Roles of H₂ uptake hydrogenases in *Shigella flexneri* acid tolerance

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Hydrogenases play many roles in bacterial physiology, and use of H₂ by the uptake-type enzymes of animal pathogens is of particular interest. Hydrogenases have never been studied in the pathogen *Shigella*, so targeted mutant strains were individually generated in the two *Shigella flexneri* H₂-uptake enzymes (Hya and Hyb) and in the H₂-evolving enzyme (Hyc) to address their roles. Under anaerobic fermentative conditions, a Hya mutant strain (*hya*) was unable to oxidize H₂, while a Hyb mutant strain oxidized H₂ like the wild-type. A *hyc* strain oxidized more exogenously added hydrogen than the parent. Fluorescence ratio imaging with dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) showed that the parent strain generated a membrane potential 15 times greater than *hya*. The *hya* mutant was also by far the most acid-sensitive strain, being even more acid-sensitive than a mutant strain in the known acid-combating glutamate-dependent acid-resistance pathway (GDAR pathway). In severe acid-challenge experiments, the addition of glutamate to *hya* restored survivability, and this ability was attributed in part to the GDAR system (removes intracellular protons) by mutant strain (e.g. *hya/gadBC* double mutant) analyses. However, mutant strain phenotypes indicated that a larger portion of the glutamate-rescued acid tolerance was independent of GadBC. The acid tolerance of the *hya* strains was aided by adding chloride ions to the growth medium. The whole-cell Hya enzyme became more active upon acid exposure (20 min), based on assays of *hyc*. Indeed, the very high rates of *Shigella* H₂ oxidation by Hya in acid can supply each cell with 2.4×10^8 protons min⁻¹. Electrons generated from Hya-mediated H₂ oxidation at the inner membrane likely counteract cytoplasmic positive charge stress, while abundant proton pools deposited periplasmically likely repel proton influx during severe acid stress.

Received 1 February 2012

Revised 16 May 2012

Accepted 19 May 2012

INTRODUCTION

Four predicted unidirectional hydrogenases have been identified in the genome of *Shigella flexneri*: two uptake (*hya*, *hyb*) and two evolving (*hyc*, *hyf*) types. No physiological studies on *S. flexneri* hydrogenases have been published, but the *S. flexneri* hydrogenases are expected to have functions and mechanisms similar to those of the described *Escherichia coli* enzymes. Still, physiological and pathogenesis differences between *S. flexneri* and other *Enterobacteriaceae* justify studies on the comparative roles of the *Shigella* enzymes. Similarly, variations in enterobacterial acid-stress responses warrant further studies on *Shigella*.

Upon H₂ oxidation by uptake hydrogenases, electrons are commonly donated to various redox potential electron

carriers at the inner membrane (Vignais & Colbeau, 2004). Due to the membrane orientation of the H₂ oxidation reactions, the model for hydrogenase-mediated proton motive force (PMF) generation involves the deposition of protons at the periplasm so that ATP production or other work can be accomplished by the cell (Brøndsted & Atlung, 1994; Vignais & Colbeau, 2004). In *Enterobacteriaceae*, it is believed that the Hya hydrogenase is the primary enzyme to recycle protons and electrons originally produced anaerobically by the formate hydrogenlyase complex (Redwood *et al.*, 2008; Zbell & Maier, 2009). One component of FHL is HycE, a hydrogen-evolving enzyme (Redwood *et al.*, 2008; Sawers, 2005).

S. flexneri causes diarrhoeal disease symptoms in humans upon ingestion of fewer than 100 bacilli (Philpott *et al.*, 2000; Jennison & Verma, 2007). *Shigella* cells enter the acidic environment of the stomach (pH 2), where they subsist (~2 h) in stationary phase but typically grow very little (Jennison & Verma, 2007). *Shigella* eventually enters the colon (pH 5.5–7.0) and invades the colonic epithelium via exterior sampling immune structures called M-cells (Ingersoll & Zychlinsky, 2006). Again, they encounter extreme

Abbreviations: AR, acid resistance; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; $\Delta\psi$, membrane potential; GDAR pathway, glutamate-dependent acid-resistance pathway; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; PMF, proton motive force; wt, wild-type.

A supplementary figure and a supplementary table are available with the online version of this paper.

acid conditions after their ingestion by macrophages (Fortier *et al.*, 1995).

