately prior to acid challenge; fresh EG, cells were grown overnight in the media indicated before being transferred to fresh EG (pH 7).

†Challenge was 1:1000 dilution of overnight culture into pH 2.5 medium for 2 h.

‡Survival is stated as a percentage of the inoculum. Results are the mean $\pm$ SEM of three experiments.

1999). In this study, the oxidative pathway of the *S. flexneri* 2457T strain SFL1001 appeared to display acid induction: resistance was detectable when cells were grown overnight at pH 5, but not when they were grown at pH 8 (Table 4, condition 1 vs condition 3). To determine whether this behaviour was due to the production of an inhibitor, as reported for *E. coli*, cells grown overnight at pH 8 were transferred to fresh EG (pH 7) prior to acid challenge. This treatment did not generate acid resistance in these cells (Table 4, condition 7), suggesting that the lack of resistance in pH 8-grown cells of *S. flexneri* 2457T was not due to an inhibitor, but that oxidatively grown cells require acid induction in order to display acid resistance in EG at pH 2.5. Additionally, Castanie-Cornet *et al.* (1999) demonstrated that the addition of 5.9 mM glutamate to cells grown overnight at pH 8 could activate oxidative-pathway acid resistance. Table 4, conditions 5 and 6, shows that the addition of 5.9 mM glutamate to SFL1001 stationary cells had no effect on the levels of acid resistance.

Detection of non-glucose-repressed, glutamate-independent acid resistance in SFL1001

Many previous studies of *S. flexneri* and *E. coli* have stated that the oxidative resistance pathway is in fact glucose repressed, and not observed in cells grown to stationary phase in media containing glucose (Lin *et al.*, 1995, 1996). It should be noted that the glucose contained within the acid-shock medium EG does not appear to play a role in glucose repression, suggesting that it is stationary-phase growth in the presence of glucose that is important. In those studies, it appears that overnight growth in LBG at pH 5 suppresses the oxidative pathway, such that no protection is observed when cells are acid-shocked in EG (pH 2.5). The absence of glutamate in the acid-shock media should prevent the GDAR pathway from operating, making these acid test conditions essentially a negative control for both pathways. However, as shown in Table 4, condition 8, nearly 60% cell survival was possible for *S. flexneri* 2457T under these conditions. When cells were

grown similarly at pH 5 in LBG but with limited oxygenation, which favours the GDAR system, no acid resistance was detected (Table 4, condition 9). This result seems to suggest that the response is due to the oxidative pathway, which prefers oxygenated growth and can operate in acid-shock minimal media without glutamate supplementation. If this is the case, the pathway is not repressed by the overnight fermentative growth of the cells in glucose-containing media. As *S. flexneri* 2457T requires growth factor supplementation in minimal media, it was necessary to determine whether the presence of the supplements was contributing to this novel resistance. Similar resistance was seen when EG (pH 2.5) was not supplemented with the *S. flexneri* growth supplements methionine, tryptophan or nicotinic acid, demonstrating that the resistance was not dependent on the presence of these growth supplements (data not shown). This resistance, observed in cells grown to stationary phase overnight in LBG (pH 5) and acid-shocked in EG (pH 2.5), will be referred to as non-glucose-repressed, glutamate-independent acid resistance, or acid-resistance pathway 4 (AR4) for convenience. To determine the contribution of either the oxidative or the GDAR pathway to AR4, mutants were constructed in acid-resistance-related genes known to contribute to the GDAR and oxidative pathways of *S. flexneri* 3136.

Role of RpoS in SFL1001 acid resistance

It is believed that the oxidative acid-resistance pathway is RpoS-dependent, and RpoS appears to be important, but not essential, for operation of the GDAR pathway in *S. flexneri* (Small *et al.*, 1994; Waterman & Small, 1996). However, in *S. flexneri* 2457T, the *rpoS* gene contains a frameshift mutation that results in a truncated protein missing the last 89 amino acids (Fig. 1) (Wei *et al.*, 2003). The *rpoS* gene of SFL1001 was sequenced to confirm the presence of the frameshift mutation in SFL1001, and aligned to the full-size *rpoS* gene sequence of *S. flexneri* 301 published by Small *et al.* (1994) (Fig. 1).

Fig. 1. (a) Nucleotide sequence of the *S. flexneri* 2457T *rpoS* gene, sequenced from SFL1001, aligned with the published sequence of *S. flexneri* 301 *rpoS* (NCBI accession number P35540). The SFL1001 2457T *rpoS* sequence correlates with the published 2457T sequence, except that there is a missing G at 675 bp. (b) *S. flexneri* 2457T RpoS predicted truncated protein sequence. The amino acids in bold type are 100% homologous to the sequence of *S. flexneri* 301 RpoS. The frameshift results in the altered amino acids (indicated by underlined type), creating a stop codon (indicated by the first asterisk) 255 aa into the protein.

