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Acid Resistance in Enteric Bacteria

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***Shigella* species require a uniquely small inoculum for causing dysentery. One explanation for the low infective dose is that *Shigella* species are better able to survive the acidic conditions encountered in the stomach than are other enteric pathogens. We have tested *Shigella* species, *Escherichia coli*, and *Salmonella* species for the ability to survive at pH 2.5 for at least 2 h. Most isolates of *Shigella* and *E. coli* survived this treatment, whereas none of the *Salmonella* isolates were able to do so. The ability of *Shigella* species to survive at low pHs does not require the presence of the large virulence plasmid or growth at 37°C but is strikingly dependent on growth phase. We have also found that *Shigella* isolates exposed to acid lose the ability to invade epithelial cells.**

Enteric pathogens share an oral route of infection. However, *Shigella* species have a uniquely low infective dose. Human volunteer studies show that 10 to 500 shigellae are sufficient to cause dysentery in healthy adults (10). In contrast, in similar studies the infective dose of *Vibrio cholerae* is 10^8 organisms (5) and that of *Salmonella* species is 10^5 to 10^{10} organisms (1, 18). Epidemiological studies support a low infective dose for shigellosis. Although foodborne infection occurs, person-to-person transmission is the predominant mode of transmission (10, 15, 21). Outbreaks of salmonellosis are most often associated with ingestion of common food in which bacteria have undergone extensive replication before ingestion, though some epidemiological data estimate a lower infective dose for *Salmonella typhi* than that found in volunteer studies (1).

Early studies described the sterilizing role of low gastric pH in establishing a gastric barrier to infection (6, 13). In experiments with human volunteers, the infective dose of *V. cholerae* was lowered from 10^8 to 10^4 organisms by administering the inoculum with sodium bicarbonate (5), intestinal colonization of oral vaccine strains was markedly enhanced by feeding strains with sodium bicarbonate (9, 17, 23), and epidemiological data reveal an association between achlorhydria and salmonellosis (6). Such evidence points to a major role for low gastric pH in determining the infective dose. If so, one would expect *Shigella* species to be more acid resistant than other enteric pathogens. Studies on the acid sensitivity of enteric pathogens yield conflicting results, possibly because of differences in assay conditions and strains used (11, 11a, 12, 14, 27).

We have studied the ability of *Escherichia coli*, *Shigella* species, and *Salmonella* species to survive exposure to acid. Bacteria were exposed to Luria-Bertani (LB) broth (25) which was acidified with HCl. Assay conditions were based on those of the normal fasting stomach, i.e., a pH less than 3.0 and a gastric emptying time of less than 2 h (6, 13). Acid resistance was defined as the percent survival of an inoculum exposed to pH 2.5 for 2 h. Strains in which ≥10% of the inoculum survived after exposure to pH 2.5 for 2 h were considered acid resistant. In most cases, acid-sensitive isolates exhibited less than 0.001% survival after exposure to acid. Data from this study are shown in Table 1. Nine of twelve (75%) isolates of *Shigella* species tested were acid resistant (range, 13 to 102% survival; average, 55%), as were many *E. coli* isolates. Eight of ten freshly isolated normal-flora *E. coli* strains tested (80%) were acid resistant (range, 25 to 113% survival; average, 50%). Among laboratory strains of *E. coli* K-12 tested, only HB101 and RR1 were acid sensitive. Six of nine (66%) isolates of enteroinvasive *E. coli* (EIEC) were acid resistant (range, 17 to 70% survival; average, 32%). In contrast to *Shigella* species and *E. coli*, all *Salmonella* species tested were extremely acid sensitive. Less than 0.001% survival was found for all *Salmonella* isolates tested. Isolates of *Salmonella* were acid sensitive when tested at pH 3.0 as well (data not shown).