The ability of *S. flexneri* to express stationary phase acid-resistance (AR) mechanisms in the stomach contributes to the bacterium's unusual pathogenicity (Lin *et al.*, 1995). Two AR pathways have been identified in *S. flexneri*: AR1 requires oxygenation of cultures, and AR2 is glutamic acid-dependent and glucose-repressed (Castanie-Cornet *et al.*, 1999; Jennison & Verma, 2007). However, cells grown anaerobically are the most acid-resistant. It is clear that the AR2 provides the most effective AR, allowing cells to persist in the human stomach at pH levels below 3 (Coldewey *et al.*, 2007). The genes involved in AR2 include decarboxylases (*gadA* and/or *gadB*) and an antiporter (*gadC*). Glutamate is taken into the cell via GadC, and decarboxylated via GadA/B, which consumes an intracellular proton; the product, gamma aminobutyric acid (GABA), is exported out of the cell through the action of GadC (Richard & Foster, 2004). The net result is a neutralization of the cytoplasm, aiding acid tolerance.

Another AR mechanism used by enteric bacteria involves chloride ion channels to prevent proton buildup in the cytoplasm (Gut *et al.*, 2006). The exchange of a chloride ion for a proton in the cell decreases the intracellular pH, but also counterbalances the excess intracellular positive charges accumulated due to the decarboxylation products (Foster, 2004); the exchange allows the cell to eventually recover an internally negative membrane potential.

Deletion of all uptake-type hydrogenases in *Salmonella enterica* yields an avirulent strain with respect to mouse mortality (Maier *et al.*, 2004), and H₂ has been postulated to be an important energy source for many enteric bacteria *in vivo* (Maier, 2003). The large intestine is a rich source of H₂, but the gas is found (dissolved in the bloodstream) at micromolar levels in many tissues colonized by pathogens, and no known mechanism for animal hosts to utilize the

gas have been described. Therefore, H₂ uniquely represents (to pathogens) an energy source for which competition with host cells is not a factor. Activity studies on *E. coli* Hya have revealed that the *hya* operon is anaerobically induced under acid conditions (King & Przybyla, 1999; Trchounian & Trchounian, 2009). Our study focuses primarily on the Hya hydrogenase in *Shigella* acid tolerance.

METHODS

Bacterial strains and growth conditions. *S. flexneri* 2a 2457T strain ATCC 700930 was used. *Salmonella enterica* Typhimurium JSG210 containing the recombinase plasmid pKD46, and *E. coli* pCP20 and *E. coli* pKD4 were used for lambda red recombination. Strains with temperature-sensitive plasmids (pKD46 and pCP20) were grown aerobically at 30 °C with ampicillin (100 µg ml⁻¹). pKD4 containing the kanamycin-resistance cassette was maintained at 37 °C. Growth was performed in Luria–Bertani broth (LB; pH 7.0, 10 g NaCl l⁻¹) supplemented with 0.4% (v/v) glucose in 165 ml stopper-sealed serum vials. The liquid volume was 20 ml and the atmosphere contained an anaerobic mix (10% H₂, 5% CO₂, 85% N₂). Single mutant deletions are annotated as ALZ44 (Δ *hya*), ALZ47 (Δ *hyb*) and MMM01 (Δ *hyc*). The double mutants are designated MMM02 (Δ *gadBC*) and MMM03 (Δ *hya* Δ *gadBC*). The strains are shown in Table 1.

Lambda red mutant constructs. Genes for both large and small subunits of the hydrogenase proteins were deleted. The procedure was adapted from Datsenko & Wanner (2000). The temperature-sensitive recombination plasmid pKD46 was electroporated into background strain 2457T. pKD46 was induced with 0.1 M L-arabinose at 30 °C. PCR constructs containing the large and small subunit deletions were transformed into pKD46-induced *Shigella* cells. After 1 h at 37 °C, cells were plated onto LB + kanamycin (25 µg ml⁻¹) at 37 °C overnight. Transformants were PCR-verified for 1.6 kb Kan cassette insertion. pCP20 containing the flip-recombinase enzyme (Cherepanov & Wackernagel, 1995; Datsenko & Wanner, 2000) was electroporated into the Kan cassette-containing mutants and plated (LB agar). Colonies were then plated on LB at 37 °C to expel pCP20. The final mutant constructs were kanamycin- and ampicillin-sensitive and each

