***Shigella* and *Escherichia coli* Strategies for Survival at Low pH**

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INTRODUCTION

A fascinating aspect of *Shigella* pathogenesis is the small inoculum required to cause shigellosis. DuPont *et. al.* (5) summarized data from volunteer studies in which subjects were administered *Shigella* species in milk. The results of these studies showed that 500 *S. flexneri*, *S. dysenteriae*, or *S. sonnei* were sufficient to cause shigellosis and in some cases ingestion of as few as 10 organisms resulted in disease. This is an infective dose far lower than that reported in similar studies for most other enteric pathogens. The parameters involved in determining the infective dose of a pathogen are likely to be numerous. The route of infection, the specific virulence determinants encoded by the pathogen, the ability of the organism to avoid host immune defenses as well as the innate resistance of the host would all be expected to play a role. In general however, diarrheal disease is associated with a high infective dose for most pathogens, presumably because the low pH of the stomach provides a major barrier to intestinal colonization. Although a good deal of evidence supports the concept of a gastric barrier to intestinal colonization (4, 9, 11, 15), the mechanisms by which enteric pathogens survive exposure to pH have only been addressed in detail in the last few years. To begin dissecting determinants of low infective dose in shigellosis *in vitro* assays have been used to dissect the genetics and bacterial physiology of survival at low pH.

Most enteric bacteria are neutrophiles able to grow only within a fairly narrow pH range between 5.5-and 8.0. The pH of the intestines generally falls within this range and thus provides a good habitat for neutrophiles such as *Shigella* species, *Salmonella* species and *Escherichia coli*. Very few of the large and diverse population of oral bacteria reach the intestines despite the fact that they are being constantly swallowed (4, 9, 11) It is

becoming increasingly clear, however, that enteric bacteria can survive exposure to pH much lower than that required for growth. An excellent review by John Foster on the ability of enteric bacteria to withstand acid stress has appeared in the past few years (2). Much of this work has come from the Foster laboratory and has focused on the acid tolerance response (ATR) of *Salmonella typhimurium* (2, 13, 16). The ATR system is defined by testing exponentially growing bacteria in minimal media, and requires prior exposure to moderately low pH for full expression. Even under ideal *in vitro* conditions, however, *Salmonella* species do not survive below pH 3.0 (13). In contrast, *Escherichia coli* and *Shigella* species are able to survive exposure to pH as low as 2.0 for 2 hours (13, 18, 21, 22) without pre-exposure to acid. This data suggests that these organisms have unique mechanisms for protection at extremely low pH.

The genetics and physiology of survival at low pH

Survival at extremely low or high pH requires that organisms maintain a cytoplasmic pH (pH_i) compatible with normal enzyme function. For neutrophiles this pH is usually no lower than pH 5.0. Exposure to acid also damages many cellular structures which presumably must be protected or repaired in order for bacteria to recover following acid shock. Although a number of proteins are induced by exposure to low pH, little is known about the function of specific acid shock proteins. On the other hand, a good deal is now known about some of the mechanisms by which bacteria maintain a normal pH_i in the face of low external pH (2). A basic strategy used by *S. typhimurium*, *E. coli* and *Shigella* species is to neutralize incoming H^+ by coupling H^+ transport with amino acid transport. In these systems, the decarboxylation of an amino acid in the cytoplasm is coupled to the formation of a product in which H^+ is consumed. The resulting product is then exported from the cell via a specific antiporter and exchanged for the incoming amino acid (Fig. 1). A number of decarboxylation systems exist, each of which has a specific amino acid substrate.

Acid resistance in *E. coli*

E. coli has several systems for protecting organisms against acid stress (2, 20). Proteins involved in these systems include lysine decarboxylase (encoded by *cadA*), arginine decarboxylase (encoded by *adi*) and ornithine decarboxylase (*speC*). In addition *E. coli* has a glutamate decarboxylase encoded by duplicate genes, *gadAB* which share 98% DNA homology but which map to separated regions of the chromosome (19). The decarboxylases listed above encode biodegradative enzymes which are

induced at low pH. Under conditions of fermentative growth at low pH in the presence of the required amino acid, these proteins may make up as much as 12% of *E. coli*'s total cellular protein (20). In addition, separate biosynthetic enzymes exist for arginine and ornithine decarboxylases in many isolates of *E. coli*. These are constitutively expressed at low levels and are less likely to play a significant role in protection from low pH (20). The striking redundancy in *E. coli* for systems which protect against low pH suggests that this phenotype is an important one.

