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Characterization of Aromatic- and Purine-Dependent *Salmonella typhimurium*: Attenuation, Persistence, and Ability to Induce Protective Immunity in BALB/c Mice

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Stable transposon-generated auxotrophic mutations in *aroA*, *purA*, and *purE* or *aroA* and *purA* together were introduced into *Salmonella typhimurium* strains which were virulent in mice. Strains harboring any of these mutations were attenuated when tested in BALB/c mice. *purE* strains were less attenuated than *aroA* or *purA* strains. Both *aroA* and *purA* mutants persisted for several weeks in the livers and spleens of the mice after intravenous infection, although the numbers of viable cells detected at various times after infection differed. *aroA* strains persisted at a higher level than *purA* strains and were effective live vaccines given intravenously or orally. *purA* strains were ineffective as oral vaccines and were poor intravenous vaccines. Strains harboring both *aroA* and *purA* mutations together were ineffective vaccines when administered orally or intravenously, even though they persisted in the livers and spleens of the mice for long periods after intravenous infection.

The current inactivated whole-cell typhoid vaccine, although in widespread use, has shown variable efficacy in controlled field trials and can cause serious side effects in some recipients (1, 18, 20, 23). In the murine model with salmonellae that were virulent in mice, live vaccines have been shown to confer greater and longer-lasting immunity than killed vaccines (5, 6). In view of this, attempts are being made to develop live oral vaccines against typhoid, based on genetically attenuated strains of *Salmonella typhi*. An example of such a vaccine strain is *S. typhi* Ty21a, a *galE* mutant of *S. typhi* Ty2, which has been used as a live oral typhoid vaccine in human field trials (12, 22). Although Ty21a has shown reasonable efficacy in field trials, the strain was isolated by chemical mutagenesis and work is under way to construct attenuated vaccine strains carrying defined genetic lesions.

Prompted by the work of Bacon et al. (2, 3) with undefined purine and *para*-aminobenzoic acid-dependent mutants of *S. typhi*, Hoiseth and Stocker (8) constructed auxotrophic *S. typhimurium* strains carrying genetically defined nonreverting lesions in the *aroA* gene. *aroA* mutants are dependent on aromatic compounds, including aromatic amino acids, *p*-aminobenzoic acid, and 2,4-dihydroxybenzoate, for growth. *S. typhimurium aroA* mutants were found to be highly attenuated when tested in inbred mice and were excellent live oral vaccines against murine salmonellosis (8, 11, 14, 15). Stocker and colleagues (13) went on to construct strains of *S. typhi* harboring nonreverting lesions in *aroA* and *purA*, a gene involved in purine biosynthesis. The *S. typhi aroA purA* strains were fed to human volunteers and were found to be attenuated, causing no serious clinical reactions (13).

Although salmonella *aroA* derivatives have been well characterized in the murine typhoid model, little work has been carried out on the behavior in mice of salmonellae that are virulent in mice after introducing either purine mutations alone or both *aroA* and purine mutations. Stocker and colleagues (16, 17) recently showed that salmonella derivatives harboring defined purine mutations were attenuated, but they did not determine if the strains were effective oral vaccines.

To make a thorough comparison of the immunogenic properties of different auxotrophic derivatives, we constructed a series of isogenic mutants of *S. typhimurium* strains harboring nonreverting lesions in *aroA*, *purA*, and *purE* or both *aroA* and *purA* and characterized these mutants in BALB/c mice. Our results are reported here.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth conditions. The bacterial strains used in this study are shown in Table 1. Bacteriophage P22 HT105/1 *int* mutant (7) was obtained from Tim Foster, Trinity College, Dublin, Ireland. Bacteria were routinely cultured on L agar or in L broth (7). Minimal medium (7, 15) was supplemented with nutrients and antibiotics at the appropriate concentrations. Solid media contained 1.6% (wt/vol) Noble agar (Difco, East Molesey, Surrey, United Kingdom).