Table 1. Strains, constructs and plasmids used in this study

Strain, construct or plasmid	Description	Source or reference
Strain		
<i>S. flexneri</i> 2a	2457T wt	ATCC 700930
Mutant constructs		
Δ <i>hya</i> ::FRT ALZ44	<i>hya</i> deletion with FRT site (S1040–S1041)	This study
Δ <i>hyb</i> ::FRT ALZ47	<i>hyb</i> deletion with FRT site (S3242–S3244)	This study
Δ <i>hyc</i> ::FRT MMM01	<i>hyc</i> deletion with FRT site (S2930–S2931)	This study
Δ <i>gadBC</i> ::FRT MMM02	Deletion of AR2 Glu/GABA antiporter and Glu decarboxylase with FRT site (S1867–S1868)	This study
Δ <i>hya</i> ::FRT Δ <i>gadBC</i> ::FRT MMM03	Deletion of <i>hya</i> , Glu/GABA antiporter, and Glu decarboxylase with FRT sites	This study
Plasmids		
pKD46	λ -red recombinase, Amp ^r	Datsenko & Wanner (2000)
pKD4	Kanamycin-resistance cassette	Datsenko & Wanner (2000)
pCP20	Flp recombinase, Amp ^r	Cherepanov & Wackernagel (1995), Datsenko & Wanner (2000)

contained a 'scar' site from the Kan cassette removal. Table S1 shows the primers used.

Hydrogenase assays. *S. flexneri* wild-type (wt) and mutant constructs were grown overnight (shaking at 200 r.p.m.) in 165 ml vials (20 ml LB) to stationary phase (16–18 h). H_2 concentrations were determined using a microelectrode probe (Maier *et al.*, 2004). A Unisense H_2 -50 probe (Unisense) was used. The cell samples (7 ml) were removed anaerobically via syringe for addition to the (anaerobic) amperometric assay chamber, or for acid treatment prior to assay (see below). For the Fig. 1 study, a 10 ml sample was placed into a beaker and swirled for 10–15 s in air, and then (7 ml) evaluated aerobically for amperometric hydrogen consumption/evolution (Maier *et al.*, 2003; Zbell & Maier, 2009). Exposure to air inactivates the H_2 evolution enzymes (Zbell & Maier, 2009). For studying the effects of acid, stationary phase cells were added to vials (previously sparged with anaerobic mix) containing acid challenge medium, which was composed of buffered citric acid phosphate (Mclivaine), pH 2.5. Oxyrase for Broth (Oxyrase Inc.) was added to the vials to maintain anoxic conditions, and the amperometric assay chamber was sealed throughout the procedure. Initial H_2 concentrations were noted after electrode stabilization (20–30 s), and then H_2 levels were recorded for 1 min. For H_2 assays upon acid challenge, samples were loaded directly from the acid-challenge milieu into the amperometric chamber (i.e. without washing), so the H_2 levels (i.e. activities) were measured while cells were suspended in the acid medium. After calculating nmoles of H_2 consumption or evolution in one minute, for specific activity calculations the cell number was determined from a standard curve of cell number versus OD₆₀₀. Oxyrase-only controls oxidized a negligible amount of H_2 .

Acid survival. The acid challenge procedure was adapted from one described earlier (Jennison & Verma, 2007). Wt and mutant constructs (*hya*, *hyb*, *hyc*, *gadBC* and *hya gadBC*) were grown to stationary phase (16–18 h) in LB (pH 7.0). The challenge medium atmosphere was adjusted by sparging with anaerobic mix. Stationary phase cells (10^7 c.f.u. ml⁻¹) were added to the acid challenge medium, which was composed of buffered citric acid phosphate (Mclivaine),

Fig. 1. Microelectrode hydrogenase assay of *S. flexneri* strains. Hydrogenase activity was measured by hydrogen consumption under anaerobic conditions (atmosphere composed of 10% H_2 , 5% CO_2 and 85% N_2). Strains are wt (2457T), *hya* (ALZ44), *hyb* (ALZ47) and *hyc* (MMM01). Error bars, SD determined from five to eight replicate recordings of H_2 removal by individual strains, after inactivating H_2 evolution enzymes by brief O_2 treatment (Zbell & Maier, 2009). Asterisks indicate significant differences from wt at $P < 0.01$ (*) and $P < 0.05$ (**) based on Tukey's HSD method.

pH 2.5. After the indicated incubation period, serial dilutions were plated (LB) and colonies were counted after overnight growth.

For assessment of AR2 influence, 1.5 mM L-glutamic acid (Sigma) was added to the challenge medium upon cell inoculation.

For NaCl acid survival, 1% NaCl (w/v) was either supplemented into LB broth and/or added to challenge medium (pH 2.5), where indicated.

[¹⁴C]Glutamate uptake and amino acid pool analysis.

