

Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent

(intracellular parasitism/transposon mutagenesis/virulence determinants/phagocytic cells)

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ABSTRACT *Salmonella typhimurium* is a facultative intracellular pathogen capable of surviving within phagocytic cells of the reticuloendothelial system. To identify the genes important for intracellular survival, 9516 independent Tn10 insertional mutations were isolated in a virulent strain of *S. typhimurium*. By using an *in vitro* assay for survival within macrophages, 83 Tn10 mutants have been identified that have a diminished capacity for intracellular survival (designated MS or macrophage survival mutants). All of the MS mutants are less virulent than the parent strain *in vivo*, demonstrating that, for *Salmonella*, survival within the macrophage is essential for virulence. Thirty-seven of the MS mutants have been characterized as to their phenotype, including several mutations that confer sensitivity to specific microbiocidal mechanisms of the macrophage.

Phagocytic cells are one of the first lines of defense in the body against invading organisms (1); they engulf and kill nonpathogenic and some pathogenic microbes by oxygen-dependent and -independent mechanisms (2, 3). Intracellular pathogens have evolved means to evade killing by professional phagocytes, thereby allowing them to survive within phagocytic cells (4). Although intracellular pathogens are a clinically important group of microorganisms that cause lingering diseases, such as tuberculosis and typhoid fever, little is known about the molecular mechanisms they employ to interfere with normal phagocyte function.

Salmonella typhimurium is a facultative intracellular pathogen of mice (5) and the leading cause of gastroenteritis in man in the United States (6). Although *S. typhimurium* has been well characterized both physiologically and genetically, only a limited amount of data is available on the mechanisms employed by *S. typhimurium* for survival within the macrophage. It has been reported that *S. typhimurium* survives and may replicate within the phagolysosome (7). Lipopolysaccharide (LPS or endotoxin) on the surface of the bacteria is a well-characterized virulence factor (8). Also, high molecular weight plasmids have been associated with virulence in pathogenic strains of *Salmonella* (9, 10); however, the plasmid functions that contribute to virulence have not been identified.

To better understand intracellular parasitism, we have constructed a bank of Tn10 mutants of a mouse virulent strain of *S. typhimurium* and screened individual mutants for intracellular survival *in vitro*. This paper reports the identification and preliminary characterization of 83 mutants (designated MS or macrophage survival mutants) of *S. typhimurium* that are attenuated in intracellular survival *in vitro* and in the mouse. Further characterization of the MS mutants will certainly contribute to the understanding of pathogenic mechanisms

employed by this and other clinically important intracellular pathogens.

MATERIALS AND METHODS

Strains. ATCC strain 14028, designated 14028s, was the virulent strain of *S. typhimurium* used in this study. A rough derivative of this strain (14028r), possessing a complete LPS core, was the parent strain for the Tn10 mutants. SL3235 (*aroA*; ref. 11) was the representative avirulent strain for comparison of survival levels.

Macrophages. Peritoneal macrophages from BALB/c mice (Research Institute of Scripps Clinic) were elicited with 1.5 ml of Brewer thioglycollate medium (Difco) injected intraperitoneally and harvested 4 days later as described (12). Macrophages were seeded into 96-well microtiter plates at a concentration of 2×10^5 peritoneal exudate cells per well in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and 100 μ g of gentamicin per ml. The macrophages were incubated at 37°C in 5% CO₂ in air for 2 hr and the medium was changed to remove nonadherent cells. Macrophages were used 0.5–7 days after harvesting.

Isolation of Tn10 Mutants. Tn10 insertions were made by mating a temperature-sensitive *F'*_{ts}114 *lac*⁺ *zsf*-20::Tn10; ref. 13) into 14028r at 32°C and selecting *Lac*⁻, tetracycline-resistant derivatives on MacConkey lactose plates (14) at 42°C. To ensure that the mutations were independent, each *Lac*⁻, tetracycline-resistant mutant was purified from an isolated *Lac*⁺ colony grown at 32°C.

