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Effect of a *purA* Mutation on Efficacy of *Salmonella* Live-Vaccine Vectors

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We made Δ*aroA*, Δ*purA*, and Δ*aroA* Δ*purA* derivatives of a strain of *Salmonella dublin* and isolated a nalidixate-resistant mutant of each construct. An inoculum of each of the nearly isogenic nalidixate-resistant auxotrophs was administered to BALB/c mice by gavage. The ability of each strain to colonize, invade, persist in tissues, and evoke serum and mucosal antibody responses to the lipopolysaccharide of the parent strain was examined. Only the Δ*aroA* strain colonized, invaded, persisted, and (more importantly) evoked sustained significant serum and mucosal antibody responses. Neither the Δ*purA* nor the Δ*aroA* Δ*purA* strain showed any of these abilities. These observations demonstrate that the *purA* defect, which causes a requirement for adenine, reduces the live-vaccine efficacy of attenuated *Salmonella* strains and may limit the effectiveness of *Salmonella* strains as carriers of heterologous antigens. These findings may be important in the selection of attenuated *S. typhi* strains for use in humans either as antityphoid live vaccines or as vectors for antigens of other pathogens.

A variety of techniques, including use of live oral vaccines, have been employed to deliver antigens to the gut-associated lymphoid tissue in an attempt to initiate production of specific secretory immunoglobulin A (IgA) antibodies. One recent approach has been to employ avirulent derivatives of *Salmonella* strains as carriers for plasmids which code for virulence determinants of heterologous mucosal pathogens (recently reviewed by Clements [2]). Antigens expressed by these strains would presumably be delivered directly to the antibody-forming cells in the gut-associated lymphoid tissue. This has been shown to be an effective means of stimulating significant levels of specific mucosal secretory IgA directed against the carrier strain and the heterologous antigen and has been shown to stimulate production of serum antibodies as well (4).

A number of investigators have employed a variety of *Salmonella* mutants for this purpose, including *galE* mutants, which lack the enzyme UDP-galactose-4-epimerase (3, 10–12, 16, 25, 26), and *aroA* mutants, which have specific nonreverting deletions in the common aromatic biosynthetic pathway leading to chorismic acid (1, 4, 8, 13, 16, 17, 20–24). Deletion mutants of *Salmonella typhimurium* lacking adenylate cyclase and cyclic AMP receptor protein have also been examined as carriers for antigens of *Streptococcus mutans* in the development of a potential anticaries vaccine (5–7).

Strains of *Salmonella typhi* attenuated by auxotrophic characters have also been constructed as live-vaccine candidates (9) for immunization against typhoid fever. Two strains recently tested in volunteers (15) each had an *aroA* deletion, expected to cause complete loss of virulence by itself, and, as an additional safety factor, a deletion at *purA*. Unlike mice given an *aroA* (deletion) mutant of *Salmonella dublin* orally, volunteers who received an oral dose of even 10^{10} CFU of either of the two Δ*aroA* Δ*purA* live-vaccine strains, 541Ty (Vi positive) or 543Ty (Vi negative), developed no or only very low titers of humoral antibody to the O antigen of the vaccine strain. It is not clear why the *S. typhi* live vaccine failed to cause the expected humoral antibody response to the O antigen (or to the H or the Vi antigen); it might have resulted either from the strains used having two attenuating nutritional requirements instead of the single mutation to aromatic dependence in the *S. dublin* live vaccine tested in mice or just from the Δ*purA* mutation, which was not tested in the mouse experiments of Clements et al. (4). To test these possibilities, we used three nonvirulent derivatives of an *S. dublin* strain virulent for mice, one with an *aroA* deletion, one with a *purA* deletion, and one with both deletions. We tested the ability of each strain to colonize, invade, persist in tissues, and evoke serum and mucosal (gut) antibody responses to the O antigen in mice given one or another of the three nearly isogenic strains by gavage.