[¹⁴C]Glutamate uptake was measured as described previously (Lamichhane-Khadka *et al.*, 2010; Mehta *et al.*, 2005). Uniformly labelled glutamate (Sigma) was added to either exponential or stationary phase *Shigella* cultures, and glutamate uptake was determined at numerous points. Amino acid pools were determined as follows. Stationary phase cells were subjected to French press disruption and then sonication. The extract (containing PMSF and maintained at 0–4 °C) was subjected to ultracentrifugation to remove the membranes. The supernatant was removed and concentrated by lyophilization. The physiological samples containing the amino acid pools were sent to the Molecular Structure Facility at the University of California, Davis, where the samples were analysed for full amino acid content by use of a Hitachi L-8800 amino acid analyser.

Membrane potential ($\Delta\psi$) measurement. The fluorescent cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) was used to stain viable *Shigella* cells for subsequent visualization via confocal microscopy. The fluorescence shift was used to approximate the $\Delta\psi$ of cells (Lamichhane-Khadka *et al.*, 2010). JC-1 dissociation shifts the red emission to green and indicates a decrease in $\Delta\psi$ (Jovanovic *et al.*, 2006; Lamichhane-Khadka *et al.*, 2010). Cultures were grown (with H_2) to stationary (16–18 h) phase, and samples were prepared as described previously (Lamichhane-Khadka *et al.*, 2010). For the negative control, a chemical protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), was used (dissipates $\Delta\psi$). The negative control samples were incubated with JC-1 (10 μ g ml⁻¹ final concentration) for 28 min. Then 40 μ M CCCP (stock solution dissolved in DMSO and used at 1:2000 dilution) was added for an additional 2 min (JC-1 still present). Samples were centrifuged at 18 000 *g* for 2 min and the pellet was resuspended in 250 ml fresh permeabilization buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM glucose). Ratios were taken from five fields with an average of 100 cells per field. Four independent experiments were conducted with similar results. Ratios are expressed in green/red; a lower ratio indicates a higher $\Delta\psi$ (Jovanovic *et al.*, 2006).

Statistical measurements and precision. One-way analysis of variance (ANOVA), Tukey's HSD test for independent samples or Student's *t* test was used to compare data significance. Asterisks above bars in graphs indicate a significant difference compared with wt except for Fig. 4, where the difference was in comparison with (untreated for the same time point) the *hya* strain: $P < 0.01$ (*) and $P < 0.05$ (**), or for Fig. 5, $P < 0.02$ (*). All experiments were performed at least one additional time, with results similar to those presented herein.

RESULTS

Hydrogenase activities associated with gene-targeted mutants grown in fermentative conditions

The strains were grown in fermentative conditions and then assayed in conditions favouring H_2 oxidation and not

H₂ evolution (see Methods). In many separate experiments we found that the *hya* mutant (*hya*) was unable to oxidize H₂ (see Fig. 1). Indeed, *hya* showed slight hydrogen evolution, undoubtedly due to residual activity of the Hyc (H₂-evolving) system. In contrast, the *hyb* mutant strain (*hyb*) had almost wt levels of H₂ oxidation. The hydrogenase assays confirm that in anaerobic fermentative conditions Hya is responsible for hydrogen oxidation, while Hyb contributes little and cannot replace Hya in H₂ oxidation. As expected, the strain lacking the evolving enzyme Hyc showed high levels of H₂ oxidation. Only in this strain can the full H₂ oxidation activity be measured, as the contribution from H₂ produced by Hyc is eliminated.

Acid challenge

The Hya hydrogenase has been implicated as playing a role in *Salmonella* Typhimurium AR (Zbell & Maier, 2009). The *Salmonella* strain lacking Hya both is acid-sensitive and has a reduced ability to survive in RAW 264.7 macrophages compared with the wt (Zbell *et al.*, 2008). After fermentative growth with glucose in LB to stationary phase, individual deletion mutant strains of *Shigella* were subsequently severely acid-challenged in a buffered medium (pH 2.5). The results are shown in Fig. 2. The viable cell counts decreased over the 6 h period for the wt and the three hydrogenase mutants. Compared with initial cell counts, the wt had 65 and 38 % survival at 4 and 6 h, respectively. In contrast, a dramatic drop (almost 100-fold) in survival after just 2 h was observed for the *hya* mutant. At 4 h, ~0.01 % of the *hya* cells remained. No *hya* cells were recovered at 6 h post acid-challenge; its survival ability was statistically significantly less than that of all the other strains at all sampling periods (2, 4, 6 h). The *hyb* strain was slightly more acid-sensitive than wt; it was statistically significantly different from wt only at the 2 h time point.

