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Tests of the Virulence and Live-Vaccine Efficacy of Auxotrophic and *galE* Derivatives of *Salmonella choleraesuis*

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Aromatic compound-dependent (*aro*) derivatives of three mouse-virulent strains of *Salmonella choleraesuis* (*Salmonella cholerae-suis*) were constructed and shown to be nonvirulent for mice (intraperitoneal [i.p.] 50% lethal dose [LD_{50}], $>5 \times 10^6$ CFU). A *pur* derivative and a *thy* derivative, each of a different virulent parent, remained moderately virulent (i.p. LD_{50}s for BALB/c mice, ca. 10^5 and 5×10^4 CFU, respectively). Tested as live vaccines i.p., the *aro* strains were ineffective in salmonella-susceptible BALB/c and C57BL/6 mice but were somewhat effective in salmonella-resistant CBA/J mice and in outbred CD-1 mice. The *pur* and *thy* strains were effective as live vaccines in BALB/c mice when given in sublethal doses. Two previously isolated nonvirulent *galE* derivatives of *S. choleraesuis* (i.p. LD_{50} in BALB/c mice, $>10^6$ CFU) were also ineffective as live vaccines in BALB/c and C57BL/6 mice. The main antigenic difference between *S. choleraesuis* (O-6,7) and *S. typhimurium* (O-4,12) is in O-antigen character, thought to largely determine the specificity of protection in salmonellosis. Paired, nearly isogenic O-6,7 and O-4,12 derivatives were constructed from an *aro S. typhimurium* strain of proven efficacy as a live vaccine. Used as live vaccines, the O-4,12 member protected BALB/c mice against challenge with virulent *S. typhimurium*, whereas the O-6,7 member did not protect against virulent *S. choleraesuis*. However, BALB/c mice vaccinated with the O-6,7 member and mice vaccinated with an *aro S. choleraesuis* strain were protected against challenge with a moderately virulent (LD_{50}, 5×10^4 CFU) O-6,7 derivative of an *S. typhimurium* strain.

Salmonella choleraesuis (*Salmonella cholerae-suis*) is the cause of swine paratyphoid, a widespread diesease of pigs (2, 3, 11, 16, 32). Human infections with this organism are clinically distinctive, with a high incidence of septicemia and multiple focal abscesses but a low rate of intestinal involvement and a case fatality rate of 16 to 20% (19, 27, 38).

No vaccine is known to be effective in the control of *S. choleraesuis* infections. A rough variant of *S. choleraesuis* isolated by H. W. Smith (30) has been used as a live vaccine in Europe but is not in use in the United States. Live attenuated strains of salmonella (5, 8, 12, 13, 24, 33–35) and killed organisms given with complete Freund adjuvant (5, 33, 35) are generally effective as vaccines. In some systems, even repeated administration of killed organisms without adjuvants does not protect effectively (5). Salmonella strains of proven efficacy as live vaccines include those deficient in aromatic biosynthesis (*aro* mutants) and those deficient in the enzyme UDP-glucose-4-epimerase (*galE* mutants). Aromatic compound-deficient mutants of *S. typhimurium* and of *S. dublin* constructed by S. K. Hoiseth and B. A. D. Stocker were highly effective as live vaccines in both mice and calves (13, 24, 29). *galE* mutants of *Salmonella typhimurium* were also effective as live vaccines in mice (8) and calves (39). A *galE* mutant of *Salmonella typhi* designated Ty21a (9) is currently undergoing trials in humans and has proven effective as a live vaccine against typhoid.

This study was intended: (i) to test the effects on the virulence of *S. choleraesuis* of auxotrophic mutations that reduce the virulence of other salmonella species; (ii) to evaluate nonvirulent auxotrophic strains and also two previously isolated *galE* mutants (21) for efficacy as live vaccines in mice. The auxotrophic derivatives tested comprised those requiring aromatic compounds (*aro*), purine (*pur*), and thymine (*thy*).

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MATERIALS AND METHODS

Media. The complete media used were Oxoid blood agar base (code CM55; Oxoid Ltd., London, England), Oxoid nutrient broth (code CM67), Difco MacConkey agar (Difco Laboratories, Detroit, Mich.), and Difco MacConkey agar base supplemented with the desired carbohydrate source (5 g/liter). The defined medium used was that of Davis and Mingioli (4), with glycerol (5 ml/liter) and sodium citrate (0.5 g/liter) as carbon sources. In some experiments, these carbon sources were replaced by galactose (5 g/liter) to make *o*-galactose agar.

Phages and genetic methods. A high-transducing derivative of bacteriophage P22, P22 HT105/1 *int* (28), was used for transductions between strains of *S. typhimurium*. No known transducing phages adsorb to smooth strains of *S. typhimurium* (O group B) and *S. choleraesuis* (O group C_1). Rough derivatives of *S. typhimurium* of the types *rfaG*, *rfaH*, *galU*, and *galE*, however, adsorb phage P1 (6, 22), and preliminary tests showed, as expected, that *galE* mutants of *S. choleraesuis* also adsorb the phage. Transductions between *S. typhimurium* and *S. choleraesuis* were therefore carried out with P1 between strains first made *galE* or carrying any rough mutation conferring P1 sensitivity. Lysates of donors were made with phage P1::Tn*9* *c*(ts), a derivative of P1kc carrying the chloramphenicol resistance transposon, Tn*9* (15), and a temperature-sensitive repressor (26). It cannot lysogenize bacteria or persist as a prophage at 37°C but can do so at 30°C. Bacteria were lysogenized by selection for conversion to chloramphenicol (12 μg/ml) resistance at 30°C, and phage was propagated by heat induction of a lysogen. A lysogen was grown in broth at 30°C with shaking, shifted to 42°C for 30 min, and then shifted to 37°C for 1 h, both with shaking. This treatment induced the lytic cycle. A lysed culture was shaken for 5 min with chloroform, clarified by centrifugation, and sterilized by filtration.