The expression of amino acid decarboxylases is highly regulated. Most of these systems are induced at low pH and stationary growth phase. The genetic determinants underlying regulation are not fully known in most cases. However, the expression of glutamate decarboxylase (*gadA/B*) as well as expression of *gadC* which encodes a putative glutamate/ γ -amino butyrate antiporter is at least partially dependent on the stationary phase sigma factor, σ^S (17, 21). In *E. coli*, expression of arginine decarboxylase (*adi*) appears to be dependent on anaerobic growth as well as acid induction though neither the genetics nor physiology of this system is well understood (13).

The systems described above are expressed preferentially during fermentative growth and require the presence of specific amino acids. Thus they are not operative in minimal media. In addition to the decarboxylase systems, an acid tolerance response (ATR) has been reported for *E. coli* which functions during exponential growth phase under oxidative growth conditions. Expression of this phenotype requires prior exposure to acid and thus is similar to the ATR response described for *S. typhimurium* (2). Although the phenotype has been characterized, the genetic basis for this system is unknown.

In summary, *E. coli*, like *S. typhimurium* has a number of systems for protection against acid stress and the vast majority of clinical isolates of *E. coli* tested display a high level of acid resistance (3, 10, 21). Although not every isolate will contain all of the decarboxylase systems, the vast majority of fresh clinical isolates of *E. coli* express lysine and glutamine decarboxylases. One notable exception to the above is enteroinvasive *E. coli* which lack lysine decarboxylase activity. Finally over 50% of clinical *E. coli* isolates contain ornithine decarboxylase activity (12). Since this data comes from clinical laboratory testing which is based on phenotypic rather than genotypic assessment, no distinction is made between biosynthetic and biodegradative enzymes. However, in most cases overnight cultures of bacteria are tested. These conditions would favor expression of the induced form of the enzyme which is present in much higher amounts

than the biosynthetic form. It should be noted, however, that many laboratory strains of *E. coli* K-12 as well as the *E. coli* B/K-12 hybrid, are extremely acid sensitive. In cases where this has been investigated, acid sensitivity has usually resulted from a lesion in σ^S (22). Presumably laboratory maintenance does not require either the ability to withstand low pH and/or other stresses against which possession of σ^S protects.

Genetics and physiology of acid resistance in *Shigella* species.

Early investigators seeking to understand the basis of the extremely low infective dose for shigellosis attempted to correlate *in vitro* acid sensitivity with infective dose (6, 8, 15). The results of these studies were inconsistent. When bacteria were tested for acid resistance in media such as milk, Luria broth, open well water, or gastric juice, *Shigella* survived exposure to low pH better than other pathogens tested such as *Salmonella* species. However, when bacteria were tested in sterile water or phosphate-buffered saline, little difference in acid resistance was found between different species. Further, under these conditions, the level of acid resistance expressed was extremely low. This suggested that some component in the assay media might be required for acid resistance. This is consistent with the presence of an amino acid decarboxylase system. However lysine decarboxylase activity is conspicuously lacking in *Shigella* species, and all species except for *S. sonnei* lack ornithine decarboxylase. The prevalence of arginine decarboxylase in *Shigella* species is unknown though its existence has been demonstrated in some isolates. Thus *Shigella* lacks many of the genes utilized by *E. coli* to withstand acid shock and yet is equally able to survive exposure to very low pH.

