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Role of Acid Tolerance Response Genes in *Salmonella typhimurium* Virulence

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The *atp* and *fur* genes are involved in the acid tolerance response of *Salmonella typhimurium*. An *atp*::Tn*10* mutant was avirulent in the mouse typhoid model when assayed by oral and intraperitoneal routes. However, a *fur* mutant was completely virulent by the intraperitoneal route. No relevant differences in intracellular survival or invasion rates were observed for the two mutants in macrophages and epithelial cells. These data indicate that separate acid tolerance response genes may have different roles in *S. typhimurium* virulence.

Enteric pathogens such as *Salmonella typhimurium* are exposed to variety of acidic environments within the host prior to infection of the intestinal epithelium (23). *S. typhimurium* is also an intracellular parasite that survives within macrophages and multiplies inside nonphagocytic cells (4, 6–8, 14, 17, 19, 20). Recent reports have described the existence of a global regulatory system in *S. typhimurium*, the acid tolerance response (ATR) (10–12), that provides protection from low pH. The physiological function of the ATR is correlated with the ability to overcome severe acid stress (an external pH less than 4.0) when previous exposure to a mildly acidic pH (an external pH between 5.5 and 6.0) has occurred (11). Several gene products belong to this system, including the Mg^{2+}-dependent proton-translocating ATPase, which is encoded by the *atp* (formerly *unc*) operon, and the Fe^{2+}-binding regulatory protein encoded by the *fur* gene (10, 12). Strains with mutations in these two genes are extremely sensitive to acidic pH and do not survive at pH 3.3, even if they have been previously adapted to a mildly acidic pH (10, 12). However, the role of these genes in virulence has not been established.

We used phage P22 transduction to move the *atp* and *fur* mutations of *S. typhimurium* SF342 (LT2 *atp-102*::Tn*10*) and JF2043 (LT2 *fur-1 zbf-5123*::Tn*10 iroA1*::MudJ) into the mouse-virulent strain SL1344 (18). The two new strains, PGP184 (SL1344 *atp-102*::Tn*10*) and FGP258 (SL1344 *fur-1 zbf-5123*::Tn*10*), were tested for acid sensitivity by the procedure of Foster and Hall (11). Briefly, bacteria were grown in minimal glucose medium (24) to a density of 10^8 cells per ml. The pH of the medium was adjusted to pH 5.8, and bacteria were permitted to adapt for one generation. Unadapted bacteria were grown to the same density at pH 7.7. Then the pH of both cultures was adjusted to pH 3.3, and viability was monitored over time. Both *atp* and *fur* mutants exhibited a significant decrease in viability (greater than 5 log units) when the cells were exposed to highly acidic conditions (pH 3.3) for 4 h (Fig. 1A). This acid sensitivity at 4 h was unchanged (5-log-unit decrease in viability) when the mutants were previously adapted to a mildly acidic pH (Fig. 1B), demonstrating that both mutant strains were unable to trigger the ATR. The *atp* mutant was more acid sensitive at short times of exposure when previously adapted at pH 5.8. This may indicate that *atp* mutants could be presensitized during adaptation either by increasing the proton potential leak or by producing an imbalance of the ATR that make the cells more susceptible to acid stress. The wild-type strain SL1344 retained 80 to 90% viability at pH 3.3 when it had been previously exposed to a mildly acidic pH (Fig. 1B). When the ATR of the virulent wild-type strain SL1344 was compared with the ATR described with the laboratory *S. typhimurium* wild-type strain LT2 (11, 12), it appeared that the virulent strain was more acid resistant. Nevertheless, our results clearly show that the *atp* and *fur* mutations also confer an acid-sensitive phenotype in a virulent genetic background.