TABLE 1. Bacterial strains[a]

Strain	Description	Source or reference
38_1	*S. choleraesuis* wild type	R. Griffith (11)
110	*S. choleraesuis* wild type	R. Griffith (11)
117	*S. choleraesuis* wild type	R. Griffith (11)
UCD108-11	*S. typhimurium* wild type	B. Smith (29)
M7471	*S. typhimurium* FIRN wild type	This lab (22)
SH566	*S. abony strA* HfrH3	P. H. Mäkelä
SL1479	UCD108-11 *hisC527* CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)]	This lab (29)
SL2805	38_1 *galE710*	21
SL2808	38_1 *galE710* CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10*	This study
SL2812	38_1 *galE710* CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10* (F'-8-*gal*)	This study
SL2815	38_1 *thy*	R. Griffith (11)
SL2822	38_1 CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10*	21
SL2829	38_1 *galE710* Gal^s	21
SL2852	117 *galE717* Gal^r	This study
SL2848	UCD108-11 *galE712* CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10* C-*rfb*$^+$ (O-6,7$^+$) B-*rfb*$^-$	This study
SL2856	UCD108-11 CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10* C-*rfb*$^+$ (O-6,7$^+$) B-*rfb*$^-$	This study
SL2863	117 CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10*	This study
SL2864	110 CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10*	This study
SL2880	100 Δ*purHD343 purH887*::Tn*10*	This study
SL2888	UCD108-11 *hisC527* CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10*	This study
SL4381	UCD108-11 C-*rfb*$^+$ (O-6,7$^+$) B-*rfb*$^-$	This study
SL5224	UCD108-11 *galE712 hisC527* CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-903*::Tn*10*	This lab
SL5225	UCD108-11 *galE712 hisC527* CRR426[*aroA544*::Tn*10* (Tc^s, nonrev)] *zbj-904*::Tn*10*	This lab

[a] Symbols and abbreviations: CRR, complex rearrangement mutation (in the present case, Tn*10*-mediated deletion of at least part of the transposon and deletion or inversion of at least part of gene *aroA*); Δ, deletion mutation; Tc^s, tetracycline sensitive; nonrev, nonreverting; *zbj*. . .::Tn*10*, silent insertion of Tn*10* at minute 20 of the chromosome.

Bacterial strains. All strains are listed in Table 1. Mouse-virulent swine isolates of *S. choleraesuis* subsp. Kunzendorf, 38_1, 110, and 117 (11), were received from Ron Griffith of Iowa State University, Ames, Iowa. A mouse- and calf-virulent strain of *S. typhimurium*, UCD108-11 (29), was obtained from Bradford Smith of The University of California at Davis. *S. typhimurium* M7471 was of the FIRN biotype (22). The O-antigenic composition of these bacteria is: strain 38_1 (O-6_2,7); strain 110 (O-6_1,7); strain 117 (O-$6_1$7); strain M7471 (O-4,5,12); and strain UCD108-11 (O-1,4,12).

Isolation of *galE* mutants and construction of *aroA* strains of *S. choleraesuis*. *galE* mutants were isolated by screening spontaneous 2-deoxygalactose-resistant mutants for sensitivity to phage C21 (21). The nonreverting transposon-generated *aroA* mutation introduced into the *S. choleraesuis* strains was that of *S. typhimurium* UCD108-11 *aro* live-vaccine strain SL1479 (29). SL5224 is a *galE* mutant of strain SL1479 given by transduction a Tn*10* insertion, *zbj903*::Tn*10*, in silent DNA closely linked to its *aroA* mutation. Phage P1::Tn*9* *c*(ts) grown on SL5224 was used to evoke tetracycline-resistant transductants, some of which were *aro*, from a *galE* mutant of *S. choleraesuis* 38_1, SL2805, by the drop-on-lawn procedure (17). A phage P1::Tn*9* *c*(ts) lysate of one such transductant was used to construct *zbj903*::Tn*10* *aroA* derivatives of *galE* mutants of *S. choleraesuis* strains 110 and 117. To obtain smooth *aro* vaccine strains, the *galE* defects in all these derivatives were corrected by transduction of *gal*$^+$, by using P1::Tn*9* *c*(ts) propagated on an *rfaH gal*$^+$ strain of *S. typhimurium* (6, 22).

SL5225 is derived from SL1479 and has the same *galE* mutation as SL5224 but carries linked to its *aroA* mutation Tn*10* insertion *zbj904*::Tn*10*, instead of *zbj903*::Tn*10*. The *aroA* live-vaccine strain of *S. typhimurium* used, SL2888, was derived by transducing SL5225 to *gal*$^+$ with P22HT 105/1 *int* propagated on another strain of *S. typhimurium*. For this transduction, SL5225 was grown in nutrient broth supplemented with galactose (5 g/liter) to enable it to synthesize smooth lipopolysaccharide and adsorb P22.

Construction of a purine-requiring derivative of *S. choleraesuis* 110. A strain of *S. typhimurium* with a deletion of parts of the *purD* and *purH* genes (10), which is cotransducible with a Tn*10* insertion in the remaining segment of the *purH* gene, is available in this laboratory (B. A. D. Stocker, unpublished data). A P22 HT105/1 *int* lysate of this strain was used to transduce the *purHD* deletion with the *purH*::Tn*10* insertion into a *galE* derivative of *S. typhimurium* biotype FIRN, by selection for tetracycline resistance; the recipient was grown in nutrient broth with galactose (5 g/liter) to enable it to synthesize smooth lipopolysaccharide. A P1::Tn*9* *c*(ts) lysate of a tetracycline-resistant transductant found to be purine requiring and nonreverting and inferred to be Δ*purHD343 purH887*::Tn*10* was used to transduce the *purH887*::Tn*10* and presumably also the *purHD343* deletion into SL2853, a *galE* derivative of *S. choleraesuis* 110. A smooth, *gal*$^+$ revertant of one such transductant was obtained by selection for galactose utilization on *o*-galactose agar supplemented with 1% nutrient broth; it was numbered SL2880.