Construction of vaccine strains containing nonreverting auxotrophic mutations. Vaccine strains were constructed by transducing Tn*10*-generated auxotrophic mutations from *S. typhimurium* LT2 with bacteriophage P22 HT105/1 *int* into either HWSH or SL1344 *S. typhimurium* that are virulent in mice and selecting for tetracycline resistance (Tc^r) as described elsewhere (7, 8). Tetracycline-sensitive (Tc^s) derivatives of Tn*10* mutants were selected on Bochner medium (4). Tc^s isolates which exhibited a stable auxotrophic requirement were used in vaccination experiments. No prototrophic revertants were detected under conditions which would identify one revertant in 10^{11} cells. Strains harboring mutations in both *aroA* and *purA* were selected by transducing the *purA*::Tn*10* mutation into a stable *aroA* mutant and followed by selection for stable *purA* mutants as described above. Genetically manipulated strains were routinely tested for serological characteristics with anti-H and

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TABLE 1. Strains of *S. typhimurium* used in this study

Strain	Auxotrophy	Source or reference
LT2 *aroA554*::Tn*10*	*aroA*	J. Roth
LT2 *purA874*::Tn*10*	*purA*	J. Roth
LT2 *purE884*::Tn*10*	*purE*	J. Roth
SL1344	*his*	B. A. D. Stocker (11)
SL3261	*aroA his*	B. A. D. Stocker (11)
SL1344 *purA*	*purA his*	This laboratory
SL3261 *purA*	*purA aroA his*	This laboratory
HWSH[a]	Prototroph	H. Williams-Smith
HWSH *aroA*	*aroA*	This laboratory
HWSH *purA*	*purA*	This laboratory
HWSH *aroA purA*	*aroA purA*	This laboratory
HWSH *purE*	*purE*	This laboratory
C5	Prototroph	C. Hormaeche (9)

[a] A strain isolated from a calf dying of salmonellosis, which was virulent in mice and calves.

anti-O diagnostic sera provided by Wellcome Diagnostics, Dartford, Kent, United Kingdom. Lipopolysaccharide structure was examined by using silver-stained polyacrylamide gels (21).

Infection of mice and enumeration of bacteria in murine organs. Innately *Salmonella*-susceptible BALB/c mice of 8 to 10 weeks of age, bred in the animal unit at Wellcome Research Laboratories from breeders originally purchased from OLAC (1976) (Blackthorn, Bicester, Oxfordshire, United Kingdom) were used throughout. Livers and spleens were homogenized as previously described (9). Viable counts were performed on these homogenates with L agar as growth medium and are expressed in the figures as geometric means ± two standard errors of the mean, for four mice per point. The intravenous (i.v.) 50% lethal dose (LD_{50}) for virulent strains was obtained by injecting groups of five mice with serial 10-fold dilutions, prepared in phosphate-buffered saline (pH 7.2), of overnight L broth cultures grown at 37°C without shaking. To determine LD_{50} values for attenuated strains, 200 ml of overnight L broth cultures were harvested by centrifugation and suspended in phosphate-buffered saline to give a concentration of 10^9 to 10^{11} bacteria per ml. This was serially 10-fold diluted in phosphate-buffered saline, the top dose being 0.2 ml of the undiluted suspension given i.v. or orally, with 8 to 10 mice per group. Deaths were recorded over the following 4 weeks, and the LD_{50} was calculated by the method of Reed and Muench (19). For i.v. inoculation, mice were injected with 0.2 ml of bacterial suspension into the tail vein. For oral inoculation, bacteria were administered in 0.2-ml volumes to lightly ether-anesthetized mice by gavage as described previously (15).

RESULTS

Attenuation of virulent *S. typhimurium* strains by auxotrophic mutations. All auxotrophic derivatives had a much higher LD_{50} than the parental strains (Table 2). The *purA* and *aroA purA* derivatives were more attenuated than the *aroA* derivatives which had LD_{50}s in good agreement with published data (8, 11, 15). The HWSH *purE* strain was considerably less attenuated than the other auxotrophic derivatives, though the measured LD_{50} was found to vary considerably between experiments. For example, sometimes mice given as few as 100 organisms died, whereas others given up to 10^4 to 10^5 organisms survived. Aromatic or purine mutants did not kill the mice even when doses as high as 10^{10} organisms were given orally.