Early work from our laboratory showed that *Shigella* species were resistant to pH as low as 2.0 and that this phenotype required expression of the stationary sigma factor σ^S (10, 18). Attempts to find σ^S -regulated genes involved in acid resistance using transposon mutagenesis led to the identification of *gadC*, which encodes a putative antiporter for a glutamate decarboxylase system, and *hdeA*, which encode two small periplasmic proteins of unknown function (22). Insertions in *gadC* and *hdeA* rendered *S. flexneri* acid sensitive and this sensitivity could be complemented by the cloned genes. Although *Shigella* species like *E. coli* are normally very sensitive to low pH when grown in minimal media, addition of glutamate to minimal media increases survival 10,000 fold. Addition of sodium chloride along with glutamate increased acid resistance by an additional 60% compared to that of glutamate alone (22). The gene encoding GadC lies upstream of *gadB*, one of two genes encoding glutamate decarboxylase. Data from translated sequence suggests that *gadC* encodes an antiporter

with significant homology to CadB, the lysine antiporter found in *E. coli*. The model proposed for the mechanism by which the glutamate decarboxylase system protects *Shigella* from low pH is entirely homologous to that for lysine decarboxylase. In the presence of low external pH, glutamate and possibly H^+ is transported into the cell via GadC and decarboxylated by the *gadA/B* gene product to form γ -amino-butyrate which is then exported via GadC. H^+ is removed from the cytoplasmic environment by this reaction and internal pH is stabilized. Although expression of *gadC* and *gadB* is completely dependent on σ^S , there is much about this system that remains unknown. Preliminary evidence suggests that the circuit from σ^S to *gadB* and *gadC* involves the participation of other genes. Whether the *hdeA/B* locus is part of this pathway is unknown. Like *gadB* and *gadC*, *hdeA* expression is σ^S -dependent. However, in the absence of H-NS, the *hde* operon is transcribed by σ^{70} . Thus this operon is negatively regulated by the nucleoid protein H-NS (22).

Distribution of amino acid decarboxylases in nature

The amino acid decarboxylases are widely distributed in procaryotes and eucaryotes (see Table). Therefore it is surprising to see that glutamate decarboxylases, though common in eucaryotes, appear to be uncommon

Table: Inducible Degradative Amino Acid Decarboxylases in Bacteria

	arg	glu	lys	orn
<i>E. coli</i> (12, 22)	+	+	+	+
enteroinvasive <i>E. coli</i>	?	+	—	+
<i>Shigella</i> (12, 22)				
<i>S. flexneri</i>	?	+	—	—
<i>S. dysenteriae</i>	?	+	—	—
<i>S. boydii</i>	?	+	—	—
<i>S. sonnei</i>	?	+	—	+
<i>Salmonella enteritidis</i> (2)	+	—	+	+
<i>Vibrio cholerae</i> (12)	?	—	+	+
<i>Pseudomonas species</i> (12)	+	—	+	+
<i>Clostridium perfringens</i> (1)	?	+	—	?
<i>Lactobacillus lactis</i> (17)	?	+	?	?
<i>Bacteroides fragilis</i> (1)	?	+	?	?
<i>Eubacterium limosum</i> (1)	?	+	?	?

arg=arginine decarboxylase/hydrolase, glu=glutamic acid decarboxylase,
lys=lysine decarboxylase, orn=ornithine decarboxylase

among procaryotes (7,14). Not only are *gadBC* lacking in enterobacteriaceae other than *Shigella* species and *E. coli*, they are also absent from other gram negative bacteria assayed such as *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* (14). Until very recently, *gadA/B* and *gadC* had only been identified in *Shigella* species and *E. coli*. However, a glutamate decarboxylase enzyme had been purified from *Clostridium perfringens* (7). In addition, glutamate decarboxylase activity has been detected in a number intestinal anaerobes such as *Bacteroides fragilis*, *Eubacterium limosum*, and *Clostridium barati* (1). In these cases, however, evidence rests solely on biochemical enzymatic activity and it is impossible to say whether the genes found in *E. coli* and *Shigella* species are involved in the phenotype described.

