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Genes *aroA* and *serC* of *Salmonella typhimurium* Constitute an Operon

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Genetic analysis of *aroA554*::Tn*10* derivatives of two mouse-virulent *Salmonella typhimurium* strains, "FIRN" and "WRAY," and of a nonreverting derivative of each constructed for use as a live vaccine, showed the site of the insertion among mapped *aroA* point mutants. The WRAY live-vaccine strain gave no *aro*$^+$ recombinants in crosses with *aroA* point mutations to one side of the insertion, indicating a deletion from Tn*10* through the sites of these point mutations. The FIRN live-vaccine strain gave wild-type recombinants with all tested point mutants; it probably has a deletion or inversion extending from Tn*10* into *aroA* but not as far as the nearest point mutation. Some tetracycline-sensitive mutants of *aroA554*::Tn*10* strains required serine and pyridoxine, indicating loss of *serC* function, and some that were found to be SerC$^-$ did not produce gas from glucose, indicating a loss of *pfl* function. These results show the gene order *pfl-serC-aroA*, as in *Escherichia coli*. Ampicillin enrichment applied to pools of tetracycline-sensitive mutants of strains with Tn*10* insertions near *aroA* (i.e., *zbj*::Tn*10* strains) yielded Aro$^-$ SerC$^-$ Pfl$^-$, Aro$^-$ SerC$^+$ Pfl$^+$, and Aro$^-$ SerC$^-$ Pfl$^+$ mutants but none which were Aro$^+$ SerC$^-$. All of the mutants are explicable by deletions or inversions extending clockwise from *zbj*::Tn*10* into or through an operon comprising *serC* (promoter-proximal) and *aroA*. Such an operon was also shown by the identification of two Tn*10* insertions causing phenotype Aro$^-$ SerC$^-$, each able to revert to Aro$^+$ SerC$^+$ by precise excision. *serC* corresponds to the open reading frame promoter-proximal to *aroA* that was identified elsewhere by base sequencing of a cloned *aroA* segment of *S. typhimurium* (Comai et al., Science 221:370–371, 1983). Both serine and chorismate are precursors of enterochelin; this may be why *serC* and *aroA* are in a single operon.

We have described the construction of strains of *Salmonella* sp. with nonleaky, nonreverting blocks in the common aromatic biosynthesis pathway. They were isolated as secondary deletion or deletion-inversion mutations in strains made *aroA554*::Tn*10*. Such strains proved to be nonvirulent because, we believe, of their requirement for *p*-aminobenzoate and for 2,3-dihydroxybenzoate (DHB) (which are not available in mammalian tissues); some of them have been found effective as live vaccines in mice and calves (7, 15, 21).

The safety of a live-vaccine strain depends on its inability to revert to virulence. We therefore undertook genetic characterization of two nonreverting *aro* live-vaccine strains by crosses with 10 *aroA* mutants mapped by Nishioka et al. (12). One vaccine strain, SL3261, proved to be deleted for one segment of gene *aroA*. This gene, at 19 min on the linkage map (17), specifies 3-enolpyruvateshikimate-5-phosphate synthetase, which catalyzes a reaction of the aromatic biosynthesis pathway. In the course of our examination of tetracycline-sensitive mutants with Tn*10*-generated deletion or inversion mutations extending from *aroA554*::Tn*10* or from silent insertions of Tn*10* near *aroA*, we encountered mutants requiring serine and pyridoxine. This is the phenotype of *Escherichia coli* with mutations of gene *serC* (3, 20), which is located at about 20 min (thus near *aroA*) and determines the enzyme phosphoserine aminotransferase (EC 2.6.1.52). Genetic analysis of these and other mutants indicated that in *Salmonella typhimurium*, genes *aroA* and *serC* are in the same operon, with *serC* being promoter proximal. We later identified two Tn*10* insertions causing requirement both for aromatic metabolites and for serine and pyridoxine, i.e., phenotype Aro$^-$ SerC$^-$. We describe these two insertion mutants first, because they prove the existence of the *serC aroA* operon and thus simplify the analysis of the deletion and inversion mutants. In the Discussion section we speculate about a possible reason for the grouping of *serC* and *aroA* into an operon, so permitting their coregulation.