The ability of attenuated and virulent strains to grow and persist in the livers and spleens of BALB/c mice after i.v. inoculation was determined. Parental strains grew rapidly after i.v. administration of 10^2 to 10^3 organisms, reaching levels of 10^8 to 10^9 organisms per organ by days 5 and 6 and resulting in death of all the mice infected (data not shown). *S. typhimurium* strains harboring *aroA* mutations grew much less efficiently and produced growth curves similar to those detected with other routes of infection (Fig. 1a). At around day 7 of infection, marked splenomegaly was observed (Table 3) which peaked at day 14.

S. typhimurium purA strains grew poorly in vivo compared with the parental strains (Fig. 1b and c). A total of 100 BALB/c mice were infected i.v. with 1.6×10^6 SL1344 *purA* or 1.3×10^6 HWSH *purA*. Day 1 counts dropped by a factor of 10^2 for both strains as opposed to a factor of 10 for the *aroA* mutants After this, both strains exhibited a similar pattern of persistence, although SL1344 *purA* but not HWSH *purA* showed an increase in viable counts in livers and spleens over the first few days. Both strains were then cleared from livers and spleens but at a slower rate than *aroA* derivatives. SL1344 *purA* and HWSH *purA* induced minimal changes in spleen weight (Table 3).

Figure 1d shows the persistence of the *S. typhimurium* HWSH *aroA purA* mutant. A total of 100 BALB/c mice were infected i.v. with 2×10^5 HWSH *aroA purA*. Day 1 counts again showed clearance of the inoculum by a factor of 10^2. Bacterial counts continued to decline over the first 7 days of infection. After this, levels of persistence of between 5×10^2 and 1×10^3 organisms per organ were detected for 4 weeks, followed by a slow clearance over the next 6 weeks. After the sixth week of infection, individual mice had no detectable organisms in either the liver or spleen, but never both organs. At 10 weeks after infection, low numbers of bacteria were still detectable in livers and spleens. At no stage in the infection was splenomegaly detected (data not shown).

A total of 100 BALB/c mice were injected i.v. with 3.3×10^2 HWSH *purE* organisms (Fig. 2). Day 1 counts showed an initial decrease by a factor of 10. The bacteria then grew rapidly in livers and spleens up to day 14 when, in most cases, the counts declined. Of the mice infected, 10% died during the experiment and in many mice, large abscesses were observed on livers and spleens. At 119 days after infection, the organism still persisted in livers and spleens at around 10^3. Minimal medium plates supplemented with liver or spleen homogenate from uninfected mice supported growth of the *purE* mutant.

Protection of mice after i.v. or oral challenge. Mice immunized i.v. with the auxotrophic mutants were challenged i.v.

TABLE 2. Results obtained by infecting BALB/c mice i.v. with various auxotrophic derivatives of two highly virulent *S. typhimurium* strains[a]

Strain	Log LD_{50}
SL1344	<1
SL3261	7.1
SL1344 *purA*	7.7
SL3261 *purA*	8.7
HWSH	<1
HWSH *aroA*	7.4
HWSH *purA*	8.6
HWSH *aroA purA*	8.9
HWSH *purE*	3.8

[a] All LD_{50}s were calculated after 28 days, except for HWSH *purE*, which was calculated after 56 days.