To determine whether this altered RpoS protein was still important for the acid-resistance pathways of *S. flexneri* 2457T, in particular the non-glucose-repressed, glutamate-independent resistance, an *rpoS* mutant was produced for SFL1001. A Western blot was performed with whole-cell lysate to determine whether the truncated RpoS is in fact expressed by SFL1001, and also to confirm the successful disruption of *rpoS* in SFL1629 (Fig. 2). A truncated RpoS of approximately 30 kDa was detected for SFL1001, which was not found in SFL1629, confirming that the gene disruption had been successful in SFL1629. The full-sized RpoS of *E. coli* MG1655 was observed as a 38 kDa band. RpoS proteins of 30 and 38 kDa were observed for the complemented strains SFL1647 and SFL1648, respectively, as expected. Although roughly the same number of cells was loaded on the 1D SDS-PAGE gel for each strain, there was a large discrepancy in the expression levels of RpoS. The plasmid-based RpoS levels were higher, as would be expected, as there are multiple copies of the gene within each cell, with expression of RpoS by SFL1647 being extremely high for unknown reasons.

Acid-resistance assays were performed on the strains grown in LB-MES (pH 5) and LBG (pH 5), and challenged in EG (pH 2.5) or EG (pH 2.5) supplemented with 0.012 % glutamate (Fig. 3). The *rpoS* disruption did not have a significant effect on either the GDAR (condition 3, Fig. 3) or the oxidative resistance pathway (condition 2, Fig. 3), as SFL1629 did not display any attenuation in acid resistance. Likewise, there was no change in AR4 resistance (condition 1, Fig. 3). It seems that the truncated RpoS of *S. flexneri* 2457T has little or no importance in the regulation of the *S. flexneri* 2457T acid-resistance pathways.

Activity of SFL1001 truncated RpoS measured by catalase assay

To determine the activity of the truncated RpoS of SFL1001, it is possible to measure the activity of RpoS

Fig. 2. Western blot showing the expression of RpoS. B1401, MG1655 *E. coli* strain expressing a full-sized RpoS protein; SFL1001, 2457T with truncated RpoS; SFL1629, SFL1001 with an insertion disrupting *rpoS*; SFL1647, SFL1629 complemented with the SFL1001 truncated RpoS; SFL1648, SFL1001 complemented with the MG1655 RpoS. The expected sizes for the full-sized RpoS and 2457T truncated RpoS are 38 and 30 kDa, respectively. Approximate band sizes were determined from the closest protein ladder bands of the Fermentas prestained PageRuler.

Fig. 3. Effect of RpoS disruption in *S. flexneri* 2457T strain SFL1001 on acid-challenge survival. Cells were grown overnight in the media indicated and diluted 1 : 1000 into the acid-challenge media; survival is stated as a percentage of the inoculum. Results are the mean $\pm$ SEM of four experiments. Glt, glutamate.

using a rapid semi-quantitative catalase assay, in which an RpoS mutant will display a reduced level of bubbling, although some bubbling will still be observed as the cell possesses a second hydrogen peroxidase (HPI) that is not regulated by RpoS (Subbarayan & Sarkar, 2004). *E. coli* MG1655, which possesses a full-sized (330 aa) RpoS, had a rapid, vigorous bubbling phenotype, while *S. flexneri* 2457T, which contains a truncated (255 aa) RpoS, displayed reduced oxygen production, with slow bubbling observed. Furthermore, the *rpoS* mutant SFL1629 was only able to slowly decompose the H_2O_2 , with sluggish bubbling observed after 30 s. SFL1647, the 2457T truncated-*rpoS*-complemented strain, was also severely attenuated in catalase activity, displaying bubbling similar to that observed for SFL1629, while the *E. coli* full-sized-*rpoS*-complemented *S. flexneri* strain produced vigorous bubbling, similar to that observed for MG1655. Therefore, the reduction in SFL1001 catalase activity in comparison to the catalase activity displayed by the full-sized *E. coli* RpoS suggests that the truncated RpoS is reduced in its function.