Construction of *aro* and *aro*$^+$ *S. typhimurium* strains with O antigen 6,7. The *rfb* region of the salmonella chromosome determines O-antigen structure. We replaced the group B-*rfb* region of SL5225 (UCD108-11 *galE aroA hisC*) with that of *S. choleraesuis* 38_1 (C_1-*rfb*) by cotransduction with *his*$^+$ by using phage P1::Tn*9* *c*(ts) propagated on SL2808 (*S. choleraesuis* 38_1 *galE aroA*). *his*$^+$ transductants acquiring the group C_1-*rfb* region were identified by agglutination in

O-6,7 but not in O-4,12 antiserum. A *his*$^+$ C_1-*rfb*$^+$ transductant of SL5225 was subsequently made smooth by transduction to *gal*$^+$ by using a P1::Tn*9* *c*(ts) lysate of an *rfaH* strain of *S. typhimurium*. One clone determined by phage pattern, galactose fermentation, and nutritional tests to be a *his*$^+$ C_1-*rfb*$^+$ *gal*$^+$ derivative of SL5225 was numbered SL2856. Its UCD108-11 ancestry was confirmed by its lysogeny for an O1-converting phage, antibiogram, and plasmid profile. SL2856 should be like a typical *aroA* derivative of *S. typhimurium* UCD108-11 in phenotype except for lipopolysaccharide character, for which it should resemble *S. choleraesuis*. Compared with *S. choleraesuis*, however, SL2856 was shown to synthesize very little enterobacterial common antigen (ECA), a bacterial surface component and virulence factor for salmonella (36); this was determined by indirect hemagglutination (20). Almost complete absence of ECA in such hybrids is expected because in *S. choleraesuis* the *rfe* locus alone, at minute 84, suffices for synthesis of normal levels of ECA, whereas in *S. typhimurium*, both *rfb* and *rfe* functions are required so that a combination of B-*rfe* and C-*rfb* loci, as in SL2856, results in the synthesis of minimal amounts of ECA (20). When used as a live vaccine, however, such a strain should elicit protection specific for *S. choleraesuis* since the specificity of protection in salmonellosis is determined in a large part by the O antigen (18).

SL4381 is an *aro*$^+$ but O-6,7 derivative of UCD108-11. For construction of this strain, SL2856 (UCD108-11 *aroA*) was crossed with *Salmonella abony* SH566 *strA* HfrH3. Tetracycline-resistant (25 μg/ml) *aro*$^+$ recombinants of SL2856 were selected on minimal agar. A clone identified as an *aro*$^+$ derivative of SL2856 was numbered SL4381. The intraperitoneal (i.p.) 50% lethal dose (LD_{50}) of SL4381 for BALB/c mice was determined to be 4×10^4 CFU. Its relatively low virulence compared with its UCD108-11 ancestor (LD_{50}, <5 CFU) presumably results in part from change in O antigen from 4,12 to 6,7 (37) and in part from its inability to make normal levels of ECA (36).

Animal studies. Specific-pathogen-free BALB/c mice were from the Department of Radiology, Stanford University, Stanford, Calif.; CD-1 mice were from Charles River Breeding Laboratories, Inc., Wilmington, Mass.; CBA/J mice were from Jackson Laboratory, Bar Harbor, Maine; and C57BL/6 mice were from The Institute for Medical Research, San Jose, Calif. Bacteria were grown overnight in nutrient broth at 37°C without shaking. Mice were inoculated i.p. with 0.1 ml of appropriate dilutions in normal saline. For LD_{50} determinations (23), animals were generally observed for 30 days, and mice used for protection determination were observed for at least 45 days. All dead animals were autopsied for gross pathology, especially of the liver. Cultures of livers and spleens were also done for bacteria.

TABLE 2. Virulence i.p. of *S. choleraesuis* derivatives in BALB/c mice

Strain	Genetic defect	LD_{50} (CFU)	
		Derivative	Parent (approx)
SL2822	38_1 *aro*	$>6 \times 10^6$	100
SL2863	117 *aro*	$>3 \times 10^6$	500
SL2864	110 *aro*	$>5 \times 10^6$	200
SL2852	117 *galE* Gal^r	10^6	500
SL2829	38_1 *galE* Gal^s	$>4 \times 10^6$	100
SL2815	38_1 *thy*	5×10^4	100
SL2880	110 *pur*	10^5	200

TABLE 3. Efficacy as live vaccine of *S. choleraesuis* 38_1 *aro* (SL2822) in Ity^s (BALB/c and C57BL/6) mice

Mouse strain and expt no.	Day(s) of vaccination (3×10^6 to 6×10^6 CFU i.p.)	Challenge with strain 38_1 or homologous parent		Deaths/total; days
		Day	Dose, CFU (no. of LD_{50}s)	
BALB/c				
1	0, 21	35	6×10^3 (60)	5/5; 8, 9, 9, 10, 11
	0, 21	35	6×10^5 (6,000)	5/5; 6, 6, 7, 7, 10
2	0, 14, 24	36	2×10^5 (2,000)	3/8; 8, 8, 13[a]
C57BL/6				
3	0	21	10^4 (100)	4/4; 7, 9, 10, 10
	0, 21	35	10^4 (100)	6/9; 9, 10, 12, 14, 20, 23[a]
4	0, 10, 21	35	6×10^4 (600)	7/8; 7, 9, 9, 9, 9, 9, 15[a]

[a] Data from two separate experiments have been combined.

RESULTS

Virulence of auxotrophic and *galE* derivatives of *S. choleraesuis*. Nonreverting *aroA* derivatives of three mouse-virulent (i.p. LD_{50} for BALB/c mice, ca. 100 to 500 CFU) *S. choleraesuis* parents, 38_1, 110, and 117, were nonvirulent for BALB/c mice (no deaths from inocula of 3×10^6 to 6×10^6 CFU i.p.) (Table 2). This was also true of one of them, 38_1 *aroA*, SL2822, when tested in C57BL/6, CBA/J, and CD-1 mice (no deaths from 5×10^6 CFU) (data not shown). The thymine-requiring mutant of strain 38_1 and the purine-requiring derivative of strain 110 were moderately virulent, with i.p. LD_{50}s of about 5×10^4 and 10^5, respectively, in BALB/c mice (Table 2). The *galE* strains used in this study, SL2829 (38_1 *galE*) and SL2852 (117 *galE*), have been previously tested and found nonvirulent (21). Some other *galE* mutants tested in the same study were as virulent as their *gal*$^+$ parents. Thus, of the kinds of mutants used in this study, only *aroA* mutants are consistently of much reduced virulence, and, therefore, they are the best candidates for testing as live vaccines.

Tests of the protective effects of i.p. vaccination with auxotrophic derivatives in mice. The results of mouse protection studies with the three *aroA* derivatives of *S. choleraesuis* are presented in Tables 3, 4, and 6. The *aroA* strains were tested in four mouse lines which differ in innate susceptibility to salmonella infection. BALB/c and C57BL/6 mice are inbred and salmonella susceptible or Ity^s. CBA/J mice are inbred and salmonella resistant or Ity^r. CD-1 mice are an outbred stock, and batches or individual members of this line might, in theory, differ in Ity character but are probably all Ity^r.