We also confirmed the presence of the *atp* and *fur* mutations in strains PGP184 (*atp*) and FGP258 (*fur*) by two additional criteria: (i) as described for *atp* mutants (25), strain PGP184 (*atp*) was unable to grow on minimal medium plates with succinate or acetate as the only carbon source, and (ii) when we transduced an *iroA*::MudJ fusion (*iroA* is a gene repressed by *fur* in the presence of iron [13]) into strain FGP258 (*fur*), the new strain, FGP72.01 (SL1344 *iroA*::MudJ *fur-1 zbf-5123*::Tn*10*), turned red on MacConkey-lactose indicator plates either with iron (60 μM $FeCl_3$) or without iron (50 μM diethylenetriaminepentaacetic acid [DTPA], an iron chelator). Strain FGP72 (SL1344 *iroA*::MudJ) was white with iron (repressed) and red without iron (activated). These results are the same as previous data obtained with identical constructions in the LT2 genetic background (13).

We next determined whether the acid-sensitive phenotype of the *atp* and *fur* mutants altered virulence when tested in the mouse typhoid model. We infected susceptible BALB/c mice with different doses of *atp* and *fur* mutants and the parental strain by both oral and intraperitoneal (i.p.) routes (Table 1). PGP184 (*atp*) was avirulent by both oral and i.p. routes. For both the oral and i.p. routes, 50% lethal doses (LD_{50}s) were more than 3 log units greater than those of the parental strain, suggesting that the proton-translocating Mg^{2+}-dependent ATPase is required for *S. typhimurium* virulence.

In contrast to the highly attenuated virulence phenotype observed with the *atp* mutant, strain FGP258, containing the *fur* mutation, exhibited an LD_{50} almost 2 log units higher after oral administration, whereas no changes were detected in the LD_{50} after i.p. administration (Table 1). To determine whether a possible reversion of the *fur* phenotype could

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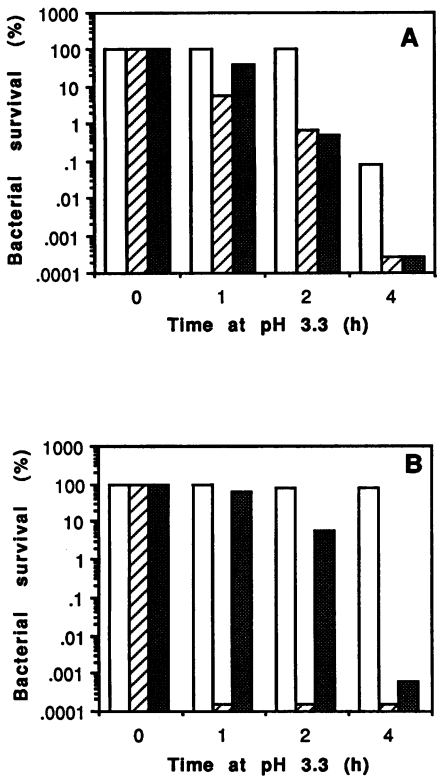

FIG. 1. Susceptibility of the *atp* (▨) and *fur* (■) mutants of *S. typhimurium* wild-type SL1344 (□) to low pH. Strains PGP184 (*atp-102*::Tn*10*) and FGP258 (*fur-1 zbf-5123*::Tn*10*) were grown in minimal E glucose medium, and the ATR was monitored as described in the text. (A) Unadapted cells grown at pH 7.7 and challenged at pH 3.3 for the times indicated. (B) Adapted cells grown at pH 7.7, adapted to pH 5.8 for one generation, and further challenged at pH 3.3 for the times indicated.

occur during infection, we tested eight individual isolates recovered from the livers and spleens of convalescent mice infected with the *fur* mutant for ATR. All eight isolates showed an ATR similar to that of FGP258, which was used to inoculate the mice (data not shown), confirming that the *fur* mutant that killed mice was still acid sensitive. We also used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to test the outer membrane proteins of these eight isolates grown in minimal M9 medium in the presence (100 μM $FeSO_4$) or absence (100 μM DTPA) of iron. All isolates exhibited the three characteristic outer membrane proteins (OM1, OM2, and OM3) which are induced by iron limitation (3) and constitutively expressed in *fur* mutants (5) (data not shown). These data indicate that no reversion occurred during infection. Since it has also been reported that enterobactin, another *fur*-regulated protein overproduced in *fur* mutants (13), is not a virulence factor in *S. typhimurium* (2), the attenuation observed with the orally administered *fur* mutant may indicate that any involvement of the *fur* gene in virulence is independent of enterobactin production.