Glutamate-dependent acid tolerance mediated by GadBC is known to confer AR upon *Shigella* (Jennison & Verma, 2007). Therefore a *gadBC* mutant (*gadBC*) was generated

to compare its properties with those of *hya*; the strain lacks both the *gadB* decarboxylase and the *gadC* antiporter necessary for AR2 activity. This strain unexpectedly showed more AR than the *hya* mutant, but (expectedly) was more acid-sensitive than the other strains (see Fig. 2 at 4 and 6 h points). Significant acid sensitivity (more than the other four strains in the table) was also shown by *hya* in pH 3 challenge medium (data not shown).

Hydrogenases are active in acid conditions

The roles/activities of the hydrogenases specifically during acid challenge (as opposed to in LB medium) were of interest. For example, it was possible that H₂ metabolism during pre-challenge growth had the major effect in conferring subsequent acid tolerance, and that H₂ metabolism enzyme activities in acid were negligible due to the acid shock. However, we found that the whole-cell activities in acid were even greater than in LB (see Table 2). In pH 2.5 conditions, the wt, *hyb* and *hyc* strains oxidized considerable hydrogen, while the *hya* strain evolved some hydrogen in the anaerobic and acid stress conditions. In line with the hydrogen oxidation results (Fig. 1), *hyb* has a phenotype similar to that of the wt strain. As expected, the *hyc* strain showed the highest H₂ consumption, because only in that strain could the full H₂ disappearance (i.e. oxidation) be monitored. After 5 or 20 min in acid, the *hyc* strain took up more than 200 nmoles of H₂ per minute per 10⁹ cells. This means more than 2.4×10^8 H⁺ ions are produced per cell per minute.

The Hya enzyme (uptake activity) was stimulated by acid. In one experiment the *hyc* strain had an activity of 74 ± 10 nmoles of H₂ per minute per 10⁹ cells for cells taken directly from LB (*n*=5) and 228 ± 33 nmoles of H₂ per minute per 10⁹ cells (*n*=9) for the same cell culture placed into the acid shock medium. Hydrogen evolution in *hya* undoubtedly originates via the Hyc enzyme. H₂-uptake enzymes normally consume all Hyc-evolved H₂, so that *Enterobacteriaceae* H₂ metabolism is interconnected (Sawers,

Fig. 2. Viability upon acid challenge of *Shigella* cultures at pH 2.5. c.f.u. yields from serial dilutions at different post-inoculation times are shown. Error bars, SD determined from at least three replicates from multiple dilutions. Control experiments in the same buffer but at pH 6.5 showed no viability loss for strains. Asterisks indicate a significant difference ($P < 0.01$) compared with wt calculated using Tukey's HSD test.

Table 2. Amperometric hydrogen measurements of strains subjected to acidic (pH 2.5) conditions

H₂ metabolism activity (uptake or evolution) of each strain was measured after subjection to acid challenge under strictly anaerobic conditions. Values are mean $\pm$ SD determined from four to six replicate recordings of H₂ level changes in a 1 min period.

Strain	Specific activity after indicated time period in acid (pH 2.5) [nmol H ₂ min ⁻¹ (10 ⁹ cells) ⁻¹]	
	5 min	20 min
wt	150 $\pm$ 37 H ₂ uptake	180 $\pm$ 23 H ₂ uptake
<i>hya</i>	51 $\pm$ 19 H ₂ evolution	44 $\pm$ 5 H ₂ evolution
<i>hyb</i>	113 $\pm$ 31 H ₂ uptake	151 $\pm$ 54 H ₂ uptake
<i>hyc</i>	276 $\pm$ 66 H ₂ uptake	228 $\pm$ 23 H ₂ uptake

2005; Zbell & Maier, 2009). For *E. coli* it has been noted that Hya is transcriptionally activated in acid conditions and Hyb more so in alkaline conditions (King & Przybyla, 1999; Park *et al.*, 1999). Hydrogenase activity comparable with that of wt *E. coli* was observed in a *hybC* deletion mutant at pH 5.5, while no hydrogenase activity was observed in a *hyaB* mutant (Trchounian *et al.*, 2012). The uptake activity that we observe in the *hyc* strain is due to both Hya and Hyb enzymes. However, Hya must play the major uptake role, as the *hyb* mutant (contains Hyc and Hya) oxidized all Hyc-produced H₂ and some exogenous H₂. The *hya* mutant could not even oxidize all the H₂ produced by the formate hydrogen lyase system (Table 2). Therefore the *hya* mutant evolved H₂.