SL2822, strain 38_1 *aroA*, was not effective as a live vaccine in BALB/c mice. Mice vaccinated i.p. with two doses of this strain (3×10^6 CFU per dose) at 21 days apart and challenged 15 days after the second dose all died from i.p. challenge with 60 or 6,000 LD_{50}s of strain 38_1 (Table 3, experiment 1). Three doses of ca. 5×10^6 CFU given at intervals of 12 days protected only five of eight mice against challenge with 2,500 LD_{50}s of 38_1 given 12 days after the third dose of live vaccine (Table 3, experiment 2).

The results of tests in BALB/c mice with the 110 *aroA* (SL2864) and 117 *aroA* (SL2863) strains were similar to those obtained with 38_1 *aroA* (SL2822). In each case, none of four mice vaccinated i.p. (3×10^6 to 6×10^6 CFU) with two

TABLE 4. Efficacy of strain 38_1 *aroA* (SL2822) as a live vaccine[a] in CBA/J (Ity^r) and CD-1 (outbred) mice

Mouse strain and expt no.	Challenge i.p. with strain 38_1		Deaths/total; days
	Day	Dose, CFU (no. of LD_{50}s)	
CBA/J			
5	21	6×10^5 (200)	0/5
	21	6×10^6 (2,000)	3/5; 6, 7, 7
	Control	6×10^5 (200)	5/5; 6, 7, 7, 8, 8
6	43	6×10^5 (200)	1/5; 7
	Control	6×10^5 (200)	5/5; 6, 6, 6, 7, 7
CD-1			
7	21	6×10^6 (120)	0/9[b]
	21	6×10^7 (1,200)	2/9; 9, 10[b]
	Control	6×10^6 (120)	5/8; 7, 10, 10, 14, 15[b]

[a] Mice were vaccinated i.p. with 5×10^6 CFU of SL2822 on day 0; control mice received no vaccination.
[b] Data from two separate experiments have been combined.

doses of the live *aroA* strain, 21 days apart, survived challenge with 140 to 350 LD_{50}s of the homologous virulent parental strain 14 days after the second vaccination (data not shown).

To test the live-vaccine efficacy of SL2822 (38_1 *aroA*) in another Ity^s mouse line, C57BL/6 mice were injected i.p. with one to three doses of 4×10^6 CFU and were then challenged with strain 38_1. All of four mice vaccinated once i.p. died within 10 days after challenge, 21 days later, with 100 LD_{50}s of 38_1 (Table 3, experiment 3). Four similarly vaccinated mice challenged with 10,000 LD_{50}s died even more rapidly (data not shown). Of nine mice vaccinated twice i.p., only three survived challenge with 100 LD_{50}s of 38_1 administered 14 days after the second vaccination (Table 3, experiment 3). Vaccinating the C57BL/6 mice three times before challenge did not improve protective efficacy; of eight mice vaccinated three times i.p. at 10-day intervals, seven died when challenged with 600 LD_{50}s of 38_1 given 14 days after the third vaccination (Table 3, experiment 4).

In tests of SL2822 as a live vaccine in CBA/J (Ity^r) mice, all animals were vaccinated only once i.p. with 5×10^6 CFU. In the first test, mice were challenged i.p. 21 days later with 38_1. All of five mice survived 200 LD_{50}s, whereas three of five mice died from challenge with 2,000 LD_{50}s (Table 4, experiment 5). In the second test, similarly vaccinated mice were challenged on day 43 postvaccination instead of on day 21. All of five mice died from challenge with 2,000 LD_{50}s (data not shown); four of five survived 200 LD_{50}s (Table 4, experiment 6); and all of five survived 20 LD_{50}s (data not shown).

Three tests were done with CD-1 (outbred) mice. The design and results of the first two tests were quite similar, so the data are presented together (Table 4, experiment 7). Nine of nine mice vaccinated i.p. with 5×10^6 CFU of SL2822 survived challenge 21 days later with 120 LD_{50}s of 38_1; of nine mice similarly vaccinated but challenged with 1,200 LD_{50}s of 38_1, two died. By comparison, of eight control mice injected i.p. with 120 LD_{50}s of 38_1, five died. In the third experiment, mice vaccinated only once i.p. were kept for 43 days before challenge with 200 LD_{50}s of 38_1. Four of seven vaccinated mice survived this challenge, compared with only one of seven control animals (data not shown).

Tests of the protective effects of i.p. vaccination with nonvirulent *galE* mutants of *S. choleraesuis*. None of five BALB/c mice vaccinated i.p. with 4×10^6 CFU of SL2829 (38_1 *galE*) survived challenge, 21 days later, with 70 or 7,000 LD_{50}s of 38_1 (data not shown). Four BALB/c mice vaccinated three times i.p. with the same *galE* strain and challenged with only 250 LD_{50}s of 38_1 all died (Table 5, experiment 8). The results of testing SL2829 in C57BL/6 mice were similar to results obtained with BALB/c. Neither one (data not shown), two, nor three i.p. injections of SL2829 (each dose, $>10^6$ CFU) protected C57BL/6 mice against challenge with 40 or 500 to 5,000 LD_{50}s of 38_1 (Table 5, experiment 10).

The second *galE* strain of reduced virulence, SL2852 (117 *galE*), also did not protect BALB/c mice when used i.p. as a live vaccine. Of four mice given two doses of the vaccine strain ($>10^6$ CFU per dose), three died from challenge with 140 LD_{50}s of the parental strain (Table 5, experiment 9); the same result was obtained with the four mice vaccinated three times but challenged with the same dose of strain 117. Thus, the *galE* mutants of two *S. choleraesuis* strains, though nonvirulent, proved ineffective as live vaccines when given i.p. in two or three doses to Ity^s mice.