Macrophage Assay for Avirulence. The Tn10 mutants were grown overnight in 0.2 ml of LB broth (15) in microtiter dishes. The overnight cultures were diluted and about 10⁵ bacteria in 50 μ l of DMEM were added to each well of macrophages previously washed with 200 μ l of Hank's balanced salts (GIBCO). The plates were incubated for 1 hr, after which gentamicin (final concentration, 200 μ g per ml) was added in 200 μ l of DMEM to inactivate extracellular bacteria. After 2 hr, the medium was changed to DMEM with 10 μ g of gentamicin per ml. After 18–24 hr of incubation, the macrophages were lysed with 0.5% sodium deoxycholate in normal saline, and an aliquot of the lysis mix was plated on LB agar. Any mutant showing a decrease in number of viable intracellular bacteria relative to the parent by a factor of at least 2 was selected as a possible MS mutant and retested *in vitro*. Those mutants that had reproducibly lower survival levels generally showed a decrease in viable intracellular bacteria by a factor of at least 4 compared to the parent.

RESULTS

Development of the *in Vitro* Screen for Survival in Macrophages. The *in vitro* screen for intracellular survival devel-

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Abbreviations: MS mutant, macrophage survival mutant; LPS, lipopolysaccharide.

oped was based on the assay of Lissner *et al.* (16) but was modified for the large-scale screening of mutants. To determine optimal conditions for the assay, survival of known virulent and avirulent strains of *S. typhimurium* was determined in macrophages from mice that varied in their susceptibility to *S. typhimurium* infection. Several virulent *S. typhimurium* strains were compared. ATCC 14028 was selected for this study because it retains its virulence *in vitro*, which is not true of more commonly used strains. SL3235 (11), an *aroA* mutant, was the representative avirulent strain. SL3235 requires the products of the aromatic amino acid biosynthesis pathway for growth, including *p*-aminobenzoic acid and 2,3-dihydroxybenzoic acid, which are not available in mammalian tissue.

Salmonella species possess O antigen as the outer component of LPS (17) and lose expression of O antigen, exhibiting a rough colony morphology at a relatively high frequency (10^{-4} to 10^{-5} ; ref. 18). A rough variant of virulent strain 14028s (designated 14028r) was isolated and its virulence properties were characterized. The LD₅₀ for 14028r was similar to that for 14028s in BALB/c mice [<30 organisms by intraperitoneal injection, as determined according to the method of Reed and Muench (19)], although its LD₅₀ in resistant (A/J) mice was higher (10^5 organisms for 14028r, 10^4 organisms for 14028s; data not shown). The rough strain survives almost as well as the smooth strain in BALB/c macrophages *in vitro* (Fig. 1). In addition, the rough variant was phagocytized at a more consistent rate than the smooth strain when not opsonized, resulting in more reproducible bacterial counts. Thus, 14028r was chosen as the starting strain for the mutagenesis.

The survival of *S. typhimurium* strains 14028r and SL3235 was assayed in thioglycollate-elicited peritoneal macrophages isolated from BALB/c (susceptible to *S. typhimurium* due to a macrophage defect; ref. 20), CBA/N (susceptible to *S. typhimurium* due to a B-cell defect; ref. 21), and A/J (resistant; ref. 21) mice. 14028r shows a decrease in viability

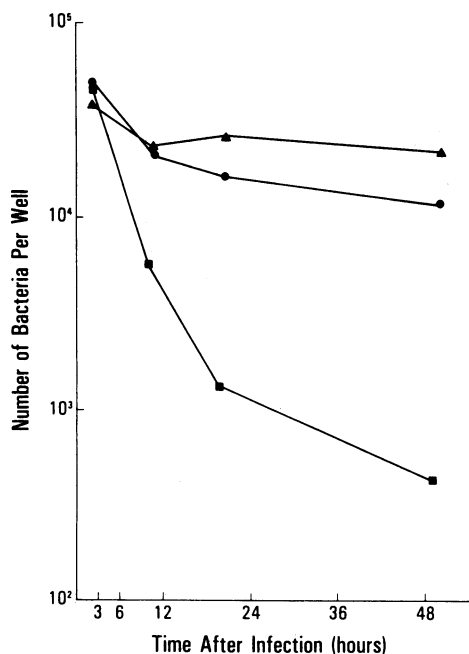


FIG. 1. Survival of known virulent and avirulent strains in BALB/c macrophages *in vitro*. Macrophages were infected as described for the *in vitro* macrophage assay, except that multiple wells were infected with each strain. Phagocytosis time was 2 hr and antibiotic treatment was for 2 hr. At the times indicated, representing time after addition of bacteria, duplicate wells were used to determine the number of viable bacteria. ■, SL3235; ●, 14028r; ▲, 14028s.