Influence of the GDAR pathway

The AR2 pathway is the most efficient *S. flexneri* acid-combating pathway at pHs below 2.5 (Richard & Foster, 2004). It is dependent upon efficient glutamate uptake. Therefore we thought that Hya might play a role in providing membrane PMF to facilitate amino acid uptake,

a role attributed to one of the *Salmonella* hydrogenases (Lamichhane-Khadka *et al.*, 2010). If glutamate transport was facilitated by Hya, causing high internal glutamate pools, one might also expect that adding high levels of glutamate would diminish the strong acid sensitivity of the *hya* strain. To address this, 1.5 mM L-glutamic acid was added to the challenge medium vials along with the various strains (see Fig. 3).

Fig. 3 shows that the wt was mildly acid-sensitive, with 63 % of the cells surviving at 4 h and 31 % of the cells surviving 6 h of acid challenge. From an initial cell level of 10⁷ cells ml⁻¹, less than 0.1 % of the *hya* strain cells were recoverable after 4 h of acid challenge. As before (Fig. 2), at the 6 h point no cells were recovered. However, glutamate supplementation greatly aided recovery of the *hya* mutant; it achieved wt (no glutamate added) survival levels (2 and 4 h) or nearly so (6 h). Glutamate supplementation had little effect on (aiding) acid survival of the wt (data not shown). The double mutant *hya gadBC* was also acid-challenged with and without glutamic acid. Although glutamate aided recovery of this strain, viable cell recovery

Fig. 3. Influence of added glutamate on acid-challenged *Shigella*. Acid challenge at pH 2.5 was conducted as described in the text. Glutamic acid (1.5 mM) was added to the challenge buffer where indicated. No *hya* or *hya gadBC* isolates were recoverable at 6 h post-challenge (no glutamate added samples). Error bars, SD determined from multiple dilutions and from 12 independent samples. Asterisks indicate a significant difference ($P < 0.01$) compared with wt calculated using Tukey's HSD test.

Fig. 4. NaCl effects on acid tolerance of *hya*. Treatment 1, 1 % NaCl was added to the challenge medium (pH 2.5); treatment 2, 1 % NaCl was added to the overnight growth medium (LB broth); treatment 3, 1 % NaCl was added to both overnight and challenge media. As with the glutamate acid rescue, *Shigella* strains were grown anaerobically in LB to stationary phase, and then added to pH 2.5 buffer. At the indicated times, serial dilutions were plated. The significance of values is based on comparison with the untreated *Hya* mutant; *, $P < 0.01$.

after glutamate addition was nearly 67-fold less (see 4 h time point) than for the *hya* strain; therefore, most but not all of the glutamate-dependent rescue of *hya* is due to the GDAR system.

Additional experiments (data not shown) included ^{14}C -labelled glutamic acid uptake assays and comparative intracellular amino acid pool analysis to try to explain the *hya* phenotype. Increased glutamate accumulation during growth in the pre-shock medium would aid AR2-mediated AR. [^{14}C]Glutamate was added to *Shigella* (wt and *hya*) at various growth phases in an H_2 -containing atmosphere, and the rate of glutamate uptake over minutes and hours was measured using liquid scintillation spectrometry. The results (data not shown) were not supportive of the hypothesis that *Hya* plays a direct role in transporting glutamate.

Amino acid pools of both the wt and *hya* mutant were also analysed to determine whether the reason behind the phenotypic differences in survival was due to more robust amino acid accumulation in the cytoplasm by the parent strain. However, the pool analysis showed little difference between the strains; no evidence could be gleaned to conclude that *Hya* aided *Shigella* in the accumulation of glutamate or other amino acids. The glutamate-dependent rescue of the *hya* strain phenotype may be due to the GDAR system permitting extra positively charged GABA export out of the cell.

NaCl facilitates acid survival of the *hya* strain

The reversal of $\Delta\psi$ comes from both proton influx and accumulation of positively charged decarboxylation products associated with the function of AR2 (Foster, 2004). It is proposed that this acid-combating mechanism (positive internal charge) protects cells from acid by repulsion of proton movement into the cell. However, maintaining a positive electrical potential within the cell can also be detrimental, as hyperpolarization can cause additional severe