The ability of *Shigella flexneri* to survive low pH was dependent on time, pH, and growth phase but not on growth at 37°C or on the presence of the large *Shigella* virulence plasmid. At pH 2.0, 14% of the inoculum survived after 2 h; by 4 h, no survivors were found. At pH 2.5, 44% of the inoculum survived after 2 h; by 5 h, 6% of the inoculum could still be recovered (Fig. 1). It is unlikely that these bacteria were replicating, since the pH of the assay medium did not change by more than 0.05 during any experiment. When low-pH survivors were retested, they exhibited survival kinetics similar to that of the parental strain (data not shown), suggesting that survival at low pH is a stochastic characteristic of the population rather than a result of the presence of genetically altered variants.

Acid resistance was strikingly dependent on growth phase. *S. flexneri* did not become acid resistant until late exponential growth (Fig. 2). When an overnight culture of bacteria was diluted 1:1,000 in LB broth, 5 h of growth was required for expression of acid resistance. Many virulence genes in *Shigella* species and *E. coli* are regulated by the chromosomal locus *virR*. These genes are expressed at 37°C but not at 28°C (19). However, *S. flexneri* was acid resistant whether cultured at 37°C (82.3% survival) or 28°C (86.6% survival). Although acid resistance was enhanced by the presence of the virulence plasmid, the plasmid was not required in either *S. flexneri* (Fig. 3) or *Shigella sonnei* (data not shown). In addition to carrying essential virulence genes, the *S. sonnei* virulence plasmid encodes a species-specific O antigen. The fact that *S. sonnei* strains lacking this plasmid are acid resistant provides evidence that O antigen is not required for acid resistance.

Invasion of colonic cells is a prerequisite for *Shigella* virulence. Using a tissue culture assay (31), we examined the ability of acid-exposed *S. flexneri* to invade HEp-2 cells. *S.*

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TABLE 1. Survival of enteric bacteria exposed to pH 2.5 for 2 h

Isolate	% Survival[a]	Source or reference
Shigella species		
S. flexneri 1a-625	25	CA State Health Dept.[b]
S. flexneri 602	66	CA State Health Dept.
S. flexneri 2a 3136	93	CA State Health Dept.
S. flexneri 2a 700	20	CA State Health Dept.
S. flexneri M25-8	102	16
S. flexneri 4a	43	CA State Health Dept.
S. sonnei 3421	51	CA State Health Dept.
S. sonnei 482-79	83	16
S. boydii 14	80	CA State Health Dept.
S. boydii 16	3	CA State Health Dept.
S. dysenteriae 12-31	13	CA State Health Dept.
S. dysenteriae 1a-31	<0.01	CA State Health Dept.
Salmonella species		
S. typhimurium 79-80	<0.001	CA State Health Dept.
S. typhimurium 80-85	<0.001	CA State Health Dept.
S. typhimurium 3399	<0.001	CA State Health Dept.
S. typhimurium 3428	<0.001	CA State Health Dept.
S. choleraesuis 1101	<0.001	CA State Health Dept.
S. choleraesuis 2629	<0.001	CA State Health Dept.
S. entertidis 3407	<0.001	CA State Health Dept.
S. enteritidis 3419	<0.001	CA State Health Dept.
S. enteritidis 3404	<0.001	CA State Health Dept.
S. pullorum 159	<0.001	CA State Health Dept.
S. typhi 1231	<0.001	CA State Health Dept.
S. typhi 1265	<0.001	CA State Health Dept.
E. coli		
K-12 and normal-flora isolates		
HB101	<0.001	29
RR1	<0.001	29
LE 392	26	29
MC1000	26	29
DH5α	26	29
JSG	25	This work
JM2	<0.001	This work
PLCS	25	This work
DR	<0.001	This work
JS	48	This work
JB	77	This work
CL	29	This work
CF	26	This work
PP	113	This work
SF	62	This work
Enteroinvasive		
5	<0.001	Stanford Medical School[c]
11	<0.001	30
12	<0.001	CDC[d]
949	70	Walter Reed Institute[e]
164	32	Walter Reed Institute
LE32B	18	Walter Reed Institute
1150	17	Walter Reed Institute
711	20	Walter Reed Institute
Enteropathogenic		
E2348-69	13	20
B171	<0.001	28

[a] Values are averages for duplicate samples from a typical experiment. Duplicates varied from one another by less than 10%. All *Shigella* isolates were *inv*$^+$.