Contribution of GadB and GadC to SFL1001 acid resistance

As the GDAR pathway has been so well characterized, it seemed prudent to determine whether the unusual resistance behaviour observed in SFL1001 LBG (pH 5) cultures acid-shocked in EG (pH 2.5) had any link to the key components of the glutamate decarboxylase system. It seems unlikely that the GDAR pathway is functional under these conditions, as the pathway has an absolute requirement for glutamate to be present in the acid-shock

medium. Disruption mutants of *gadB* and *gadC* were produced in SFL1001, and acid-resistance assays were performed on all strains, as shown in Fig. 4. Both the *gadB* and *gadC* mutants displayed a change in their GDAR phenotype. Both strains' survival in EG (pH 2.5), with 0.012 % glutamate added (condition 3, Fig. 4a, b), were significantly reduced in comparison to SFL1001 (*t* tests, $P < 0.01$). Complementation of each gene disruption was sufficient to restore the acid resistance. There was no change to resistance levels of the oxidative pathway (condition 2, Fig. 4a, b), as expected, or in the unusual resistance phenotype for the *gadB* and *gadC* mutants (condition 1, Fig. 4a, b), suggesting that the key components of the GDAR pathway are not contributing

to the glutamate-independent non-glucose-repressed resistance observed in SFL1001. Interestingly, this resistance, which is observed in LBG (pH 5) cultures shocked in EG (pH 2.5), did not prevent the loss of acid resistance for *gadB* and *gadC* mutants in LBG (pH 5) challenged in EG (pH 2.5) containing 0.012 % glutamate (condition 3, Fig. 4a, b).

DISCUSSION

The acid-resistance phenotype appears to be very important in intestinal pathogens. Bacteria must survive exposure to gastric acid in the stomach and volatile fatty acids produced within the intestine (Giannella *et al.*, 1972). This ability to survive acidic conditions encountered during passage through the host is most likely a contributing factor to the very low infectious dose of *S. flexneri*. *S. flexneri* 2457T has been widely studied in virulence assays and challenge work, and is known for its virulent phenotype; however, it has never been examined from an acid-resistance perspective (Wei *et al.*, 2003). This study detected two acid-resistance systems, the GDAR and oxidative systems, which have been characterized elsewhere in *S. flexneri* 3136 (Lin *et al.*, 1995).

The GDAR system observed in *S. flexneri* 2457T behaved the same as the GDAR pathway of *S. flexneri* 3136 (Lin *et al.*, 1995); the pathway was acid induced, cultures requiring growth at pH 5 to generate measurable acid resistance in acidified EG supplemented with 0.012 % glutamate. Likewise, the pathway showed anaerobic induction, with higher resistance observed for cultures grown under conditions of limited oxygen availability. Additionally, the GDAR pathway was capable of operating in cells grown in minimal media at pH 5, demonstrating that the system has no requirements for complex media during overnight growth.

The oxidative system was also present in *S. flexneri* 2457T. Cultures grown in MES-buffered LB overnight were capable of surviving exposure to acidified EG in the absence of glutamate. The pathway did not operate in cells grown without oxygenation, and showed an absolute requirement for overnight growth in complex media, as also reported for *S. flexneri* 3136 (Lin *et al.*, 1995). Some properties reported for the oxidative pathway of *E. coli* were not detected in this study (Castanie-Cornet *et al.*, 1999). It has been reported for *E. coli* that the oxidative pathway does not require acid induction by overnight growth at pH 5 to induce resistance, but that the lack of resistance observed during growth at pH 8 is due to the presence of a secreted inhibitor (Castanie-Cornet *et al.*, 1999). The lack of resistance at pH 8 for *S. flexneri* 2457T was not due to the presence of an inhibitor, but instead seems simply to be due to a requirement for acidic growth in order to induce the pathway. Furthermore, glutamate activation was not observed, as the addition of 5.9 mM glutamate to pH 8 cells did not generate any oxidative resistance.

Fig. 4. (a) Acid resistance of SFL1001, SFL1650 (*gadB* mutant) and SFL1723 (*gadB*, complemented). (b) Acid resistance of SFL1001, SFL1641 (*gadC* mutant) and SFL1729 (*gadC*, complemented). Cells were grown overnight in the media indicated and diluted 1 : 1000 into the acid-challenge media; survival is stated as a percentage of the inoculum. Results are the mean $\pm$ SEM of three experiments. SFL1650 and SFL1641 displayed significantly reduced acid resistance under condition 3, when compared to SFL1001 by *t* test ($P < 0.01$). Glt, glutamate.