by a factor of only 2 after 24 hr in BALB/c macrophages compared to a decrease by a factor of 50 for the *aroA* mutant (Fig. 1). Survival of these strains in macrophages from A/J and CBA/N mice was lower but otherwise paralleled survival in BALB/c macrophages (data not shown). However, the difference in survival between the *aroA* mutant and the virulent strain was greater in BALB/c macrophages (50-fold) than in macrophages from CBA/N or A/J mice (10-fold). Hence, BALB/c macrophages were chosen for use in the *in vitro* assay.

Identification of Avirulent Mutants. Nine thousand five hundred sixteen Tn10 insertion mutants were screened in the *in vitro* assay to identify those that showed a lower level of survival in macrophages. One hundred fifteen mutants were identified as potential MS mutants. These putative mutants were tested for avirulence in the mouse by infecting two BALB/c mice with 1000 organisms by intraperitoneal injection. This dose corresponds to $100\times$ LD₅₀ for the virulent parent strain. Eighty-three of the mutants identified in the *in vitro* screen were avirulent or less virulent at this dose. Both mice survived this dose for 67 mutants, whereas one mouse survived for 16 mutants.

Thirty-two mutants identified in the *in vitro* screen as potentially avirulent were virulent in the mouse at a dose of 1000 organisms. All of these mutants either rapidly settled out of a liquid culture, indicating that they may have surface alterations that make them more hydrophobic, thus phagocytized less well (1), or were visibly less turbid after overnight culture. In either case, the lower number of cells at the 24-hr time point would be due to a lower starting number of phagocytized cells rather than intracellular killing.

The 83 mutants that are less virulent in the mouse are most likely defective in intracellular survival since they (i) are no more sensitive than the parent to gentamicin, (ii) are resistant to 0.5% sodium deoxycholate, as is the parent, and (iii) have growth rates comparable to the parent strain in complex media and minimal media (data not shown). Also, all of the MS mutants so far tested have numbers of viable intracellular bacteria that are similar to those of the parent immediately after phagocytosis and gentamicin treatment (data not shown; see Fig. 3, for examples), indicating that these mutants are phagocytized as well as the parent.

Characterization of the Avirulent Mutants. The MS mutants have been characterized with regard to several properties suggested to be associated with virulence, including auxotrophy, serum sensitivity, response to oxidative stress, plasmid-associated factors, alterations in LPS, motility, and colony morphology.

Auxotrophy. Twelve auxotrophs were identified among the 83 avirulent mutants (Table 1). The nutritional requirements for these mutants as determined on supplemented minimal media (15) were purines (4 mutants), pyrimidines (4 mutants), products of the aromatic amino acid biosynthetic pathway (1 mutant), histidine (2 mutants), and methionine (1 mutant). No guanine auxotrophs were isolated in this study. However, known *guaA* and *guaB* mutants of *S. typhimurium* strain LT2 (22) were tested for virulence *in vivo*. Both of these auxotrophs were avirulent (LD₅₀ $> 10^5$ compared to about 20 organisms for LT2; data not shown).

Selection of prototrophic revertants of the auxotrophs provided a means of ensuring that the defect associated with the transposon insertion was also the defect affecting virulence. Prototrophic revertants were selected on nonsupplemented minimal media. Eleven of the 12 auxotrophic mutations reverted to prototrophy with concomitant reversion to tetracycline sensitivity at a frequency comparable to published values for precise Tn10 excision (ref. 23; Table 1). The Met⁻ mutation (MS1654) does not appear to revert (frequency of $<10^{-10}$); thus, the avirulent phenotype could not be confirmed as associated with methionine auxotrophy. Two

revertants of each auxotroph were tested for virulence *in vivo*. All of the revertants were virulent since a dose of 1000 organisms of a prototrophic revertant was lethal to BALB/c mice. Thus, for 11 of the 12 auxotrophic mutations the defect conferring the MS phenotype was directly linked to the Tn10 insertion.