To determine whether the avirulent phenotype of the *atp* and *fur* mutants administered by the oral route was due to a massive decrease in viability during the passage through the low pH in the stomach, we repeated the oral inoculation while using 10% (wt/vol) sodium bicarbonate to buffer the stomach pH prior to bacterial administration, following the protocol described by Galán and Curtiss (15). Our results show that this pretreatment has no effect on the LD_{50} of the orally administered *atp* mutant but produces a decrease of almost 1 log unit in the LD_{50} of the orally administered *fur* mutant (Table 1). These data suggest that buffering of the low pH of the stomach does not result in drastic changes in the LD_{50} of these two acid-sensitive mutants when administered orally, and even under these favorable conditions the wild-type LD_{50} cannot be restored. This observation is supported by the absence of bacterial death in both mutants when exposed to pH 3.3 for 1 h (Fig. 1A), a period that is probably sufficient for bacteria to pass through the stomach. Therefore, the stomach pH cannot be considered the major reason for attenuation of the virulence of orally administered *atp* and *fur* mutants.

We also infected susceptible BALB/c mice with 4.5×10^8 (oral) and 4.5×10^3 (i.p.) CFU of PGP184 (*atp*) and determined the infection kinetics. At various times the livers, spleens, and mesenteric lymph nodes were isolated and homogenized, and viable bacteria were enumerated. Few or no bacteria were detected in any organ of orally infected mice, even at 1 day postinfection (Fig. 2). However, when the *atp* mutant was administrated by the i.p. route, a large number of bacteria reached the liver, spleen, and mesenteric lymph nodes (Fig. 2). After a lag period of approximately 3 days, bacteria proliferated in the three organs, and after the first week, the number of viable bacteria decreased progressively in all three organs. The kinetics of the wild-type strain SL1344 showed a rapid targeting of bacteria to the organs after administration by both routes and an increase in

TABLE 1. Phenotype of the acid-sensitive *S. typhimurium* mutants PGP184 (*atp*) and FGP258 (*fur*)

Strain	LD_{50}[a] after administration route:			Invasion of epithelial cells[b]		Replication within epithelial cells[c]		Survival in macrophage[d]
	Oral	Oral (plus bicarbonate)	i.p.	MDCK	HeLa	MDCK	HeLa	
SL1344 (wt)[e]	10^6		10	1.01 ± 0.07	1.27 ± 0.35	12.1 ± 2.0	9.1 ± 0.8	3.1 ± 0.06
PGP184 (*atp*)	$>2 \times 10^9$[f]	$>4 \times 10^8$[f]	$>2 \times 10^4$[f]	1.26 ± 0.30	0.84 ± 0.15	24.7 ± 3.6	3.9 ± 0.4	0.93 ± 0.49
FGP258 (*fur*)	7.8×10^7	8×10^6	<50	0.25 ± 0.05	0.54 ± 0.15	13.1 ± 3.3	6.8 ± 0.2	2.82 ± 0.58

[a] Determined as described previously (22).
[b] Percentage of bacteria added that were resistant to treatment with 100 μg of gentamicin per ml after infecting HeLa cells (30 min) or MDCK cells (60 min). Values are the mean of two experiments with triplicate samples.
[c] Ratio of viable intracellular bacteria quantitated at 8 h and 2 h postinfection. Values are the mean of two experiments with triplicate samples.
[d] Ratio of viable intracellular bacteria at 16 h and 2 h postinfection. Values represent the mean of two experiments with duplicate samples.
[e] wt, wild type.
[f] Higher doses used, with 100% survival of infected mice.