MATERIALS AND METHODS

Bacterial strains and phages. The main strains used are listed in Table 1. Most of the *aro*::Tn*10* strains were from the collection of auxotrophic Tn*10* insertion mutants of John Roth (University of Utah). The *aroA* point mutants (12) were obtained from K. Sanderson (Salmonella Genetic Stock Centre, University of Calgary). The general transducing phage used was an *int* (integration-deficient) mutant of a "high-transducing" variant, P22 HT105/1 (19), of phage P22. Phage λNK55 (9) was used for Tn*10* mutagenesis.

Culture media and cultural methods. The nutrient agar and nutrient broth used were Oxoid blood agar base (code CM55) and Oxoid nutrient broth no. 2 (code CM67). The defined medium used was that of Davis and Mingioli (5) with glycerol at 5 ml/liter as a carbon and energy source; it was used either as liquid or solidified with either New Zealand agar (15 g/liter; Davis Gelatin Co., Christchurch, New Zealand) for routine purposes, or Difco Noble agar (20 g/liter), for tests of requirement of minor aromatic metabolites. Required amino acids were added to ca. 20 μg/ml, usually by spreading; *p*-aminobenzoic acid, DHB, and other vitamins were added to ca. 1/10 this concentration. Except where otherwise stated, cultures were incubated at 37°C, without shaking in the case of broth cultures. Because of the accumulation of

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TABLE 1. Main bacterial strains[a]

Strain no.	Relevant genotype[b] and/or phenotype	How obtained[c] or source (reference)
SL1027	LT2 *metA22 trpD2*, etc.	(22)
SL1346	S2337/65 (WRAY) *hisG46 aroA554*::Tn*10*	(7,21)
SL2409	As SL1346 but DEL*409* [*aroA554*::Tn*10* (Tc^s $SerC^-$)]	From SL1346 by Bochner sel.
SL2411	As SL1346 but INV*411* [*aroA554*::Tn*10* (Tc^s $SerC^-$)]	From SL1346 by Bochner sel.
SL2412	As SL1346 but CRR*412* [*aroA554*::Tn*10* (Tc^s)]	From SL1346 by Bochner sel.
SL2415	As SL1346 but DEL*415* [*aroA554*::Tn*10* (Tc^s $SerC^-$ Pfl^-)]	From SL1346 by Bochner sel.
SL2416	As SL1346 but CRR*416* [*aroA554*::Tn*10* (Tc^s)]	From SL1346 by Bochner sel.
SL2417	As SL1346 but DEL*417* [*aroA554*::Tn*10* (Tc^s $SerC^-$ Pfl^-)]	From SL1346 by Bochner sel.
SL2418	As SL1346 but CRR*418* [*aroA554*::Tn*10* (Tc^s)]	From SL1346 by Bochner sel.
SL2421	As SL1346 but INV*421* [*aroA554*::Tn*10* (Tc^s $SerC^-$)]	From SL1346 by Bochner sel.
SL2422	AS SL1346 but CRR*422* [*aroA554*::Tn*10* (Tc^s)]	From SL1346 by Bochner sel.
SL2438	As SL1027 but *zbj-903*::Tn*10* (P22)	From SL1027 by transduction
SL2439	As SL1027 but *zbj-904*::Tn*10* (P22)	From SL1027 by transduction
SL2440	As SL1027 but CRR*436* [*zbj-904*::Tn*10* (Tc^s Aro^- $SerC^-$ Pfl^-)]	From SL2439 by Bochner/Amp. sel.
SL2441	As SL1027 but CRR*437* [*zbj-904*::Tn*10* (Tc^s Aro^-)]	From SL2439 by Bochner/Amp. sel.
SL2444	As SL1027 but *zbj-903*::Tn*10*	From SL1027 by transduction
SL2445	As SL1027 but *zbj-904*::Tn*10*	From SL1027 by transduction
SL2447	LT2 aro^+ *zbj-905*::Tn*10*	From *aroA102* by transduction
SL2454[d]	As SL1027 but CRR*442* [*zbj-903*::Tn*10* (Tc^s Aro^- $SerC^-$)]	From SL2444 by Bochner/Amp. sel.
SL2456	As SL1027 but DEL*444* [*zbj-903*::Tn*10* (Tc^s Aro^- $SerC^-$ Pfl^-)]	From SL2444 by Bochner/Amp. Sel.
SL2459	As TS736 but *aroA-(serC)1123*::Tn*10*	From TS736 by λ::Tn*10* mutagenesis
SL2462	As SL1027 but CRR*446* [*zbj-904*::Tn*10* (Tc^s Aro^- $SerC^-$ Pfl^-)]	From SL2445 by Bochner/Amp sel.
SL3218	M7471 (FIRN) (ColE1-K30) *leu-1051 malB479 cysI1173 hisC527 aroA554*::Tn*10*	(7,21)
SL3235	As SL3218 but CRR*401* [*aroA554*::Tn*10* (Tc^s)]	(7,21)
SL3261	As SL1346 but DEL*407* [*aroA554*::Tn*10* (Tc^s)] Fus^r [e]	From SL1346 by Bochner sel. (7,21)
TS736	As SL1027 but *hspLT6 hspS29 ilv-452 galE496 his-6165* Δ*malB*/F'112 (*lam*B^+)	P. Liljestrom (16)
TT472	LT2 *aroA-(serC)1121*::Tn*10*	J. Roth
TT1455[f]	LT2 *aroA554*::Tn*10*, Gas^-	J. Roth