Serum sensitivity. Virulence of *S. typhimurium* has been correlated with serum sensitivity and the activation of com-

Table 1. Phenotypic characterization of MS mutants

Phenotype	Mutant	LD ₅₀ *	Auxotrophy		
			Requirement	Reversion frequency	
Auxotroph	MS879	>10 ⁶	Purine	1 × 10 ⁻⁷	
	MS5485	>10 ⁶	Purine	4 × 10 ⁻⁸	
	MS6060	>10 ⁶	Purine	5 × 10 ⁻⁹	
	MS7083	>10 ⁶	Purine	9 × 10 ⁻⁸	
	MS941	≈10 ⁴	Histidine	1 × 10 ⁻⁷	
	MS7898	≈10 ⁴	Histidine	1 × 10 ⁻⁷	
	MS1896	≈500	Pyrimidine	1 × 10 ⁻⁸	
	MS5464	≈500	Pyrimidine	1 × 10 ⁻⁸	
	MS7206	≈10 ³	Pyrimidine	1 × 10 ⁻⁷	
	MS7771	≈10 ³	Pyrimidine	1 × 10 ⁻⁹	
	MS6468	>10 ⁶	Aromatic [†]	1 × 10 ⁻⁹	
	MS1654	≈10 ⁵	Methionine	<10 ⁻¹⁰	
	Serum hypersensitive	MS5996	>10 ⁵		
		MS7953	>10 ⁵		
MS9020		>10 ⁵			
Altered response to oxidative stress	MS290	>10 ⁵			
	MS9187	≈10 ⁴			
	MS4290	≈10 ⁵			
Tn10 in plasmid	MS3575	>10 ³			
LPS alteration [‡]	MS4184	>10 ³			
Nonmotile	MS3	>10 ⁴			
	MS317	>10 ³			
	MS827	>10 ³			
	MS2894	>10 ³			
	MS4061	>10 ³			
	MS4228	≈10 ³			
	MS5706	≈10 ³			
	MS7207	≈10 ³			
	Altered colony morphology [§]	MS2606	>10 ³		
		MS3493	>10 ³		
		MS3792	>10 ³		
MS4916		>10 ³			
MS6290		>10 ³			
MS6632		>10 ³			
MS7530		>10 ³			
MS8282		>10 ³			
MS8483		>10 ³			

No phenotype differences have been found to date for the following mutants: MS1, MS2, MS4, MS949, MS1321, MS1352, MS1405, MS1413, MS1446, MS1521, MS1523, MS1592, MS1601, MS1633, MS1648, MS1668, MS1965, MS1996, MS2097, MS2101, MS2221, MS3010, MS3620, MS3747, MS3803, MS4179, MS4252, MS4347, MS4556, MS4602, MS4675, MS4840, MS5076, MS5278, MS5711, MS7281, MS7342, MS7481, MS7812, MS8009, MS8135, MS8194, MS8216, MS8467, MS8537, MS8880.

*Groups of five BALB/c mice were administered various doses of a mutant by intraperitoneal injection. LD₅₀ was calculated according to Reed and Muench (19). Those indicated as >10³ were not tested other than the initial screening in mice.

[†]Products of the aromatic amino acid biosynthetic pathway.

[‡]Based on bacteriophage typing. Bacteriophages used were P22, C21, Br2, Br60, FO, Ffm, 6SR. 14028r allows all of these bacteriophage except P22 and C21 to form plaques.

[§]All of these mutants allow the same bacteriophages as the parent to form plaques.

plement (24, 25). In addition, it has been reported that complement may be involved in intracellular killing (26). Each mutant was tested for survival in 20% normal rabbit serum. Fig. 2 shows survival curves for the three mutants that were identified as more serum sensitive than the parent. All three mutants survived normally in heat-inactivated serum (Fig. 2), suggesting complement as the sensitizing factor.

Response to oxidative stress. Phagocytic cells generate toxic oxygen radicals as part of the microbiocidal response (2). Bacteria produce enzymes that inactivate some of these compounds. In *S. typhimurium*, these include two superoxide dismutases, one of which is inducible by exposure to H₂O₂ (27), and several forms of catalase (28). All of the mutants had similar constitutive levels of the two superoxide dismutases as determined by the method of Beauchamp and Fridovich (29), indicating that none of the insertions is in the structural genes for superoxide dismutase.