In contrast to the behaviour reported for both *E. coli* and *S. flexneri* 3136, it was determined that 2457T is capable of displaying resistance when grown in LBG at pH 5 and acid-shocked in EG at pH 2.5 without glutamate supplementation. According to the literature, these acid-shock conditions are effectively a negative control for both the GDAR and oxidative pathways (Castanie-Cornet *et al.*, 1999; Lin *et al.*, 1995, 1996). The GDAR system should not be able to operate under these conditions, as there is no glutamate present in the acidified EG. Likewise, the oxidative system should be repressed by the presence of glucose in the overnight growth medium. When the test cultures were grown in LBG (pH 5) without oxygenation there was a substantial drop in resistance, suggesting that this glutamate-independent, non-glucose-repressed pathway (AR4) is induced by oxidative growth. Interestingly, this pathway was also recorded, but not discussed, by de Jonge *et al.* (2003). In their case, *E. coli* O157 grown in LBG (pH 5) showed substantial survival in EG (pH 2.5). However, no explanation was given for this unusual resistance. Whether this pathway is novel for *S. flexneri* 2457T and certain *E. coli* strains, or if it is the known oxidative pathway functioning in the presence of glucose, remains unclear. To further clarify this unusual acid-resistance phenotype, mutants of *S. flexneri* 2457T were constructed in genes known to be important to the oxidative and GDAR pathways, respectively.

As mentioned above, AR4 appears to be induced by oxidative growth. Consequently, to explore whether this resistance is from the known oxidative pathway which is not being repressed by glucose as expected, a disruption was constructed in the *rpoS* gene. RpoS is considered essential for the oxidative pathway to operate, and although also involved in the GDAR pathway, it is not essential, as resistance can still be detected from the GDAR system in an *rpoS* mutant in *E. coli*, and resistance is still present in *S. flexneri* 3136 at pH 5 (Castanie-Cornet *et al.*, 1999; Lin *et al.*, 1995; Small *et al.*, 1994). The *S. flexneri* 2457T *rpoS* mutant did not show a reduction in the non-glucose-repressed, glutamate-independent acid resistance observed for LBG (pH 5) cultures. However, there was also no defect or significant reduction in either the GDAR or the oxidative pathway for the *rpoS* knockout. It appears that the *rpoS* gene does not play an essential role in the regulation of any acid-resistance pathway in *S. flexneri* 2457T. Interestingly, this may be connected to the observation that 2457T possesses a truncated RpoS protein, caused by a frameshift mutation within the gene.

This study has shown by Western blot analysis that the RpoS protein missing the last 89 amino acids is still produced in 2457T, but it is unclear whether it is still functional. The truncation means that the protein is missing the end of the fourth domain of the conserved σ^{70} family structure, which is involved in -35 promoter recognition of target genes (Lonetto *et al.*, 1992). The loss of the fourth-domain-encoding region of RpoS in *E. coli* is sufficient to render the protein effectively inactive

(Ohnuma *et al.*, 2000; Subbarayan & Sarkar, 2004). In an attempt to determine whether the 2457T RpoS is functional, a catalase assay was performed. *S. flexneri* 2457T clearly had reduced catalase activity when compared to *E. coli* MG1655, which carries a full-sized RpoS. This activity was also reduced in the *rpoS* disruption mutant SFL1629 and in the *rpoS* complemented strain SFL1647, carrying the 2457T *rpoS* gene. It was possible to create strong catalase activity in *S. flexneri* 2457T by expressing the full-length *E. coli* RpoS in SFL1648. These results seem to suggest that the truncated *S. flexneri* RpoS protein is at least reduced in its activity, although further research is required to confirm this.

In order to clarify any possible role of the GDAR system in AR4 resistance, disruptions were also constructed in the *gadB* and *gadC* genes. GadB is a glutamate decarboxylase, responsible for the conversion of intracellular glutamate to GABA. A non-polar mutation was constructed in *gadB* to maintain expression of *gadC*, which lies downstream from *gadB*. The *gadB* knockout showed a significant reduction in GDAR, but resistance was not reduced sufficiently for it to be classified as acid sensitive (<10 % survival). This is most likely due to the presence of the functional glutamate decarboxylase isoform *gadA*, which is located elsewhere in the chromosome. Castanie-Cornet *et al.* (1999) have demonstrated that in *E. coli* K12 only one Gad isoform is required for measurable acid resistance. Conversely, Waterman & Small (2003) have shown that a *gadB* mutant in *S. flexneri* M25-8A is completely attenuated in the GDAR pathway. The *gadB* disruption had no effect on the AR4 resistance in *S. flexneri* 2457T. The complementation of the *gadB* mutant with a plasmid-based copy of *gadB* was sufficient to restore the reduced acid-resistance levels for the GDAR pathway, demonstrating that the non-polar mutation had been successful in maintaining the downstream *gadC* expression.