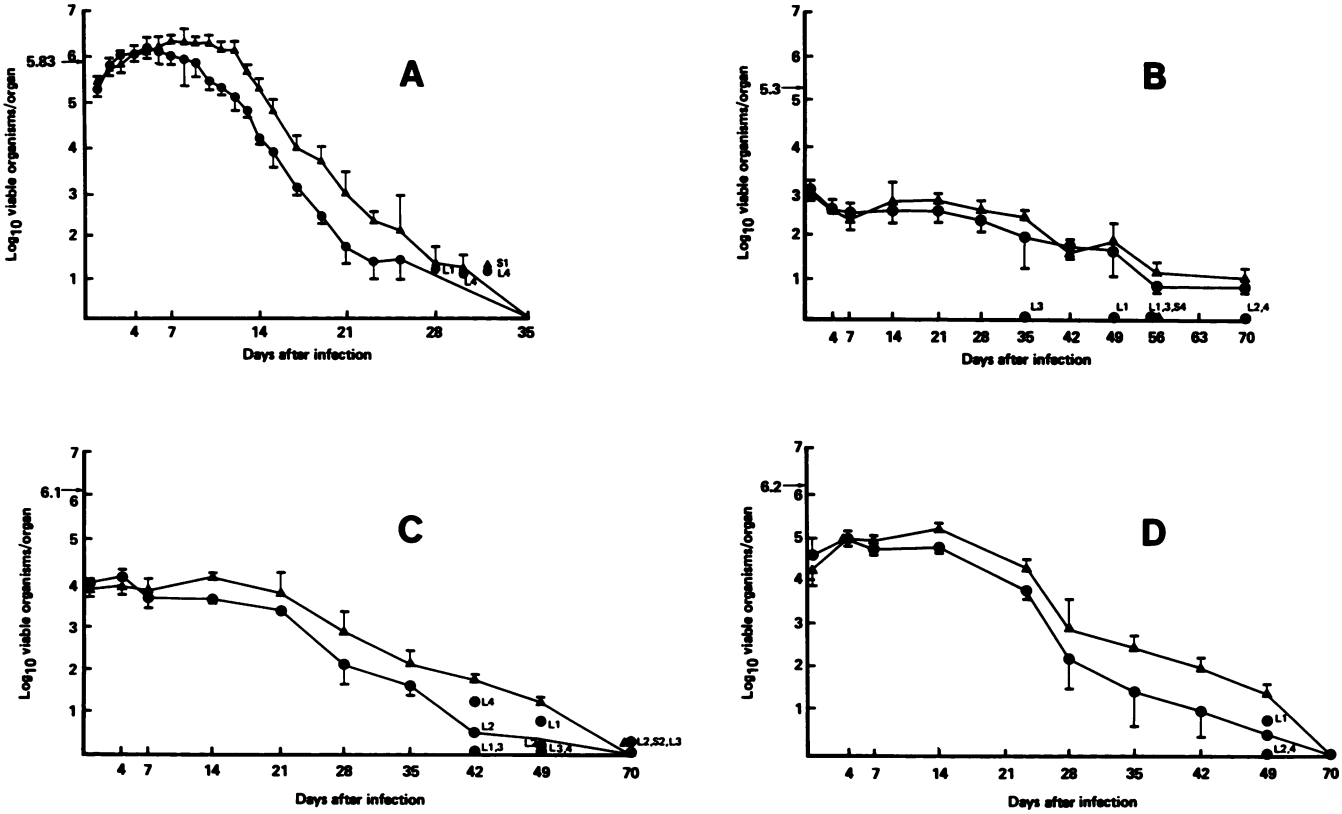

FIG. 1. Colonization of livers (●) and spleens (▲) of BALB/c mice after i.v. infection with 6.3×10^5 HWSH *aroA* (A), 2×10^5 HWSH *aroA purA* (B), 1.3×10^6 HWSH *purA* (C), on 1.6×10^6 SL1344 *purA* (D). Each point represents the geometric mean ± two standard errors for four mice. Counts in individual mice when one or more of the group of four organs had no detectable salmonellae are indicated (▲S1, mouse 1 spleen; ●1L, mouse 1 liver).

with virulent salmonellae to determine the level of protection induced by the mutants. SL3261 and HWSH *aroA* infections both induced solid protection against an i.v. challenge with their respective virulent parent strains on days 28 and 70 postimmunization (Table 4). At day 28, SL1344 *purA* also gave good protection against i.v. challenge with its virulent parent strain SL1344. However, the HWSH *aroA purA* double mutant was very poorly protective against HWSH wild-type challenge at day 28.