[b] Sharon Abbot, California State Department of Health, Berkeley.

[c] Peter O'Hanley, Stanford Medical School, Stanford, Calif.

[d] CDC, Centers for Disease Control, Atlanta, Ga.

[e] David Taylor, Department of Enteric Disease, Walter Reed Army Institute for Research, Washington, D.C.

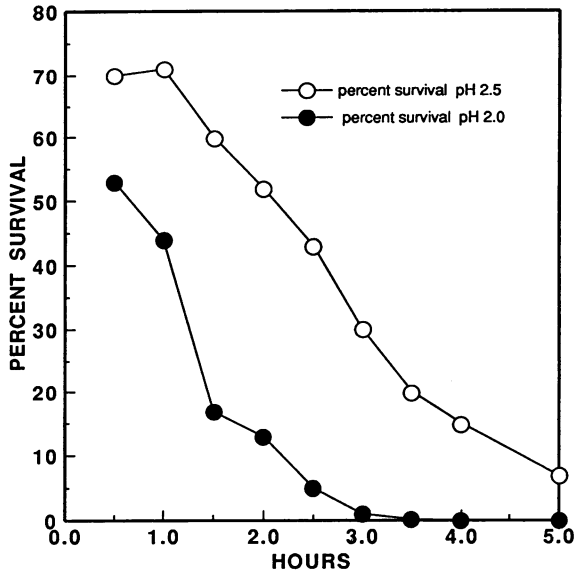

FIG. 1. Kinetics of acid resistance. Cells (10^4) from an overnight culture of *S. flexneri* 3136 were added to LB broth which had been acidified with HCl to either pH 2.0 or pH 2.5. Viable counts were obtained at 30-min intervals, and the percent survival of the initial inoculum was determined.

flexneri exposed to pH 3.0 for 2 h lost the ability to invade cells. Less than 0.001% of an inoculum of acid-treated bacteria was recovered from a gentamicin-treated, lysed monolayer of HEp-2 cells after an invasion assay, compared with recovery of 15% of an inoculum of an untreated *S. flexneri* control. Growth for 4 h at pH 7.0 restored the ability to invade cells.

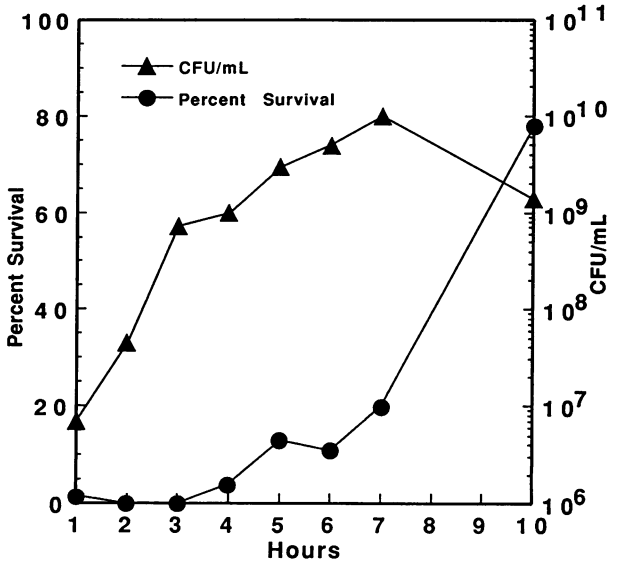

FIG. 2. Growth phase induction of acid resistance. Cells from an overnight culture of *S. flexneri* 3136 were diluted 1:1,000 and grown with shaking at 37°C. Aliquots were withdrawn over a 12-h period and assayed for the ability to survive in LB broth at pH 2.5 for 2 h. Viable counts from the test inoculum were compared with those obtained after incubation in acidified LB broth to determine percent survival.