A *gadC* disruption was also constructed for *S. flexneri* 2457T. As expected, this strain became acid-sensitive for the GDAR pathway and *gadC* plasmid complementation restored the acid resistance. However, the *gadC* mutant was still capable of surviving exposure to EG (pH 2.5) after growth in LBG (pH 5). Thus, it seems that this unusual acid resistance is perfectly operational when the GDAR pathway has been disabled and does not seem to be linked to this system. Interestingly, AR4 resistance did not rescue the *gadB* or *gadC* mutants, which had been rendered deficient or reduced in acid resistance under growth conditions that favour the GDAR system. The AR4 resistance phenotype could be detected when the *gadB* and *gadC* mutants are grown in LBG (pH 5) media and shocked in EG at pH 2.5. However, when the cells were grown as above and acid-shocked in EG (pH 2.5) containing glutamate, the pathway did not appear to contribute any resistance, since if this was the case, the *gadB*- and *gadC*-related reductions in acid resistance would not have been observed. It may be possible that this unusual pathway is in some way repressed by glutamate.

It still remains unclear whether the oxidative pathway is contributing to this novel resistance in a non-glucose-repressible manner. It was hoped that the previously reported absolute dependence of the oxidative pathway on RpoS could be used to determine whether the oxidative system is responsible for this non-glucose-repressible, glutamate-independent resistance. In contrast, it appears that RpoS is not important in the *S. flexneri* 2457T acid-resistance phenotype. RpoS, the σ^S subunit of RNA polymerase, is strongly induced on entry into the stationary phase and is the main regulator of the stress response in *E. coli* (Weber *et al.*, 2005). The loss of RpoS from *E. coli* strains, both laboratory and natural, is quite common (King *et al.*, 2006). It appears that loss of RpoS can actually convey a selective advantage for *E. coli*, in which *rpoS* deficiency may be advantageous under conditions of nutrient deprivation, may increase amino acid scavenging ability in stationary-phase cells, and even give cells a competitive advantage in mouse colonization experiments (Atlung *et al.*, 2002; Finkel & Kolter, 1999; Zinser & Kolter, 1999). In this case, the *S. flexneri* 2457T strain SFL1001 has not lost expression of *rpoS*, but instead produces a truncated protein with reduced activity, as demonstrated in a catalase assay. The maintenance of acid-resistance levels for the *rpoS* mutant of *S. flexneri* 2457T suggests that the acid-resistance pathways of the strain are able to operate independently of RpoS. All earlier reports on the loss of RpoS in *E. coli* and *S. flexneri* have shown that the oxidative pathway is consequently reduced in acid resistance, making this the first study to demonstrate RpoS independence for this pathway (Castanie-Cornet *et al.*, 1999; Notley-McRobb, 2002; Price *et al.*, 2000).

The acid resistance of 2457T includes an unusual acid-resistance phenotype. This phenotype is not related to the operation of the GDAR, as it does not display a dependence on glutamate in the acid-shock medium or require GadB and GadC. It is not clear whether this resistance is the oxidative pathway operating in the presence of glucose, or if an entirely independent pathway is present. Little is known about the oxidative pathway of *S. flexneri*; however, in a recent study a *fur* mutant of *S. flexneri* SA100 was found to be defective in the oxidative pathway (Oglesby *et al.*, 2005). It would be interesting to determine if a *fur* mutant of *S. flexneri* 2457T would also be defective in oxidative acid resistance and, furthermore, determine if disruption of oxidative resistance also eradicates the novel non-glucose-repressed, glutamate-independent acid resistance.

This study is believed to be the first report on the stationary-phase acid-resistance pathways of *S. flexneri* 2457T. Two acid-resistance systems, the GDAR and the oxidative pathways, reported elsewhere for *E. coli* and *S. flexneri* 3136, were both detected in the *S. flexneri* 2457T strain SFL1001. Furthermore, SFL1001 cells grown overnight under fermentative growth conditions and acid-shocked in minimal medium in the absence of glutamate, an acid test often described as a negative control for both

pathways, were capable of surviving acid challenge. It is unclear whether this resistance is due to the oxidative pathway operating in a non-glucose-repressible manner, or if a novel pathway is present in *S. flexneri* 2457T. The construction of *gadB* and *gadC* mutants ruled out any contribution by the GDAR pathway, whilst further characterizing the GDAR properties of *S. flexneri* 2457T. Interestingly, study of the role of *rpoS* in the oxidative pathway and the unusual acid-resistance phenotype revealed that the frameshift present in the *rpoS* gene results in expression of a truncated RpoS protein, which may be reduced in activity and is not essential for the acid-resistance phenotype of *S. flexneri* 2457T.

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