None of the MS mutants was affected in catalase expression based on a test for O₂ production from H₂O₂ (30). In fact, one catalase mutant was identified in a screen of 3700 of the Tn10 insertion mutants by this method. This mutant is totally deficient in HPIII (31) on catalase activity gels (32) but survives normally in macrophages *in vitro* and is virulent *in vivo*, suggesting that HPIII is not required for virulence.

The MS mutants were tested for sensitivity to various oxidants. Three mutants altered in their response to the oxidants were identified and can be distinguished by their sensitivities to the five oxidants tested (Table 2). MS9187 is interesting in that it is totally resistant to *t*-butyl hydroperoxide at the concentration tested but is sensitive to the other oxidants. Nondenaturing gels were used to test these mutants for the presence of catalase, glutathione reductase, and glutathione peroxidase activities (32). All three had normal constitutive levels of these enzyme activities (data not shown).

Plasmid-associated virulence factors. High molecular weight plasmids have been associated with virulence in

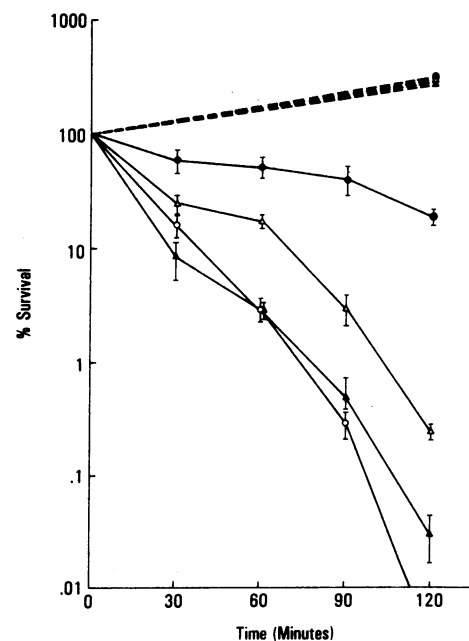


FIG. 2. Serum sensitivity of MS mutants. Bacteria were grown overnight in LB broth, diluted to about 10⁷ colony-forming units/ml in 20% normal rabbit serum in Hank's balanced salts (GIBCO), and incubated at 37°C. Aliquots were withdrawn at the times indicated and the number of viable bacteria was determined. Serum was heat-inactivated by incubating at 56°C for 30 min. Results are the average of four experiments. ●, 14028r; △, MS5996; ○, MS7953; ▲, MS9020; solid lines, 20% normal rabbit serum; dashed lines, 20% heat-inactivated normal rabbit serum.

Table 2. Sensitivity of MS mutants to oxidants

Strain	Oxidant				
	H ₂ O ₂	<i>t</i> -Butyl hydroperoxide	Cumene hydroperoxide	CdCl ₂	1-Chloro-2,4-dinitrobenzene
14028r	29	57	27	17	10.5
MS290	30	65	29	17	10.5
MS4290	31	70	33	22	10.5
MS9187	42	0	28	29	14

One-tenth milliliter of an overnight culture of each mutant was added to 3 ml of soft agar and poured onto an LB plate consisting of 25 ml of medium. Two and one-half milligrams of H₂O₂, 630 μ g of *t*-butyl hydroperoxide, 800 μ g of cumene hydroperoxide, 1 mg of CdCl₂, or 200 μ g of 1-chloro-2,4-dinitrobenzene was placed on a 0.6-cm filter disc in the center of the plate. Plates were incubated overnight at 37°C. Values are the diameter of the zone of killing in mm.

pathogenic *Salmonella*. The 83 MS mutants were analyzed for insertions in this plasmid by Southern hybridization of plasmid screens (33) using nick-translated pNK370 (contains a fragment of Tn10; N. Kleckner, personal communication) as a probe. One mutant (MS3575) was found that had a Tn10 insertion in the plasmid.