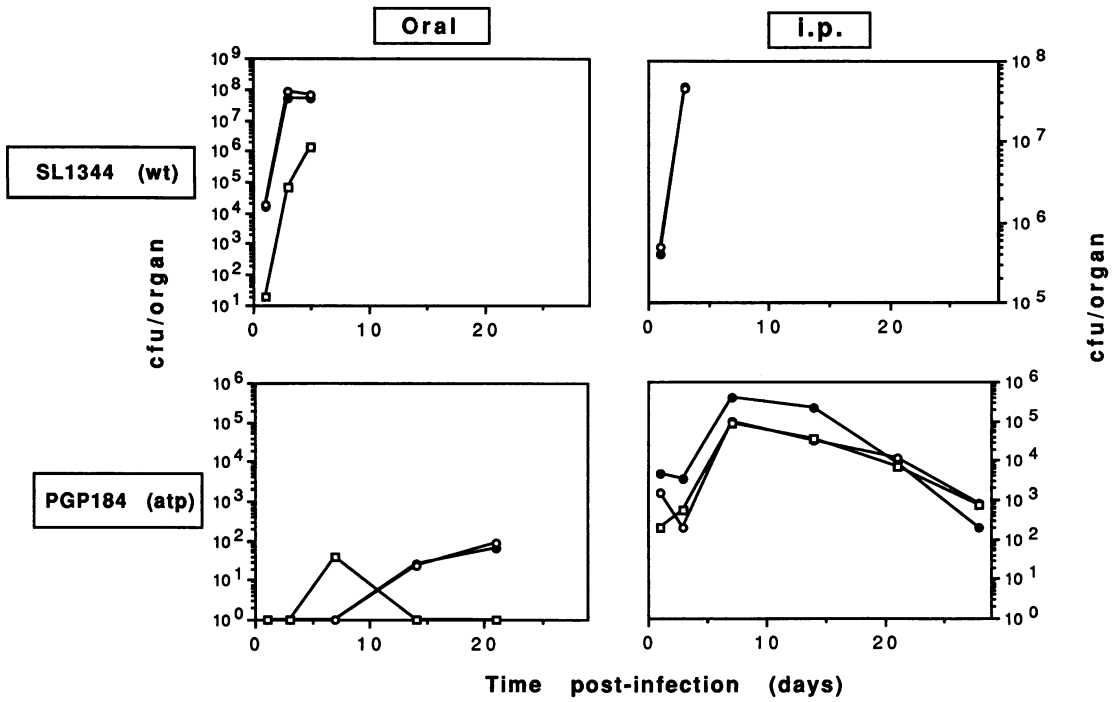

FIG. 2. Infection kinetics of *S. typhimurium* PGP184 (*atp-102*::Tn*10*) and SL1344 (wild type [wt]) in the livers (●), spleens (○), and lymph nodes (□) of mice after oral and i.p. administration. Each mouse received a dose of 4.5×10^8 CFU (oral route) or 4.4×10^3 CFU (i.p. route) of mutant PGP184 or 2×10^9 CFU (oral route) or 1.4×10^5 CFU (i.p. route) of strain SL1344 (19). The CFU present in the organs at different times were quantitated. Three mice were used at each of the different times shown.

bacterial numbers from day 1 postinfection with concomitant death of mice in 3 to 5 days (Fig. 2).

All these data suggest that (i) the *fur* mutant, although it has higher LD_{50}s than the wild-type strain after oral administration, is not as attenuated in virulence as are the *atp* mutant and other previously described acid-sensitive mutants such as *phoP*, which are defective for survival within macrophages (6, 20, 21), and (ii) the inability of the *atp* mutant to reach the liver, spleen, or lymph nodes after oral challenge of susceptible mice could be related to a deficiency in invasion of the intestinal epithelium.

