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

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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 hydrogenylase 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...
The ability of *S. flexneri* to express stationary phase acidresistance (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 (*hya*), ALZ47 (*athyb*) and MMM01 (*hya*). The double mutants are designated MMM02 (*gadBC* and MMM03 (*hya*). 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 ML-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-verifed 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

<table>
<thead>
<tr>
<th>Strain, construct or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
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<tr>
<td><em>S. flexneri</em> 2a</td>
<td>2457T wt</td>
<td>ATCC 700930</td>
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<tr>
<td><strong>Mutant constructs</strong></td>
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<tr>
<td>Δhya::FRT ALZ44</td>
<td><em>hya</em> deletion with FRT site (S1040–S1041)</td>
<td>This study</td>
</tr>
<tr>
<td>Δhyb::FRT ALZ47</td>
<td><em>hyb</em> deletion with FRT site (S3242–S3244)</td>
<td>This study</td>
</tr>
<tr>
<td>Δhyc::FRT MMM01</td>
<td><em>hyc</em> deletion with FRT site (S2930–S2931)</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgadBC::FRT MMM02</td>
<td>Deletion of AR2 Glu/GABA antiporter and Glu decarboxylase with FRT site (S1867–S1868)</td>
<td>This study</td>
</tr>
<tr>
<td>Δhya::FRT ΔgadBC::FRT MMM03</td>
<td>Deletion of <em>hya</em>, Glu/GABA antiporter, and Glu decarboxylase with FRT sites</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pKD46</td>
<td>λ-red recombinase, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
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</table>
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 (Mclvaine), 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 recorded 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 OD$_{600}$. Oxyrase-only controls oxidized a negligible amount of H$_2$. 

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 µg ml$^{-1}$ final concentration) for 28 min. Then 40 µM 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 of the hya mutant strain had 200 nmoles of H₂ per minute per 10⁹ cells. This means more than 2.4 × 10⁸ 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, 2007).

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 × 10⁸ H⁺ ions are produced per cell per minute.
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 ± sd determined from four to six replicate recordings of H₂ level changes in a 1 min period.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific activity after indicated time period in acid (pH 2.5) [nmol H₂ min⁻¹ (10⁹ cells)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>wt</td>
<td>150 ± 37 H₂ uptake</td>
</tr>
<tr>
<td>hya</td>
<td>51 ± 19 H₂ evolution</td>
</tr>
<tr>
<td>hyb</td>
<td>113 ± 31 H₂ uptake</td>
</tr>
<tr>
<td>hyc</td>
<td>276 ± 66 H₂ uptake</td>
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</table>

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...
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 14C-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. [14C]Glutamate was added to Shigella (wt and hya) at various growth phases in an H2-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 on 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 $3.5 \times 10^6$ (zero time point), $1.0 \times 10^4$ 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
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.

**Δψ in wt and hya strains**

Based on the results from the hydrogenase assays, and the predicted roles of membrane-bound hydrogenases, the electrical component (Δψ) of the PMF was assayed in the wt and hya strains. The fluorescent dye JC-1 was used to determine Δψ, a critical component of PMF. Green fluorescence indicates that the cells have a lower Δψ (see Fig S1). Wt S. flexneri had a green to red ratio of 0.064 (see Fig. 5). The hya strain had a lower Δψ, with green/red ratio of 0.876. Four independent experiments were performed and they all yielded a significantly higher Δψ 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 (hya) 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 H₂ 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 Δψ, 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 × 10⁶ 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.

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