Construction of bacterial strains. The organisms used for this study are listed in Table 1. All are nalidixate-resistant mutants of three nearly isogenic auxotrophic derivatives of a virulent, wild-type strain of *S. dublin*, SVA47 (SL5608). One derivative was attenuated by deletion Δ*aroA148*, causing a requirement for aromatic metabolites. A second deletion, Δ*purA155*, was introduced into the Δ*aroA* strain by cotransduction with a silent Tn*10* insertion, *zbj-908*::Tn*10*, to produce an *aroA purA* strain; tetracycline sensitivity was restored by a mutation, presumably a transposon-generated deletion or inversion, at *zbj-908*. The *aroA* and *aroA purA* strains had been constructed (B. A. D. Stocker and A. A. Lindberg, unpublished results) for trial as live vaccines (for protection of calves against salmonellosis) by methods similar to those used to make 541Ty and 543Ty, the Δ*aroA his* Δ*purA* strains of *S. typhi* described above. However, the *aroA* deletion introduced into the *S. dublin* strains used in this study, Δ*aroA148*, is the result of spontaneous deletion mutation, unlike the transposon-generated deletion, Δ*aroA407*, used to make the *S. typhi* live-vaccine strain. The third auxotrophic derivative of *S. dublin* SVA47 was an *aro*$^+$ transductant isolated from the Δ*aroA* Δ*purA* strain to allow testing of the effect on live-vaccine efficacy of the *purA* defect in a strain not otherwise attenuated. Spontaneous nalidixate-resistant mutants, resistant to 100 μg of nalidixic acid per ml, were isolated from each of the three auxotrophic

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TABLE 1. Bacterial strains

Strain	Genotype[a]	Source or reference
S. dublin		
SL5608	Wild type[b]	Virulent isolate from a calf
SL5621	*aroA*(*serC*)*1121*::Tn*10*	From SL5608 by transduction
SL5631	*serC*$^+$ Δ*aroA148*	From SL5621 by transduction
SL5650	Δ*aroA* Δ*purA155 zbj-908*::Tn*10*	From SL5631 by transduction
SL5653	Δ*aroA* Δ*purA* CRR[*zbj-908*::Tn*10* (Tc^s)]	From SL5650 by mutation
SL5659	*aroA*$^+$ Δ*purA* CRR[*zbj-908*::Tn*10* (Tc^s)]	From SL5653 by transduction
SL7163	Δ*aroA* Nal^r	From SL5631 by mutation
SL7164	Δ*aroA* Δ*purA* CRR[*zbj-908*::Tn*10* (Tc^s)] Nal^r	From SL5653 by mutation
SL7165	Δ*purA* CRR[*zbj-908*::Tn*10* (Tc^s)] Nal^r	From SL5659 by mutation
S. typhimurium		
TT472	LT2 *aroA*(*serC*)*1121*::Tn*10*	Hosieth and Stocker (14)
aro148	LT2 Δ*aroA148*	Nishioka et al. (18)
SL5495	Q1 Δ*purA155 zbj-908*::Tn*10*	Edwards and Stocker (9)

[a] Allele symbols are abbreviated after first mention. *aroA*(*serC*)::Tn*10*, Tn*10* insertion in proximal gene, *serC*, or promoter region of *serC-aroA* operon; *zbj*::Tn*10*, Tn*10* insertion in 92 min segment of the linkage map; CRR, Tn*10*-generated complex rearrangement mutation causing phenotypic characters indicated in parentheses (in this case, sensitivity to tetracycline).

[b] Strain SL5608 and its descendents have the nicotinate requirement typically found in *S. dublin*.

strains to be tested in mice to facilitate isolation from tissue: SL7163 (Δ*aroA*), SL7165 (Δ*purA*), and SL7164 (Δ*aroA* Δ*purA*). As only the three nalidixate-resistant mutants were used in the animal experiments described below, we refer to them below as the *aroA*, *purA*, and *aroA purA* strains rather than by their strain numbers and genotypes, which are given in Table 1.

Colonization, invasion, and persistence in mouse tissues. Studies to determine the abilities of the auxotrophic mutants to colonize the small intestine and to invade and persist in mouse tissues were conducted with groups of 20 female BALB/c mice essentially as previously described (4). On days 1, 3, 7, 8, 14, and 21 postinoculation, three to five animals from each group were sacrificed, and tissues (liver, spleen, blood, Peyer's patches, and small intestine) from each animal were removed aseptically and cultured for the presence of the infecting organism. There was evidence for colonization of the small intestines and invasion of and persistence in mouse tissues only with the *aroA* strain, which was isolated from the small intestines, Peyer's patches, livers, and spleens of animals up through day 3 postinoculation (Table 2). Thereafter, only the livers and, beginning at day 8 postinoculation, the small intestines were infected. The *aroA* strain could be isolated from the blood of only one animal on one day (day 3 postinoculation). Of the 20 animals in this group, the *aroA* strain was isolated from the small intestines of 9, from the Peyer's patches of 5, from the livers of 7, from the spleens of 2, and from the blood of 1 (Table 2).