ion stress, resulting in compromising PMF-driven functions (Foster, 2004). Another acid-combating system involving Cl^- influx (via chloride channels) has been proposed to prevent hyperpolarization due to excess positive charge stress in *E. coli* (Iyer *et al.*, 2002). The influx of the chloride (in exchange for H^+) is thought to change the internal potential from positive to less positive (Richard & Foster, 2004). Conceivably, *hya*, due to a lower ability to generate cytoplasmic electrons, could be phenotypically rescued by addition of Cl^- . To address a possible effect of Cl^- influx on the *hya* phenotype we thus challenged the wt and *hya* *Shigella* strains in pH 2.5 buffer with added NaCl. Different challenge conditions were used to address NaCl effects in AR (Fig. 4). These were NaCl added to buffer (treatment 1), NaCl added to LB growth medium (treatment 2), NaCl added to both challenge and growth media (treatment 3), and as a control no NaCl added to either LB or buffer (untreated). *hya* cells grown in LB with NaCl (treatment 2) were acid-sensitive, but survived better than the untreated sample or than the other conditions; from an initial level (all values are c.f.u. ml^{-1}) of 3.5×10^7 (zero time point), $\sim 1.0 \times 10^4$ cells were recoverable at 6 h post-inoculation. Still, this recovery was significant, in that no cells were recovered 6 h post-acid challenge in the untreated *hya* sample. In contrast, adding NaCl to LB greatly inhibited subsequent acid-challenge survival of the wt; 6 h of acid challenge caused its viable cell numbers to decrease from $\sim 4 \times 10^7$ (no NaCl in LB) to about 10^4 cells (data not shown). Compared with the inhibition of the wt, NaCl appears to aid *hya* greatly, but the inhibition of wt acid tolerance makes interpretation difficult.

The *hya* mutant was also challenged at pH 2.5 by the addition of 2 % MgCl_2 in the same manner as NaCl addition. MgCl_2 added to the overnight medium provided a similar phenotype to treatment 2. Additional NaCl in the growth medium could affect many components. For example, it has been shown that NaCl is important not

Fig. 5. Comparative $\Delta\psi$ measurements. Ratios are fluorescence emission spectra for 530/590 nm. A lower ratio indicates a greater $\Delta\psi$. Strains were incubated with JC-1. For the negative control, CCCP (dissipates PMF) was added to wt *Shigella* for 3 min prior to adding JC-1 (10 $\mu\text{g ml}^{-1}$). Error bars, SD determined from five fields (100 cells each). The wt result is statistically significantly lower than the *hya* mutant or the CCCP control based on Student's two-sample *t* test at $P < 0.02$.

only in the exchange of H^+ for Cl^- ions across the membrane but also in the activation of key enzymes in the *E. coli* AR2 system (Richard & Foster, 2007). Still, the salt could have affected many factors not directly related to the acid-combating systems.

$\Delta\psi$ in wt and *hya* strains

Based on the results from the hydrogenase assays, and the predicted roles of membrane-bound hydrogenases, the electrical component ($\Delta\psi$) of the PMF was assayed in the wt and *hya* strains. The fluorescent dye JC-1 was used to determine $\Delta\psi$, a critical component of PMF. Green fluorescence indicates that the cells have a lower $\Delta\psi$ (see Fig. S1). Wt *S. flexneri* had a green to red ratio of 0.064 (see Fig. 5). The *hya* strain had a lower $\Delta\psi$, with green/red ratio of 0.876. Four independent experiments were performed and they all yielded a significantly higher $\Delta\psi$ associated with the wt *Shigella* strain.

DISCUSSION

Hydrogenases have been previously implicated as playing roles in AR in *E. coli* and in *Salmonella*. The evolution of hydrogen by Hyd-3 (*hyc*) uses excess protons to neutralize the cytoplasm in *E. coli* (Noguchi *et al.*, 2010), and a *Salmonella hya* mutant is acid- and macrophage-sensitive (Zbell & Maier, 2009). It has been observed (King & Przybyla, 1999) that *E. coli hyb* gene expression is increased in alkaline conditions and an increase in *hya* expression is correlated with a decreased external pH. In our study, based on gene-directed mutants, we conclude that Hyc and Hyb can confer some *S. flexneri* AR, but not nearly to the

same extent as the Hya hydrogenase (Fig. 2). In our study, the strain lacking Hya was even more acid-sensitive than a strain lacking the primary AR mechanism described (AR2). The glutamate supplementation and uptake assays indicate that the major role of Hya does not lie in facilitating energy-driven GDAR function or in increasing cell pools of glutamate. Still, recovery of *hya* cells by glutamate addition could be attributed in part to an active AR2 pathway and likely to glutamate transit via other glutamate-specific or even promiscuous transporters.

Enterobacteriaceae AR is highly complex and dependent on many factors (Foster, 2004; Richard & Foster, 2004). At least two AR stationary phase pathways have been described in *S. flexneri*. Anaerobic conditions, low pH (2.5), and addition of glucose (conditions used in this study) are expected to prevent the action of the AR1 pathway described in the Introduction. The glutamic acid-dependent system is expected to function in our conditions, but another proposed *Shigella* system (Jennison & Verma, 2007) that is independent of both glucose and glutamate may also play a role in the phenotypes described here. In our experiments, addition of NaCl to LB provided significant acid protection for *hya* when cells were subsequently acid-challenged. While there was a large reduction in *hya* viability even with NaCl, without salt addition there were no recoverable *hya* cells 6 h post acid challenge. The addition of NaCl has been reported to aid acid tolerance of *E. coli* tetracycline-resistant mutants (Hung *et al.*, 2006).