Other phenotypic groups. (i) One mutant with altered LPS was identified by bacteriophage typing (34). This number may seem low in view of the many genes involved in LPS biosynthesis; however, the initial mutant isolation selected against most LPS mutations. The bacteriophage plaquing spectrum for this mutant (MS4184) corresponds with an *rfaF* mutation (34) (Table 1). Ten mutants had a different colony morphology but the same bacteriophage plaquing spectrum as the parent, suggesting that these mutants have some other surface alteration. (ii) The mutants were tested for motility by stabbing each mutant into LB medium containing 0.35% agar and looking for growth away from the stab. Based on this test, eight mutants are nonmotile. (iii) Defects in iron uptake were looked for by spotting each mutant on minimal medium containing 50–200 μ M ethylenediamine-di-*o*-hydroxyphenyl acetic acid, an iron chelator (35). Under these conditions, none of the mutants was reproducibly sensitive to low iron concentration. (iv) The low pH within a phagolysosome is thought to make the intracellular environment hostile to bacteria (1); however, none of the mutants was more sensitive to pH 4 than the parent.

Intracellular Survival of the MS Mutants. The survival of several of the MS mutants within macrophages *in vitro* was followed with time (Fig. 3). Correlations can be seen between phenotype and intracellular survival levels. The serum-sensitive mutants were among the most avirulent and had low levels of intracellular survival. MS7953 and MS9020 were similar in their sensitivity to serum (Fig. 2) as well as in their intracellular survival levels. The oxidant-sensitive mutants MS9187 and MS290 are distinct based on sensitivity to oxidants and LD₅₀; the lower LD₅₀ for MS9187 correlates with its higher *in vitro* survival level.

DISCUSSION

The microbiocidal mechanisms employed by professional phagocytes have been well studied; yet the ways in which intracellular pathogens evade these mechanisms are poorly understood. To define the mechanisms employed by intracellular pathogens for survival within macrophages, we have developed a simple *in vitro* assay and used it to identify 83 mutants of *S. typhimurium* that are attenuated for survival within macrophages *in vitro*. All of the MS mutants are less virulent than the parent strain *in vivo*, demonstrating that survival in the macrophage is essential for virulence. Thirty-two mutants were identified in the *in vitro* screen that

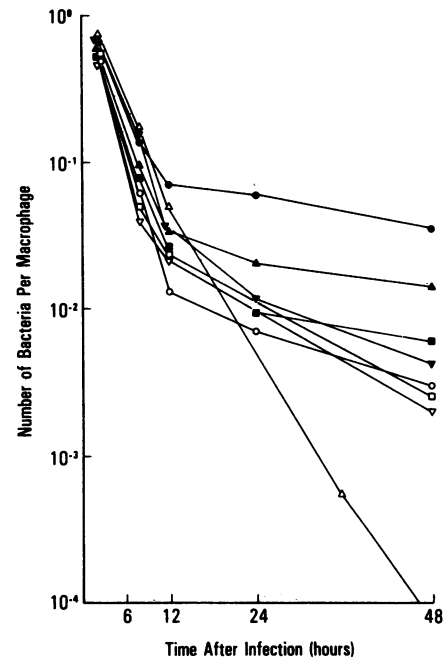


FIG. 3. Survival of MS mutants in BALB/c macrophages. Macrophages were infected as described in the legend to Fig. 1. Phagocytosis time was 1 hr and antibiotic treatment was for 1.5 hr. The number of viable macrophages was determined by staining with 0.4% trypan blue and microscopic counting with the aid of a net micrometer. ●, 14028r; ▼, MS290; ■, MS6060; ▲, MS9187; □, MS7953; ▽, MS9020; △, MS5996; ○, MS3575.

appear to be attenuated for intracellular survival but are still virulent in the mouse. All of these mutants can be explained as false positives in the *in vitro* assay. All of the MS mutants are identical to the parent for several growth characteristics; thus, the MS mutants contain defects that affect their survival within the macrophage but are normal in other respects.