To directly compare protection induced by the vaccine strains, an independent virulent *S. typhimurium*, C5, was used as the challenge strain. At day 28 postimmunization, HWSH *aroA*, SL3261, and SL1344 *purA* all protected against i.v. challenge with C5, whereas HWSH *purA* and SL3261 *purA* provided only minimal protection when compared with isogenic *aroA* mutants. HWSH *purA* and SL1344 *purA* both provided minimal protection at day 70 (Table 4).

Mice were infected orally with 10^9 to 10^{10} of the auxotrophic mutants and then challenged orally 4 weeks later

TABLE 3. Spleen weights of mice immunized i.v. with auxotrophic *S. typhimurium* mutants

Days after infection	Mean spleen wt (g) ± 2 SE for mice[a] immunized with:		
	HWSH *aroA*	SL1344 *purA*	HWSH *purA*
1	0.17 ± 0.08	0.15 ± 0.02	0.17 ± 0.02
4	0.28 ± 0.02	0.16 ± 0.02	0.14 ± 0.01
7	0.57 ± 0.05	0.23 ± 0.02	0.15 ± 0.03
14	1.05 ± 0.1	0.29 ± 0.025	0.15 ± 0.01
21	0.38 ± 0.11	0.38 ± 0.05	0.25 ± 0.025
28	0.28 ± 0.05	0.28 ± 0.04	0.24 ± 0.03
35		0.22 ± 0.03	0.18 ± 0.02
49		0.22 ± 0.02	0.20 ± 0.02

[a] Mice from infections shown in Fig. 1.

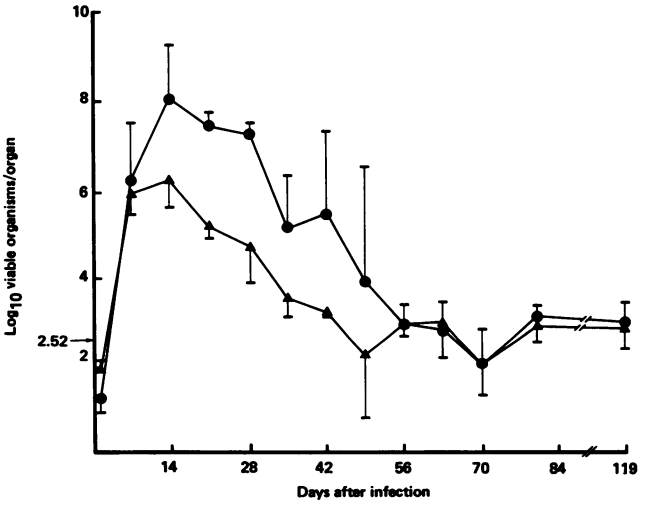

FIG. 2. Colonization of livers (●) and spleens (▲) of BALB/c mice after i.v. infection with 3.3×10^2 HWSH *purE*. Each point represents the geometric mean ± two standard errors for four mice.

with the parental virulent strains (Table 5). Unlike the *aroA* derivatives, neither the *purA* nor the *aroA purA* derivatives induced any protection orally against an oral challenge.

DISCUSSION

The data presented here support earlier observations that certain auxotrophic mutations attenuate virulent strains of *S. typhimurium*. Both *aroA* and *pur* mutations can attenuate strains but do so to differing extents. *purE* mutants are capable of significant growth in vivo, a fact which could exclude the use of such mutations in human vaccine strains. The different effects of *purE* compared with *purA* could be due to the respective positions at which the products of each gene act in the purine biosynthetic pathway. The *purE* gene product acts early in the pathway, before the IMP branch point, and it is possible that such mutants still have the capacity to scavenge or synthesize purines in vivo. This is supported by our observation that *purE* but not *purA* mutants can grow slowly in vitro on minimal agar supplemented with liver or spleen homogenates. It has recently been reported that other lesions in the genes of the biosynthetic pathway to IMP can also partially reduce the virulence of several strains of salmonella in mice (16, 17). Although *S. typhimurium aroA* and *S. typhimurium purA* derivatives both grow poorly in vivo, their growth curves also show some important differences. In the first 24 h after i.v. infection, the levels of viable *purA* mutants drop much more than *aroA* mutants when similar numbers of viable cells are administered. After the first 24 h, both *purA* and *aroA* mutants set up a persistent infection which lasts for several weeks. Our data clearly show that persistence in itself is not enough to establish immunity, as has been suggested previously (5, 10). Indeed, *purA* strains can persist for several weeks without inducing significant levels of immunity to homologous challenge. These isogenic derivatives could be useful in identifying the mechanisms responsible for protective immunity against salmonellae.