The *purA* strain was isolated from the small intestine, liver, and blood of only a single animal and only on day 1 postinoculation. Thereafter, the *purA* strain was not detected in any tissue throughout the 21 days of the study. Similarly, the *aroA purA* strain was isolated from the Peyer's patches of a single animal at day 1 postinoculation and was not subsequently detected in any tissue throughout the 21 days of the study.

TABLE 2. Colonization, invasion, and persistence in mouse tissues[a]

Strain and tissue type	No. of specimens culture positive/no. tested on day:					
	1	3	7	8	14	21
aroA						
Small intestine	3/3	3/3	0/3	1/3	1/3	1/5
Peyer's patches	3/3	2/3	0/3	0/3	0/3	0/5
Liver	2/3	1/3	1/3	1/3	1/3	1/5
Spleen	1/3	1/3	0/3	0/3	0/3	0/5
Blood	0/3	1/3	0/3	0/3	0/3	0/5
purA						
Small intestine	1/3	0/3	0/3	0/3	0/3	0/4
Peyer's patches	0/3	0/3	0/3	0/3	0/3	0/4
Liver	1/3	0/3	0/3	0/3	0/3	0/4
Spleen	0/3	0/3	0/3	0/3	0/3	0/4
Blood	1/3	0/3	0/3	0/3	0/3	0/4
aroA purA						
Small intestine	0/3	0/3	0/3	0/3	0/3	0/4
Peyer's patches	1/3	0/3	0/3	0/3	0/3	0/4
Liver	0/3	0/3	0/3	0/3	0/3	0/4
Spleen	0/3	0/3	0/3	0/3	0/3	0/4
Blood	0/3	0/3	0/3	0/3	0/3	0/4

[a] Groups of female BALB/c mice were given a single dose containing 10^{10} CFU of one or another of the three auxotrophic strains by gavage. Each group contained 20 mice.

Humoral response following immunization with the attenuated mutants. A major consideration in the selection of an appropriate live vaccine or carrier organism is the ability of that organism to evoke an appropriate immunologic response. As an indicator of that response, we examined the serum IgG and mucosal IgA responses against the lipopolysaccharide (LPS) of the parent *S. dublin* strain, SL5608. Anti-LPS was determined by enzyme-linked immunosorbent assay as previously described (4). For this study, groups of female BALB/c mice were immunized by gavage as described above with two doses, each containing 10^{10} CFU of one or another of the three auxotrophic strains, on days 0 and 4.

Mice immunized orally with the *aroA*, *purA*, or *aroA purA* strain developed serum anti-LPS antibodies and maintained them throughout the course of the experiment, 5 weeks post-primary inoculation (Table 3). There was, however, great variability between individual animals in all groups; statistical differences between values from immunized groups and control values from unimmunized animals were not consistent at 1, 2, or 3 weeks following the primary inoculation. By the end of week 5, serum anti-LPS IgG had increased from 0 to 14.1 μg/ml in animals immunized with the *aroA* strain, a value significantly greater than that obtained after immunization with either the *purA* strain (2.89 μg/ml) or the *aroA purA* strain (2.37 μg/ml).

Less initial variability was observed between animals examined for the presence of mucosal anti-LPS IgA. Mucosal anti-LPS IgA was consistently significantly higher in animals immunized with the *aroA* strain than in animals from the other two groups (Table 3). By the end of week 5,

TABLE 3. Serum and mucosal anti-LPS responses[a]

Strain	IgG concn (μg/ml) (mean ± SEM) at week:					IgA concn (ng/ml) (mean ± SEM) at week:				
	1	2	3	4	5	1	2	3	4	5
aroA	1.32 ± 0.30	4.83 ± 0.73	7.71 ± 2.78	7.66 ± 2.00	14.1 ± 4.21	2.78 ± 0.72	45.29 ± 12.72	15.28 ± 2.75	26.45 ± 7.04	27.3 ± 5.19
purA	0.47 ± 0.25	0.85 ± 0.25	2.80 ± 1.95	0.58 ± 0.22	2.89 ± 2.17	0.57 ± 0.19	1.41 ± 0.17	0.40 ± 0.19	0.17 ± 0.07	1.36 ± 0.85
aroA purA	8.05 ± 6.6	5.35 ± 3.31	5.00 ± 1.44	1.32 ± 0.91	2.37 ± 0.82	1.08 ± 0.73	0.14 ± 0.05	0.39 ± 0.16	0.21 ± 0.09	0.97 ± 0.75