To test the survival and the invasion and intracellular replication capacities of the *atp* and *fur* mutants, we used mouse resident peritoneal macrophages and two epithelial cell lines (MDCK and HeLa). These cells were infected with the mutant and wild-type strains for 20 min (macrophages), 30 min (HeLa epithelial cells), or 60 min (MDCK epithelial cells), and, after addition of 100 μg of gentamicin per ml, viable intracellular bacteria were quantitated at different times postinfection as described previously (9). The results obtained in this assay are summarized in Table 1. Bacterial viability after overnight exposure to the intracellular conditions of mouse peritoneal macrophages was monitored. After 16 h, the *fur* mutant increased by a factor of approximately 3.0 (similar to the parental strain) and the *atp* mutant remained viable during that time (ratio of viable bacteria, approximately 1.0). By using mouse peritoneal macrophages, it has been shown that *S. typhimurium* macrophage-sensitive mutants decrease in viability by approximately 3 log units after overnight exposure (4). These data suggest that neither *fur* nor *atp* mutants are defective for intracellular macrophage survival. Recent results have shown that the pH of the *S. typhimurium*-containing phagosome in cultured macrophages is close to pH 5.0 to 5.5 (1), values at which the ATR is induced (10–12). Our results support the concept that the ATR is not required for survival within macrophages.

We also screened *fur* and *atp* mutants for invasion of epithelial cells by using HeLa and MDCK epithelial cell lines. Bacteria were grown overnight in LB medium under low-oxygen conditions (without shaking). Our results showed no major differences for either mutant compared with the wild-type strain, with invasion rates of 70 to 120% for the *atp* mutant and 25 to 40% for the *fur* mutant (Table 1). Previously characterized invasion mutants of *S. typhimurium*, which show 3- to 4-log-unit decreases in invasion (16), have LD_{50}s close to 1 log unit higher than that of the wild-type strain (16). This discrepancy in invasion rate in vitro and virulence attenuation phenotypes between the two classes of mutants makes it unlikely that differences in invasion rates are the basis for oral attenuation of the *atp* and *fur* acid-sensitive mutants. We do not know at present why the *fur* mutant shows a small decrease in invasion levels of epithelial cells; however, considering that the *fur* mutation produces a drastic alteration of a large number of proteins (13), it may have some effect on a protein(s) involved in invasion. The intracellular replication data indicated that both acid-sensitive mutants are able to replicate within HeLa and MDCK cells, with growth rates greater than that of the wild-type strain (Table 1, *atp* mutant in MDCK cells).

The results of this study show that the ATR in *S. typhimurium* is not required to overcome low-pH stress in the stomachs of infected mice. The ATR regulon may be used by *S. typhimurium* in later steps of the infection either to pass through the intestinal epithelium or to disseminate from the intestinal epithelium to target organs. This latter concept is supported by the virulence of the *fur* mutant when adminis-

trated i.p. (Table 1) and the inability of the *atp* mutant to reach target organs when administered by the oral route (Fig. 2). The attenuation of the virulence of the *atp* mutant when administered by the i.p. route would imply an additional role of this gene in *Salmonella* virulence (Table 1; Fig. 2). We postulate that once bacteria are in the target organs and are growing, some changes in the nutritional source may occur and that, considering the inability of *atp* mutants to grow on dicarboxylic acids (25), bacteria cannot continue to grow.

In conclusion, our data show that the Mg^{2+}-dependent proton-translocating ATPase, encoded by the *atp* operon, is an essential virulence factor of *S. typhimurium*. The avirulence of the insertional *atp*::Tn*10* mutant used in this study was not totally correlated with that of another acid-sensitive mutant, the *fur* mutant, demonstrating that genes associated with the ATR can have different roles in *S. typhimurium* virulence.

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