Comparison of efficacy as live vaccine of *aro* derivatives of *S. choleraesuis* and *S. typhimurium*. SL2856 (O-6,7; ECA^{trace}) and SL2888 (O-1,4,12; ECA^+) are *aro* nearly isogenic derivatives of *S. typhimurium* UCD108-11 which differ in O-antigen character and also in ECA content (see Materials and Methods). BALB/c mice vaccinated with 3×10^6 to 5×10^6 CFU of SL2856 or SL2888 were compared for ability to survive challenge with virulent *S. choleraesuis* or virulent *S. typhimurium*, respectively. Vaccinated animals were expected to survive challenge with the virulent strain with which the vaccine shares O-antigen specificity, since protection in salmonellosis is largely O-antigen specific (18). Eight BALB/c mice vaccinated i.p. once with 4×10^6 CFU of

TABLE 5. Efficacy of *galE* mutants of *S. choleraesius* as live vaccine in BALB/c and C57BL/6 mice

Mouse strain and expt no.	Vaccination (3×10^6–9×10^6 CFU i.p.)		Challenge with strain 38_1 or homologous parent		Deaths/total; days
	Vaccine strain	Days given	Day	Dose, CFU (no. of LD_{50}s)	
BALB/c					
8	SL2829	0, 10, 21	35	2.5×10^4 (250)	4/4; 8, 8, 8, 13
9	SL2852	0, 32	49	7×10^4 (140)[a]	3/4; 8, 10, 11
		0, 32, 49	65	7×10^4 (140)[a]	3/4; 8, 10, 13
C57BL/6					
10	SL2829	0, 21	35	4×10^3 (40)	4/4; 5, 8, 11, 11
		0, 11, 23	38	5×10^4 or 50×10^4 (500 or 5,000)	8/8; 5, 6, 7, 7, 7, 8, 8, 15[b]

[a] Challenged with *S. choleraesuis* 117.
[b] Data from two separate experiments have been combined.

TABLE 6. Comparison in BALB/c mice of the protective effects of i.p. vaccination with *aro* strains SL2822 (*S. choleraesuis* 38_1 *aroA*), SL2856 (*S. typhimurium* UCD108-11 *aroA*, O-6,7), and SL2888 (*S. typhimurium* UCD108-11 *aroA*)

Challenge strain and expt no.	Vaccination (3×10^6–5×10^6 CFU i.p.)		Challenge i.p. with homologous parent		Deaths/total; days
	Vaccine strain	Day(s) of vaccination	Day	Dose, CFU (no. of LD_{50}s)	
S. typhimurium UCD108-11					
11	SL2888 (O-1,4,12)	0	24	0.4×10^6 or 4×10^6 (10^5 or 10^6)	0/8[a]
S. choleraesuis 38_1					
12	SL2822 (O-6,7)	0, 24	38	6×10^4 (600)	2/5; 8, 9
	SL2856 (O-6,7)	0, 24	38	6×10^4 (600)	2/5; 11, 12
SL4381 (UCD108-11 made O-6,7)					
13	SL2856 (O-6,7)	0	21	7×10^6 (200)	0/6
		0, 18	52	7×10^6 (200)	0/6
	SL2822 (O-6,7)	0	21	7×10^6 (200)	2/6; 9, 10
		0, 18	52	7×10^6 (200)	0/6
		Control		7×10^6 (200)	6/6; 3, 3, 3, 3, 5, 5
14	SL2856 (O-6,7)	0	42	7×10^6 (200)	0/6
	SL2822 (O-6,7)	0	42	7×10^6 (200)	0/6
		Control		7×10^6 (200)	6/6; 5, 5, 5, 5, 5, 6

[b] Data from two separate experiments have been combined.

SL2888 (the O-1,4,12 vaccine strain) were solidly protected against challenge with 0.4×10^6 to 4×10^6 CFU (0.1 to 1 million LD_{50}s) of *S. typhimurium* UCD108-11 (Table 6, experiment 11). By contrast, of six mice vaccinated once or twice with the O-6,7 strain, SL2856, all died from challenge with 500 to 6,000 LD_{50}s of *S. choleraesuis* 38_1 (data not shown). However, in experiment 12 (Table 6), mice similarly vaccinated with two doses of the *S. typhimurium* O-6,7 hybrid were partly protected against challenge with *S. choleraesuis* wild type (three survivors of five tested). Similar partial protection against this challenge was seen in mice which had been given two doses of the *S. choleraesuis* 38_1 *aroA* strain (Table 6, experiment 12).

Better protection was observed when the O-6,7 strain used for challenge was SL4381 (*S. typhimurium* UCD108-11 made O-6,7 and thus ECA^{trace}) instead of wild-type *S. choleraesuis* 38_1 as in experiment 12. This hybrid strain had an i.p. LD_{50} for BALB/c mice of ca. 3×10^4 CFU (data not shown) and was thus somewhat less virulent than either *S. choleraesuis* 38_1 (i.p. LD_{50}, ca. 100 CFU) or its *S. typhimurium* ancestor, UCD108-11 (i.p. LD_{50}, <5 CFU). The aim was to test whether mice immunized with an O-6,7 *aro* live vaccine, either *S. choleraesuis* SL2822 or *S. typhimurium* made O-6,7 (and ECA^{trace}), could control multiplication of a virulent O-6,7 strain derived from *S. typhimurium* though unable to control multiplication of *S. choleraesuis* wild type. When challenged with 200 LD_{50}s of SL4381, the six mice vaccinated only once with SL2856 (*S. typhimurium* O-6,7 *aro*) all survived, compared with survival of four of six mice similarly vaccinated with SL2822 (*S. choleraesuis aro*) (Table 6, experiment 13). Of the six mice, each vaccinated with two doses of either strain, all survived a challenge with 200 LD_{50}s of SL4381 given 52 days after the last vaccine dose (Table 6, experiment 13). In a second test, mice were vaccinated i.p. with single injections of SL2822 or SL2856. All the mice vaccinated with either strain survived challenge with 200 LD_{50}s of SL4381 given 42 days after vaccination (Table 6, experiment 14). Thus, challenge with an O-6,7 derivative of *S. typhimurium* revealed fairly good protection by either one or two doses of *aro* O-6,7 bacteria, either *S. choleraesuis* or *S. typhimurium* made O-6,7.