[a] All strains are *S. typhimurium*, derived from wild-type strain LT2, strain S2337/65 (WRAY) (7,21) or M7471 (FIRN) (7,21).

[b] Tn*10*-generated mutations causing loss of tetracycline resistance are indicated as CRR (complex rearrangement, nature not known), DEL (deletion), or INV (inversion), numbered in a single series; the original Tn*10* insertion is recorded within brackets, followed by notation, within parentheses, of new phenotypic traits. All the listed tetracycline-sensitive (Tc^s) mutants of *aroA554*::Tn*10* strains were nonreverting (to aro^+).

[c] Bochner sel., Selection for tetracycline sensitivity on Bochner medium (2) with DHB. Bochner/Amp sel., Selection for tetracycline sensitivity, then ampicillin enrichment for (new) auxotrophy.

[d] Strain SL2454 gave a "pale" reaction in glucose-phenol red broth.

[e] Strain SL3261 was obtained by selection on Bochner medium without added DHB; it was later found to have a mutation causing partial resistance to fusaric acid (Fus).

[f] Strain TT1455 fails to produce gas in glucose-phenol red broth, apparently from some cause other than its *aroA554*::Tn*10* character.

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aro^+ revertants in stock cultures held at room temperature, strains with *aro*::Tn*10* mutations were stored at −70°C with glycerol or reisolated after selection for tetracycline resistance, before use. Gas production was tested in Difco glucose-phenol red broth with a Durham tube.

Phage and genetic methods. Phage P22 HT105/1 *int* was propagated by overnight incubation of broth inoculated with the propagating strain and a phage inoculum, usually ca. 3×10^7 CFU and ca. 3×10^5 PFU per 10 ml of broth. If the propagating strain carried Tn*10*, the broth used was supplemented with tetracycline (25 μg/ml). Most lysates had titers of 5×10^9 to 10×10^9 PFU/ml. Phage λNK55 was propagated on an *E. coli* amber-suppressor strain. Transduction was effected by the "drop-on-lawn" method (6) with serial 10-fold dilutions of lysate for quantitation. Tetracycline-resistant transductants were selected on nutrient agar with tetracycline (25 μg/ml); aro^+ transductants were selected on defined medium. Transductant colonies were streaked out for single-colony reisolation on selective medium before tests of phenotype, etc.

The medium used to test for reversion of *aro* mutants was defined medium with a growth-limiting amount, usually 20 μg per plate, of L-tryptophan and an excess of all other required substances; plates were inoculated by flooding with broth culture. After incubation, aromatic-independent revertants were easily detected as colonies in the thin film of growth (ca. 10^{10} CFU per plate) of aromatic-dependent bacteria. Reconstruction experiments have shown that reversion at a rate of 10^{-11} per bacterium per generation would be detected by this method, with use of 10 plates (21).

Tetracycline-sensitive mutants of Tn*10*-containing strains were obtained by selection on the autoclaved chlortetracyline-fusaric acid medium devised by Bochner and his colleagues (2), or on the modified medium with glucose omitted (10). (For brevity we call this procedure "Bochner selection.") We found that *aro* strains of *S. typhimurium*, even if tetracycline sensitive, did not grow on Bochner medium, perhaps because of chelation of ferric ions by its fusaric acid component; addition of DHB at ca. 2 μg/ml (allowing synthesis of enterochelin by *aro* bacteria) permitted normal growth of *aro* tetracycline-sensitive bacteria but did not prevent the inhibition of growth of Tn*10*-

carrying bacteria, *aro* or *aro*$^+$. (Strain SL3261, the "WRAY" live-vaccine strain, had been obtained as a rare Bochner-resistant mutant on medium without DHB; it was later found to have a mutation causing partial resistance to fusaric acid.) Bochner-resistant mutants were purified by single-colony reisolation on the selection medium.