If *aroA* and *purA* mutations are combined in the same strain, the resultant strain differs significantly from strains carrying single mutations. HWSH *aroA purA* set up a long-lasting, low-level persistent infection in the mice which was quite different from that of HWSH *purA* or HWSH *aroA*. Preliminary observations suggest that SL3261 *aroA purA* sets up a similar long-lasting infection after i.v. infection. Thus, it is important that data obtained from a strain carrying a single auxotrophic mutation may not be extrapolated directly to a strain carrying two mutations in separate metabolic pathways.

Splenomegaly is a common feature of *Salmonella* infections. Killar and Eisenstein (11) reported that intraperitoneal infection of C3H lineage mice with an *aroA* mutant induced maximal splenomegaly as the mice began to clear the infection from their spleens. They associated splenomegaly with a period of nonspecific macrophage activation as assessed by immunity to *Listeria monocytogenes*. Both HWSH *aroA* (Table 3) and SL3261 (data not shown) induced gross splenomegaly after i.v. infection of BALB/c mice, whereas *purA* and *aroA purA* mutants induced minimal splenomegaly. This may indicate that in this system, the factors which induce splenomegaly may be related to the mechanisms which induce protective immunity.

The protection data obtained with oral challenge of mice strongly suggest that *S. typhimurium* strains harboring *purA* or *aroA purA* mutations make poor oral vaccines compared with *aroA* strains. To date, we have been unable to protect mice against virulent challenge with a *purA* vaccine strain administered orally. These observations may have important consequences for the design of attenuated *S. typhi* strains for use as live oral typhoid vaccines, which cannot be tested in animal models, especially since some auxotrophic derivatives of *S. typhi* are now being evaluated in humans (13).

TABLE 4. Protection of mice after i.v. challenge[a]

Immunizing strain	Challenge strain (log LD_{50})	
	Day 28	Day 70
Challenged with SL1344		
SL3261	>6.4	ND
SL1344 *purA*	4.5	ND
Unimmunized	<1.0	ND
Challenged with HWSH		
HWSH *aroA*	4.1	4.2
HWSH *purA aroA*	1.6	ND
Unimmunized	<1.0	<1.0
Challenged with C5		
HWSH *aroA*	>6.2	3.3
HWSH *purA*	2	1.49
SL3261	>6.6	5.5
SL1344 *purA*	5	2.38
SL3261 *purA*	2.8	ND
Unimmunized	<1.0	<1.0

[a] BALB/c mice were immunized i.v. with various auxotrophic *S. typhimurium* strains and LD_{50}s were calculated for the virulent *S. typhimurium* strains after i.v. inoculation 28 or 70 days later. ND, Not done.

TABLE 5. Protection of mice after oral challenge[a]

Immunizing strain	Challenge strain (log LD_{50})
Challenged with HWSH	
HWSH *aroA*	8.82
HWSH *purA aroA*	<5.22
Unimmunized	5.32
Challenged with SL1344	
SL3261	>10.3
SL1344 *purA*	6.68
SL3261 *purA*	5.58
Unimmunized	6.2

[a] BALB/c mice were immunized orally with various auxotrophic *S. typhimurium* strains and LD_{50}s were calculated for the virulent *S. typhimurium* strains after oral challenge 28 days after immunization.

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