Protection of BALB/c mice by i.p. vaccination with *thy* and *pur* derivatives of *S. choleraesuis*. As noted previously, the

TABLE 7. Protection of BALB/c mice by i.p. vaccination with *thy* and *pur* derivatives of *S. choleraesuis* strains 38_1 and 110

Expt no.	Vaccination			Challenge i.p. with homologous parent		Deaths/total
	Strain	Day	Dose (CFU)	Day	Dose (no. of LD_{50}s)	
15	38_1 *thy*[a]	0	ca. 2.5×10^4	22	6×10^4 (600)	0/7[b]
	38_1 *thy*	0	2.5×10^4	22	6×10^5 (6,000)	0/4
	Control				6×10^4 (600)	4/4
16	38_1 *thy*	0	2.5×10^4	45	6×10^4 (600)	0/3
17	110 *pur*[c]	0	5×10^3 or 5×10^4[d]	78	5×10^5 (2,500)	1/7
	Control				5×10^5 (2,500)	7/7

[a] i.p. LD_{50} of strain 38_1 *thy* for BALB/c mice is ca. 5×10^4 CFU.
[b] Data from two separate experiments have been combined.
[c] i.p. LD_{50} of strain 110 *pur* for BALB/c mice is ca. 10^5 CFU.
[d] The seven mice challenged with strain 110 comprised three mice (of four tested) which recovered after injection of 5×10^4 CFU of 110 *pur* and the four given 5×10^3 CFU of 110 *pur*, all of which survived without apparent ill effect.

thy derivative of *S. choleraesuis* 38_1 and the *pur* derivative of *S. choleraesuis* 110 were moderately virulent and thus were not suitable for use as live vaccines. They were injected i.p. into BALB/c mice in sublethal doses in order to simulate natural infection with *S. choleraesuis* and to test the level of protective immunity that such an infection could engender. In experiment 15 (Table 7), seven of the mice which survived sublethal infection (ca. 5×10^4 CFU i.p.) with the *thy* strain, SL2815, survived challenge with 600 LD_{50}s of 38_1 given 3 weeks after infection; in the same experiment, four of the survivors of *thy* infection survived challenge with 6,000 LD_{50}s of 38_1. Also, all three members of a group of mice surviving sublethal infection with the same dose of the *thy* strain survived challenge with 600 LD_{50}s of 38_1 given 45 days postinfection (Table 7, experiment 16). Tests with the *pur* strain, SL2880, gave results similar to those of the *thy* strain. Two groups of four mice were injected i.p. with the strain, one group with 5×10^4 and the other with 5×10^3 CFU. Three of the four mice injected with 5×10^4 CFU recovered after prolonged illness (overt illness, ruffled fur, or both, for 3 weeks), whereas the four mice given 5×10^3 CFU all survived without apparent ill effects. The seven surviving mice from the two groups were challenged 78 days postinfection with 2,500 LD_{50}s of strain 110 (Table 7, experiment 17). Six of the seven mice survived, whereas all seven control animals died.

DISCUSSION

Live vaccines are more effective against salmonellosis than are killed vaccines. This is thought to be because live vaccines better stimulate cellular immune response, perhaps because of longer persistence. Salmonella strains unable to make normal lipopolysaccharide because of *galE* mutation have been found to be nonvirulent for mice and calves and effective as live vaccines when given by injection or by feeding. Nearly all related salmonella studies have concerned species of O group B [(1),4,(5),12] or D [(1),9,12]. Our experiments were with mouse-virulent strains of *S. choleraesuis* of O group C_1 (O-6,7). We have reported (21) that some *galE* mutants of *S. choleraesuis* were as virulent as their wild-type parents, instead of being much less virulent as in *S. typhimurium* (O group B) or *S. dublin* and *S. typhi* (O group D). We suggested that the absence of galactose in the oligosaccharide repeat unit of the O-6,7 side chain might explain this difference. We have now shown that three *S. choleraesuis* strains given nonleaky nonreverting defects in *aroA* behaved like such mutants of groups B and D in that they showed essentially complete loss of virulence, even for genetically salmonella-susceptible Ity^s mouse lines (e.g., no ill effects from i.p. doses of ca. 5×10^6 CFU, compared with an LD_{50} of ca. 200 CFU for parental strains) (Table 2). This is the result expected if aromatic compound-dependent strains are nonvirulent because of nonavailability in host tissues of *p*-aminobenzoic acid, which is essential to such mutants.

The one purine-dependent mutant and the one thymine-dependent mutant of *S. choleraesuis* studied were of reduced virulence, with i.p. LD_{50}s in Ity^s mice ca. 500 times greater than those of their parent strains (Table 2). The *purHD343* deletion mutation introduced into *S. choleraesuis* has removed parts of two genes in the *purJHD* operon (10), needed for two steps in the de novo pathway leading to IMP; the mutation must therefore have caused a complete block in this pathway. Several workers have reported reduced virulence as a consequence of purine auxotrophy (1, 7, 14). The degree of loss of virulence associated with requirement for purine appears to depend on both the gene affected and the bacterial species. Mutants of *Bacillus anthracis* (14) and of *S. dublin* (W. McFarland and B. A. D. Stocker, unpublished data) unable to convert IMP to AMP are nonvirulent; by contrast, those responding to hypoxanthine (i.e., with a block in any of the reactions leading to IMP) or with a requirement for guanine (i.e., with a block between IMP and GMP) retained virulence, either partial (McFarland and Stocker, unpublished data) or complete (14). The hypoxanthine-responding *pur* derivative of *S. choleraesuis*, which we constructed and found to be of reduced virulence, thus behaved like hypoxanthine-responding *pur* derivatives of *S. dublin*. Thymine-requiring mutants of several salmonella species are of reduced virulence for chicks (31), and thymine-requiring mutants of *S. typhimurium* are of reduced virulence for mice (C. Spurdon and B. A. D. Stocker, unpublished data). The thymine-requiring mutant of *S. choleraesuis* we studied was likewise of moderately reduced virulence.

Of the several classes of auxotrophic strains studied, only the *aroA* were sufficiently nonvirulent to be of interest for possible use as live vaccines. However, *aro* derivatives of the three *S. choleraesuis* strains were not effective as live vaccines when given i.p. in one to three doses to BALB/c and C57BL/6 mice. Such mice died from modest challenge doses of virulent *S. choleraesuis* strains, even after two vaccinations. The only *aro* strain, SL2822, tested in three doses gave virtually no protection to C57BL/6 mice and only partial protection to BALB/c mice challenged with 600 or 6,000 LD_{50}s of its virulent parent. These results differ from the excellent protection given by *aro* and *galE* live vaccines of *S. typhimurium* and *S. dublin*, even in salmonella-susceptible mice. They suggest two possibilities. (i) *aro S. choleraesuis*, either because of its O antigen character or from some other cause, when given as live vaccine to mice does not elicit as strong an immune response as do similarly given *aro* mutants of *S. typhimurium* [O-(1),4,(5),12] and *S. dublin* (O-9,12); or (ii) *S. choleraesuis* wild-type strains (for unknown reasons) can multiply to cause death, even in mice which have mounted a strong immune response, whereas *S. typhimurium* and *S. dublin* wild type cannot.