Gram-negative bacterial hydrogenases are oriented across the cytoplasmic membrane to produce protons at the periplasm and electrons at the inner membrane, thus enabling generation of a PMF; the system is used to facilitate work in many physiological processes. In addition, from our studies we conclude that the Hya hydrogenase confers unexpectedly strong AR upon *S. flexneri*, and the reason must be related to electron and/or proton production. It is generally perceived that when cells are under extreme acid stress, protons illicitly enter the cell directly through the cell membrane or via protein channels. One role of hydrogen-utilizing hydrogenases may be to counteract positive charge stress that occurs cytoplasmically due to positively charged decarboxylation products and other positively charged molecules and ions that accumulate under acid conditions (Foster, 2004). Electron deposition at the cytoplasm due to Hya-mediated H_2 oxidation could fill this role. Such negative ion accumulation would therefore play the same role as that of Cl^- ions (Iyer *et al.*, 2002). The chloride experiments indicate that the role of Cl^- falls into the adaptation growth phase, rather than having a direct effect on counteracting protons during survival in the acid environment. Influx of chloride (in exchange for H^+) in *Enterobacteriaceae* is thought to allow negative charge back into the cell, to counterbalance excess positive charge. It is not clear whether the negative ion production hypothesis role for Hya in aiding acid tolerance is supported by the glutamate addition experiments. Still, operation of GDAR

is known to combat external proton stress, so the sensitivity of *hya* strains to acid may be related to loss of cytoplasmic proton management.

Amplifying the operation of a system that is known to reduce positive ion stress, namely the GDAR system (by adding glutamate), greatly aided survival of the *hya* strain. A positive $\Delta\psi$, measured in *E. coli* subjected to acid conditions, comes from both proton influx and the accumulation of positively charged decarboxylation products that can be associated (i.e. increased) via the function of AR2. It is proposed that this acid-combating mechanism (positive internal charge), whether due to AR2 or other metabolites, protects cells from acid by repulsion of proton movement into the cytoplasm (Foster, 2004). Such positively charged metabolites localize to the cytoplasm, but act to repel proton influx from an acid environment that exists outside the cell wall. A hydrogenase that 'splits' H_2 such that protons are deposited into the periplasm would seem to be a better system to capitalize on in combating acid via a proton-repelling mechanism. Thus, two possible mechanisms for combating acid via H_2 oxidation, one involving electron accumulation internally, and the other involving proton deposition periplasmically, are proposed for further investigation.

It has been suggested that proton concentrations inside the cell increase 1000-fold in response to a shift in external pH to ~2.5 (internal pH shift of 7.5–4.5) (Richard & Foster, 2004). It is likely that the *hya* strain dies in acid because it cannot attain a negatively charged cytoplasm when needed or because it cannot repel sufficient protons. Due to the sidedness of the H_2 oxidation reactions, protons would deposit in the periplasm, and thus not contribute to further cytoplasmic proton overload (Lengeler *et al.*, 1999). The proton deposition at the *Shigella* periplasm could be very robust: according to our H_2 uptake activity measurements (from the mutant strain unable to produce H_2 , but fully capable of using H_2), upon exogenous H_2 addition, the number of protons produced from H_2 activation could reach more than 2.4×10^8 protons per minute per cell. To put this in perspective, to change the pH within a single *E. coli* cell from pH 4.5 to 7.5 requires a net loss of 10 000 protons from the cytoplasm (Richard & Foster, 2004). In the absence of a terminal acceptor, the Hya enzyme would provide a build-up of electrons and reduced carriers on the cytoplasmic side; we propose that this is beneficial to combat proton stress and aid in a reversal of the transmembrane potential. At the same time, H_2 oxidation via Hya causing abundant proton accumulation in the periplasm would be expected to repel proton influx across the outer membrane.

ACKNOWLEDGEMENTS

This work was supported by the USA National Institutes of Health (grant 1R21AI073322). We are grateful to Dr Andrea Kwiatkowski, Young Harris College, Young Harris, GA, USA, for constructing ALZ44 and ALZ47. We also thank Dr Reena Lamichhane-Khadka

(Department of Microbiology) and Dr John Shields (Center for Advanced Ultrastructural Research), University of Georgia, Athens, for technical help with fluorescence imaging.

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Edited by: F. Sargent