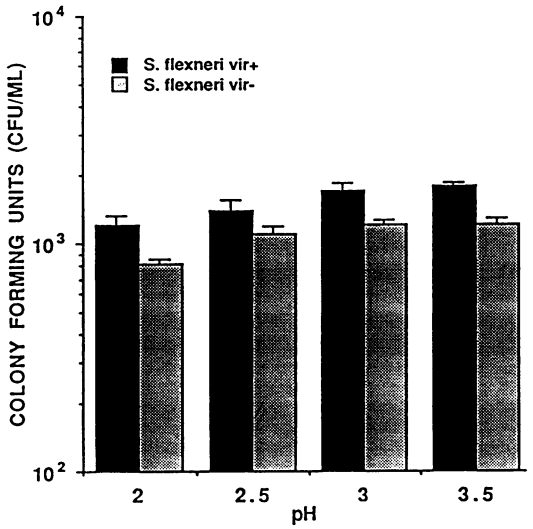

FIG. 3. Acid resistance of *S. flexneri* with (vir+) and without (vir−) the large virulence plasmid. Cells (10^4) from overnight cultures of an isogenic pair of plasmid-containing and plasmid-cured derivatives of *S. flexneri* were exposed to acidified LB broth for 3 h and viable counts were determined. Values represent the means and standard errors for triplicate samples.

How is such a small number of shigellae able to cause dysentery? The fact that *Shigella* species are facultative intracellular parasites capable of extensive intracellular replication is significant. However, although all *Shigella* species share a low infective dose, they differ considerably in the ability to enter eucaryotic cells (26). Recent work suggests that a low infective dose for enteric pathogens can be due to colonization of the oral pharyngeal cavity (2). However, three of four *Shigella* species are nonpiliated (7), no other adhesins have been identified, and a pharyngeal prodrome is absent in shigellosis. We have shown that shigellae are able to survive low pH for several hours; this ability may be a major determinant of infective dose.

Rather than being a unique *Shigella* trait, acid resistance is probably a characteristic that *Shigella* species possess because they are essentially *E. coli*. The fact that modern taxonomic criteria indicate that *Shigella* and *Escherichia* are actually the same genus (4) makes this less surprising. Acid resistance may be an adaptation *E. coli* has made as it became successfully established as normal flora in the mammalian gut. One can speculate that *Salmonella* species are more acid sensitive because they are less specifically adapted to this habitat. Although EIEC and *Shigella* species have identical virulence determinants and cause clinically indistinguishable disease (15, 33), the infective dose for EIEC has been reported to be 1,000-fold higher (8). Recent EIEC isolates from Thailand that we have tested are acid resistant (Table 1). It would be interesting to know the infective doses of these isolates. Acid resistance in *Shigella* species is not fully expressed until the stationary phase. The entrance of *E. coli* into stationary phase is accompanied by major physiological changes mediated by a global regulatory system in which a putative stationary-phase sigma factor encoded by *rpo*^{S}, formerly designated *katF*, plays a key role (3, 22, 24). We have evidence that acid resistance in *Shigella* species is similarly regulated (30).

The fact that acid-treated shigellae are noninvasive may be relevant to a distinctive feature of shigella pathology—the fact that the ileum is spared whereas the colon is filled with intracellular shigellae (32). This is particularly interesting because in vitro shigellae enter and replicate within most cell lines (26). On the basis of our studies, we propose the following model for *Shigella* infection. After shigellae leave the colon, they enter the stationary phase in response to the less favorable environment outside the host. They are thus primed for ingestion and survival through the stomach. The high percentage of organisms surviving transit through the stomach translates into a small inoculum for disease. Research into the genetic basis of acid resistance may be relevant to the development of oral vaccine strains. The identification of genetic determinants for acid resistance may lead to the construction of acid-resistant *Salmonella* and *Shigella* vaccine strains which would be effective at low dosages and would not require encapsulation or administration of bicarbonate to ensure passage through the stomach.

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