To investigate these possibilities, we constructed and used as a live vaccine an *aroA* strain of *S. typhimurium* UCD108-11 given the O-6,7 antigen of *S. choleraesuis* 38_1. The aim was to place the O antigen (believed to be the main protective immunogen in salmonella species) of *S. choleraesuis* in a proven effective live-vaccine strain. The resulting *aro* hybrid strain, SL2856, proved as ineffective as SL2822 (*S. choleraesuis* 38_1 *aroA*) in protecting BALB/c mice against challenge with strain 38_1 (Table 6). By contrast, a single dose of SL2888, the O-1,4,12 nearly isogenic sister of SL2856, effectively protected the same mouse strain against challenge with its virulent parent, UCD108-11. This result is compatible with the first hypothesis, that *S. choleraesuis* fails to elicit an adequate immune response, if this failure is a necessary consequence of its O-6,7 antigen character—but is also compatible with the second hypothesis, that virulent *S. choleraesuis* but not virulent *S. typhimurium* can cause fatal infections even in mice that have mounted a strong immune response.

Unlike the O-6,7 *aro* strains, the *pur* (SL2880) and the *thy* (SL2815) derivatives of *S. choleraesuis* effectively protected BALB/c mice against challenge with their parental strains

when used as live vaccine in sublethal doses. This demonstrates that sublethal infection with moderately virulent derivatives of *S. choleraesuis* (by implication also sublethal infection by fully virulent strains) can immunize mice effectively. Mice that survived sublethal inocula of the *thy* or *pur* strains suffered prolonged illness (ruffled fur, overt illness, or both for 2 to 3 weeks), indicating that the strains multiplied and persisted in vivo. In studies to be separately published we have shown that *pur* and *thy S. choleraesuis* given i.p. to mice multiplied greatly and persisted in their livers and spleens and that *aro* O-4,12 *S. typhimurium* multiplied only slightly but persisted, whereas *aro S. choleraesuis* or *aro* O-6,7 *S. typhimurium* organisms were rapidly killed after i.p. injection. We interpret these data to mean that elicitation of protective immunity against *S. choleraesuis* in Ity^s mice requires a degree of antigenic stimulation difficult to achieve with nonvirulent bacteria of this species because of rapid elimination of the bacteria from the reticuloendothelial system.

BALB/c mice vaccinated once or twice either with an *aro* strain of *S. choleraesuis* (SL2822) or with a hybrid *aro* strain, *S. typhimurium* UCD108-11 *aro* made O-6,7 (SL2856), were well protected against challenge with 200 LD_{50}s of SL4381 (*S. typhimurium* UCD108-11 *aro*$^+$ made O-6,7) (Table 6, experiments 13 and 14). Similar vaccination very poorly protected against *S. choleraesuis* 38_1 challenge (Table 6, experiment 12). This shows that vaccination of Ity^s mice with live *aro* O-6,7 bacteria does elicit some degree of protective immunity in Ity^s mice and so supports the hypothesis that *S. choleraesuis* can multiply in Ity^s mice even if they have developed a strong immune response to live vaccine administration.

A different picture was seen in experiments with inbred Ity^r CBA/J mice (Table 4). A single dose of SL2822 (*S. choleraesuis aro*) gave better protection in CBA/J mice than did two doses in Ity^s mice. Our data, like those of Robson and Vas (25), show that Ity^r mice are more readily immunized against salmonellosis than are Ity^s mice. CD-1 (outbred) mice also were better protected against *S. choleraesuis* by vaccination with SL2822 (*aro S. choleraesuis*) than were Ity^s (BALB/c and C57BL/6) mice. Their susceptibility to infection with strain 38_1 and the level of protective effects observed after vaccination with SL2822, however, varied between two experiments.

S. choleraesuis is a highly lethal serotype that is associated with higher case fatality rates in humans than are other salmonellae, and the infection caused in humans is mainly septicemia, with organisms being rarely isolated from the intestinal tract. Our results provide additional evidence that *S. choleraesuis* differs from other pathogenic salmonella species in several other ways. Some *galE* mutants of the organism retain high virulence, unlike those of *S. typhimurium* and *S. typhi*. The *aro* derivatives of *S. choleraesuis* strains were nonvirulent as expected but unexpectedly were not effective as live vaccines when given i.p. to Ity^s mice. Moreover, mice vaccinated with SL2822 (38_1 *aroA*), though not protected against challenge with strain 38_1, were well protected against challenge with a strain of *S. typhimurium* whose O-1,4,12 antigen had been replaced with that (O-6,7) of strain 38_1. These results suggest an unusual mode of interaction with the defense system of the host. Our data suggest two possibilities that together explain the nonprotection by vaccination with nonvirulent derivatives of *S. choleraesuis*. The first is that live bacteria with O antigen 6,7 are poorly immunogenic in mice, perhaps because of poor persistence. The second is that virulent *S. choleraesuis* strains can multiply to kill mice even though the mice have mounted a strong immunologic response.