Characterization of the MS mutants has identified phenotypes for 37 mutants (Table 1), whereas 46 MS mutants have no obvious phenotypic differences from the parent. The range of phenotypes so far identified (Table 1) combined with variations seen in the ability of the mutants to protect a mouse against a challenge with virulent *Salmonella* (P.I.F. and F.H., unpublished observation) indicate that at least 20 different genes affecting intracellular survival have been identified. The MS mutants might be arbitrarily divided into two groups: mutations that affect virulence mechanisms specific to this and possibly other intracellular pathogens and mutations in genes involved in auxiliary functions that are required for intracellular survival but also present in nonpathogens. The identification of the defect associated with avirulence in the first group will help to elucidate the mechanism of intracellular survival and contribute to the understanding of other clinically important intracellular pathogens.

Bacterial metabolism is an important factor in intracellular survival, as evidenced by this and earlier work. This study indicates that purine, pyrimidine, histidine, and aromatic amino acid auxotrophs are attenuated for survival within the macrophage *in vitro* and in the mouse. Purine auxotrophs have been reported as avirulent in *S. typhimurium* (36), *Yersinia pestis* (37), and *Vibrio cholera* (38) as have *aroA* mutants (11). The avirulence of particular auxotrophs indicates that growth and/or the biosynthesis of virulence factors are required for survival in the macrophage; a mutant requiring metabolites not available *in vivo* will be unable to metabolize and grow, hence would not survive within the macrophage.

The histidine and pyrimidine auxotrophs identified as attenuated in the *in vitro* assay were only a subset of the histidine and pyrimidine auxotrophs isolated in this study, whereas all of the purine and aromatic amino acid auxotrophs isolated were identified as reduced in virulence in the *in vitro* assay. This result suggests that only particular mutations leading to histidine and pyrimidine auxotrophy are avirulent. Mutants that are derepressed for *hisG* expression are phenotypically Pur⁻ due to depletion of ATP pools (39). Therefore, it may not be the lack of histidine that makes some histidine auxotrophs avirulent but the requirement for purines. All of the pyrimidine auxotrophs identified as attenuated had relatively high *in vitro* survival levels (data not shown) and low LD₅₀ values (Table 1). It is possible that all pyrimidine auxotrophs have similar virulence *in vivo*. The small difference in intracellular survival between the pyrimidine auxotrophs and the parent strain may have permitted some to pass through the assay undetected.

Superoxides are short-lived, toxic compounds generated in the respiratory burst by the macrophage that react with other compounds to produce a wide spectrum of microbiocidal products. Inactivation of these compounds by *Salmonella* appears to be specific to the oxidant itself; therefore, the mutants have been examined for sensitivity to a variety of oxidants. Three mutants have altered sensitivities to oxidants (Table 2). All three had normal constitutive levels of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, several enzymes involved in detoxification of oxidants. Evidence for the coordinate regulation of functions involved in the defense against oxidative stress in *S. typhimurium* has been reported (27). Exposure of bacteria to low levels of H₂O₂ induces 30 proteins and protects the bacteria from subsequent challenge with high concentrations of several oxidants. Two mutants altered at *oxyR*, a positive regulator of this system (27), are less virulent than their LT2 parent *in vivo* (P.I.F. and F.H., unpublished observation), indicating a role for this system in virulence. Further characterization of the MS mutants will determine whether they are defective in some portion of this regulatory system and the role of this pathway in intracellular survival.

Serum resistance of enteric bacteria has generally been attributed to LPS (24). 14028r is only slightly sensitive to normal rabbit serum despite the fact that it is deficient in O antigen (Fig. 2). Three MS mutants are hypersensitive to serum and show similar LPS composition to the parent strain based on phage typing. Preliminary studies with serum depleted for specific complement factors indicate that the three mutants are specifically sensitive to complement. *S. typhimurium* must therefore encode other factors required for resistance to complement in addition to LPS.

Perhaps the most important result of this work may be its implications for the development of live vaccines. Attenuated live vaccines provide the best protection to infection with intracellular pathogens but few good vaccine strains have been developed (40). Many of the MS mutants confer protection against challenge with virulent *Salmonella* in the mouse (P.I.F. and F.H., unpublished observation). The *in vitro* assay for strains attenuated in intracellular survival might be applied to other facultative intracellular pathogens. The only prerequisite would be that methodologies are available to isolate mutations in the organism. Further characterization of the protective strains isolated in this study will provide information about the parameters that are important in the development of long-term immunity to intracellular pathogens.

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