[a] Groups of 20 female BALB/c mice were immunized by gavage with two doses, each containing 10^{10} CFU of one or another of the three auxotrophic strains, on days 0 and 4. Inocula for immunization were prepared as described previously (4). Animals were sacrificed at weekly intervals over a 5-week period and analyzed for production of serum and mucosal antibodies against the LPS of SL5608 by enzyme-linked immunosorbent assay. The mean and standard error of the mean of serum and intestinal antibody concentrations were calculated for each group of mice at each time point; means of variously immunized groups were compared with the values for nonimmunized mice (all 0) by the Student *t* test. Statistical significance was considered to be $P \leq 0.05$.

mucosal anti-LPS IgA had increased to 27.3 ng/ml in animals immunized with the *aroA* strain, to 1.36 ng/ml in animals immunized with the *purA* strain, and to 0.97 ng/ml in animals immunized with the *aroA purA* strain.

These studies demonstrate that of the three strains tested, only SL7163, with the single *aroA* mutation, was able to colonize significantly, invade, and persist in tissues. More importantly, this strain was shown to consistently evoke appropriate serum and mucosal antibody responses. Neither the *purA* nor the *aroA purA* mutant demonstrated these characteristics. We do not know if the presence or lack of observed statistical significance (as determined by the Student *t* test) during the 5 weeks of the experiment correlates with biological significance. Clearly, immunization with SL7163, with the single *aroA* mutation, was more effective at eliciting appropriate antibody responses, especially at the mucosal surface.

These observations suggest that the *purA* defect, which causes an adenine requirement, reduces the live-vaccine efficacy of attenuated *Salmonella* strains and may limit the effectiveness of *Salmonella* strains as carriers of heterologous antigens. This was also suggested by the findings of O'Callaghan et al. (19) in a study characterizing aromatic- and purine-dependent *S. typhimurium* for virulence, persistence, and the ability to induce protective immunity following intravenous and oral immunization of BALB/c mice. Those authors did not measure serum and mucosal antibody responses but did demonstrate that organisms containing the single *aroA* deletion, given orally or intravenously, were more effective at protecting against intravenous challenge than were mutants containing either a single *purA* deletion or both deletions together. Our findings differed from theirs in the degree of persistence of the various mutants. In their study all three mutants persisted in livers and spleens for up to 10 weeks after intravenous inoculation. We immunized orally and found that only the *aroA* mutants were able to colonize the small intestine and to invade and persist in mouse tissues. These differences can probably be ascribed to the different routes of inoculation.

We conclude from these results that even though the *purA* mutation decreases the virulence of these organisms, it so attenuates the organisms as to make them unsuitable for use in live vaccines. It is important to note, however, that the *purA* deletion in these strains was generated by P22-mediated transduction in which strain SL5631 (*aroA*) was used as the recipient, with strain SL5495, which is *S. typhimurium* LT2 *purA155 zbj-908*::Tn*10*, as the donor. *zbj-908*::Tn*10* is a silent Tn*10* insertion at a point such that cotransduction of *purA* with it occurs at about 30% (M. F. Edwards and B. A. D. Stocker, unpublished observation). A tetracycline-sensitive mutant not detectably altered in other properties, inferred to have arisen by a Tn*10*-generated deletion-inversion mutation at *zbj-908*::Tn*10*, was subsequently isolated for use as the *aroA purA* strain. We cannot completely rule out the potential influence of *zbj-908*::Tn*10* on the observed results or the possibility that cryptic stretches of genomic DNA may have been replaced during the P22-mediated transduction. As noted previously, the methods used were similar to those used to make 541Ty and 543Ty, the Δ*aroA his* Δ*purA* strains of *S. typhi* used in the human vaccine studies, and the comparison with those strains is valid. These findings may be important, then, in the selection of an attenuated *S. typhi* strain for use in humans, either as an antityphoid live vaccine or as a vector for antigens of other pathogens.

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