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LITERATURE CITED

1. **Bacon, G. A., T. W. Burrows, and M. Yates.** 1950. The effects of biochemical mutation on the virulence of *Bacterium typhosum*: the virulence of mutants. Br. J. Exp. Pathol. **31**:714–724.
2. **Barnes, D. M., and D. K. Sorensen.** 1975. Salmonellosis, p. 554–564. *In* H. W. Dunne and A. D. Leman (ed.), Diseases of swine. Iowa State University Press, Ames.
3. **Baskerville, A., and C. Dow.** 1973. Pathology of experimental pneumonia in pigs produced by *Salmonella choleraesuis*. J. Comp. Pathol. **83**:207–215.
4. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
5. **Eisenstein, T. K., and B. M. Sultzer.** 1983. Immunity to salmonella infection. Adv. Exp. Med. Biol. **162**:260–295.
6. **Enomoto, M., and B. A. D. Stocker.** 1974. Transduction by phage P1kc in *Salmonella typhimurium*. Virology **60**:503–514.
7. **Garber, E. D.** 1956. A. nutrition-inhibition hypothesis of pathogenicity. Am. Nat. **90**:181–194.
8. **Germanier, R.** 1970. Immunity in experimental salmonellosis. I. Protection induced by rough mutants of *Salmonella typhimurium*. Infect. Immun. **2**:309–315.
9. **Germanier, R., and E. Furer.** 1975. Isolation and characterization of *galE* mutant Ty21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. J. Infect. Dis. **131**: 553–558.
10. **Gots, J. S., C. E. Benson, B. Jochimson, and K. R. Koduri.** 1977. Microbial models and regulatory elements in the control of purine metabolism. CIBA Found. Symp. **48**:23–38.
11. **Griffith, R. W., and T. T. Kramer.** 1981. Sensitivity of smooth *Salmonella choleraesuis* var. Kunzendorff field strains to antibody and complement under various conditions. Am. J. Vet. Res. **45**:59–66.
12. **Hashimoto, H., T. Honda, M. Kawakami, and S. Mitsuhashi.** 1961. Studies on experimental salmonellosis. IV. Long-lasting immunity of mouse immunised with live vaccine of *Salmonella enteritidis*. Jpn. J. Exp. Med. **31**:181–190.
13. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291**:238–239.
14. **Ivanovics, G., E. Marji, and A. Dobzoy.** 1968. The growth of purine mutants of *Bacillus anthracis* in the body of the mouse. J. Gen. Microbiol. **53**:147–152.
15. **Kondo, E., and S. Mitsuhashi.** 1964. Drug resistance of enteric bacteria. IV. Active transducing bacteriophage P1 *CM* produced by the combination of R factor with bacteriophage P1. J. Bacteriol. **88**:1266–1276.
16. **Lawson, G. H. K., and C. Dow.** 1965. The pathogenesis of oral *S. choleraesuis* infection in pigs. J. Comp. Pathol. **75**:75–81.
17. **Lyman, M. B., B. A. D. Stocker, and R. J. Roantree.** 1977. Comparison of the virulence of 0:9,12 and 0:4,5,12 *Salmonella typhimurium his*$^+$ transductants for mice. Infect. Immun. **15**: 491–499.
18. **Lyman, M. B., B. A. D. Stocker, and R. J. Roantree.** 1979. Evaluation of the immune response directed against the *Salmonella* antigenic factors O4,5 and O9. Infect. Immun. **26**:956–965.
19. **MacCready, R. A., J. P. Reardon, and I. Saphra.** 1957. Salmonellosis in Massachusetts: a sixteen-year experience. N. Engl. J. Med. **256**:1121–1128.
20. **Mäkelä, P. H., and H. Mayer.** 1974. Participation of lipopolysaccharide genes in the determination of the enterobacterial common antigen: analysis in *Salmonella* groups B and C_1. J. Bacteriol. **119**:765–770.
21. **Nnalue, N. A., and B. A. D. Stocker.** 1986. Some *galE* mutants of *Salmonella choleraesuis* retain virulence. Infect. Immun.

54:635–640.

22. **Ornellas, E. P., and B. A. D. Stocker.** 1974. Relation of lipopolysaccharide character to P1 sensitivity in *Salmonella typhimurium*. Virology **60**:491–502.
23. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**:493–497.
24. **Robertsson, J. Å, A. A. Lindberg, S. Hoiseth, and B. A. D. Stocker.** 1983. *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. Infect. Immun. **41**:742–750.
25. **Robson, H. G., and S. I. Vas.** 1972. Resistance of inbred mice to *Salmonella typhimurium*. J. Infect. Dis. **126**:378–386.
26. **Rosner, J. L.** 1972. Formation, induction and curing of bacteriophage P1 lysogens. Virology **48**:679–689.
27. **Saphra, I., and J. H. Winter.** 1957. Clinical manifestations of salmonellosis in man: an evaluation of 7,779 human infections identified at the New York Salmonella Center. N. Engl. J. Med. **256**:1128–1134.
28. **Schmieger, H.** 1972. Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119**:75–88.
29. **Smith, B. P., M. Reina-Guerra, S. K. Hoiseth, B. A. D. Stocker, F. Habasha, E. Johnson, and F. Merritt.** 1984. Aromatic-dependent *Salmonella typhimurium* as modified live vaccines for calves. Am. J. Vet. Res. **45**:59–66.
30. **Smith, H. W.** 1965. The immunization of mice, calves and pigs against *Salmonella dublin* and *Salmonella choleraesuis* infections. J. Hyg. **63**:117–135.
31. **Smith, H. W., and J. F. Tucker.** 1976. The virulence of trimethoprim-resistant thymine-requiring strains of salmonella. J. Hyg. **76**:97–108.
32. **Thomas, P.** 1977. *Salmonella choleraesuis* infection in pigs with special reference to England and Wales. Vet. Bull. **47**:731–739.
33. **Ushiba, D., R. Iwahata, K. Saito, T. Akiyama, and M. Yoshioka.** 1954. Studies on the immunity of experimental typhoid. III. A comparative study of active and passive immunizations against the infection of mice with *Salmonella enteritidis*. Keio J. Med. **3**:73–78.
34. **Ushiba, D., K. Saito, T. Akiyama, M. Nakano, T. Sugiyama, and E. Shirono.** 1959. Studies on experimental typhoid: bacterial multiplication and host-cell response after infection with *Salmonella enteritidis* in mice immunized with live and killed vaccines. Jpn. J. Microbiol. **3**:231–242.
35. **Ushiba, D., M. Yoshioka, R. Iwahata, and S. Yamagata.** 1953. Studies on the immunity of experimental typhoid. II. The effects of the immunization with killed vaccines on *Salmonella enteritidis* infection of mice. Keio J. Med. **2**:75–89.
36. **Valtonen, M. V., U. M. Larinkari, M. Plosila, V. V. Valtonen, and P. H. Mäkelä.** 1976. Effect of enterobacterial common antigen on mouse virulence of *Salmonella typhimurium*. Infect. Immun. **13**:1601–1605.
37. **Valtonen, V. V.** 1970. Mouse virulence of salmonella strains: the effect of different smooth-type O side-chains. J. Gen. Microbiol. **64**:255–268.
38. **Weissbluth, M., S. T. Shulman, B. Holson, and C. Lerner.** 1981. *Salmonella choleraesuis*: a distinctive bacterial pathogen. J. Pediatr. **98**:423–426.
39. **Wray, C., W. J. Sojka, J. A. Morris, and W. J. B. Morgan.** 1977. The immunization of mice and calves with *galE* mutants of *Salmonella typhimurium*. J. Hyg. **79**:17–24.