Very recently, a *gadCB* operon has been described in *Lactobacillus lactis* in a paper by Sanders *et al* (17). In *L. lactis*, *gadB* and *gadC* share 43% and 51% identity with the homologous genes in *S. flexneri*. As in *S. flexneri* and *E. coli*, *gadCB* is preferentially expressed by resting cells and confers resistance to low pH. Glutamate-dependent acid resistance in *L. lactis* is increased by pre-exposure to low pH. Although no information concerning the presence of σ^S in *L. lactis* is presented, an interesting finding by this group is the fact that expression of *gadCB* is positively regulated by a chloride-dependent activator *gadR*. As mentioned previously, the addition of NaCl along with glutamate results in further enhancement of acid resistance in *S. flexneri*. We originally postulated that this increase in acid resistance was due to the sodium ion. In light of the findings of Sanders *et al.* on the role of chloride in induction of the *gad* operon in *L. lactis*, it will be worth investigating whether chloride might play a role in *gadC* expression in *E. coli* and *Shigella* species. The possibility of chloride dependent regulation is interesting because of the high chloride content of gastric secretions. Since *gadR* is not present in *E. coli* the genetics of the *Shigella-E. coli* system would be different from that in *L. lactis*.

Habitat and Acid stress

The habitats occupied by *E. coli*, *Salmonella* species, and *Shigella* species overlap considerably; All may be isolated from the human intestines. However, there are also significant differences in the environmental niches exploited by these genera. *E. coli* and *Salmonella* species occupy diverse habitats including the intestines of mammals, birds, amphibians and reptiles. Although access to some of these habitats involves exposure to pH below 3.0, even among mammals this is not the rule. For example, the gastric pH of ruminants is between 4.0 and 7.0. The pH of the

chicken proventriculus and crop (the avian stomach-equivalent) is 4.5. Compared with *E. coli* and especially *Salmonella* species, *Shigella* species appear to occupy a very narrow niche, the primate intestine. Access to this environment involves exposure to very low pH.

The distribution of the degradative decarboxylases for lysine, arginine and ornithine is widespread among gram negative bacteria (12). Among the Enterobacteriaceae they can be found in *Serratia*, *Edwardsiella*, *Enterobacter*, and *Proteus* species. They are also found in nonfermentative gram negative bacteria such as *Pseudomonas*. This suggests that these enzymes may have particularly utility in specific environments encountered by the bacterium. *E. coli* and *Salmonella enteritidis* both possess a number of systems which protect against exposure to low pH, but most of these provide protection to only moderately low pH. *S. typhimurium* for example contains at least 4 different systems for protection against acid stress. Although *S. typhimurium* is far better able to grow at moderately low pH than *Shigella* species (2), it cannot survive below pH 3.0 (2, 13). In sharp contrast to this, *S. flexneri* contains only the glutamate-dependent acid resistance system encoded by *gadBC*. The fact that *gadA/B* is one of the few examples of duplicated structural genes in *E. coli* and *Shigella*, along with the fact that this system appears to be sufficient for *Shigella* species to colonize the intestinal tract is remarkable. Equally surprising is the absence of these genes in other closely related gram negative bacteria, along with their presence in gram positive and anaerobic normal intestinal flora. This suggests that the glutamate dependent acid resistance system may be uniquely valuable to organisms which colonize the human intestines.

An examination of the distribution of the glutamate decarboxylase system raises a number of interesting questions. Is the *gad* operon limited to bacteria which are found as normal flora in the human intestines? If so what are its primary functions? Sanders *et. al.* has suggested that the *gad* operon in *Lactobacillus lactis* may serve a metabolic function by generating proton motive force. Since the gut environment is anaerobic, facultative anaerobes must generate energy through fermentative processes. It is possible that the glutamate system functions as an additional source of energy for these organisms. *E. coli* and *Shigella* may have acquired and maintained this system because of the advantage it confers to growth in the intestines as well as advantage it may confer for passage through the stomach.

Finally, the data presented above rests on the use of *in vitro* assays for acid resistance. Whether this data accurately reflects *in vivo* acid resistance remains to be proven. We have constructed deletion mutants lacking σ^S

and *gadC* in *S. flexneri*. Until these strains are tested in a primate infection model, the relationship between infective dose and *in vitro* acid resistance will remain an intriguing hypothesis.

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