For Tn*10* transposon mutagenesis we used a λ::Tn*10* phage, λNK55 (9), and the Palva (13) *S. typhimurium* strain TS736, which has an F′ factor carrying *lamB*$^+$ of *E. coli* and is restriction negative for the LT and SA systems of *S. typhimurium*. Strain TS736 was grown with shaking in liquid defined medium with maltose as energy source (because without such selection the F′ was rapidly lost); $MgSO_4$, to 10 mM, was added to the log-phase culture, and then phage λNK55 was added (ca. 3×10^8 PFU/ml). After 30 min of incubation at 30°C to allow adsorption, broth was added, and the mixture was incubated at 37°C for 75 min. Plates inoculated with 0.1-ml volumes of 10× concentrated culture yielded ca. 500 tetracycline-resistant colonies per plate. Such colonies were washed off in defined medium and subjected to ampicillin enrichment.

Ampicillin enrichment was used to select for auxotrophs among (i) tetracycline-sensitive mutants of Tn*10*-containing strains, obtained by Bochner selection, and (ii) tetracycline-resistant Tn*10* insertion mutants obtained from strain TS736 by λ::Tn*10* mutagenesis. Colonies washed from selection plates were diluted in defined medium with any required supplements to give a faintly turbid suspension. This suspension was shaken at 37°C for 1 to 2 h; glucose was added to 5 g/liter, and shaking was continued until the culture was moderately turbid. Ampicillin was then added to 2.5 mg/ml, and shaking at 37°C was continued for 90 min. Volumes of 1 ml were then membrane filtered, the deposited bacteria were washed on the filter with deionized water, and the membrane filter was then placed face down on an appropriate plate, either Bochner medium with DHB or nutrient agar with tetracycline (25 μg/ml). After 20 min, the filter was removed and the bacteria were spread with a glass rod.

RESULTS

Assignment of aro::Tn*10* mutations to loci and identification of *aroA*(*serC*)::Tn*10* insertion mutations. The *aro-554*::Tn*10* insertion mutation used for construction of nonvirulent strains of *S. typhimurium* and *Salmonella dublin* (7, 21) was shown by transductional crosses to be at *aroA*. Testing of 14 Tn*10* insertion auxotrophs with unidentified (because complex) nutritional requirements showed that one of them, strain TT472, required not only aromatic metabolites but also serine and pyridoxine, i.e., was of Aro$^-$ SerC$^-$ phenotype. Strain TT472 readily gave rise to pyridoxine-independent mutants which remained serine dependent (and aromatic dependent); frequent mutational loss of pyridoxine requirement has been reported for *serC* mutants of *E. coli* (20). Strain TT472 gave rise to prototrophic, tetracycline-sensitive revertants at about the frequency expected for precise excision of Tn*10*. Lysates of TT472 applied to *aro*$^+$ recipients evoked tetracycline-resistant transductants; all of 25 tested had the Aro$^-$ SerC$^-$ phenotype of the donor. When *aroA* strains (in line LT2) were crossed, as transductional donors, to strains given the Aro$^-$ SerC$^-$ tetracycline resistance character of TT472, some transductants selected as requiring neither serine nor pyridoxine were aromatic independent and tetracycline sensitive and others were Aro$^-$ and tetracycline sensitive. This shows that the mutation of TT472 is closely linked to *aroA*; we indicate its insertion mutation as *aroA*(*serC*)*1121*::Tn*10*.

Another Tn*10* insertion mutant of Aro$^-$ SerC$^-$ phenotype was obtained by λ::Tn*10* mutagenesis. Tetracycline-resistant clones were elicited by exposure of the λ-sensitive *S. typhimurium* LT2 derivative TS736 of Palva et al. (13) to phage λNK55::Tn*10*. These clones were subjected to ampicillin treatment to enrich for mutants with new auxotrophic characters. One pool of about 1,500 tetracycline-resistant clones was subjected to ampicillin selection, allowing survival of about 10^{-4}. Of 54 tested survivor colonies, 50 required aromatic compounds, serine, and pyridoxine. A representative clone was saved as SL2459; transductants given the tetracycline resistance of SL2459 were Aro$^-$ SerC$^-$. We indicate this insertion mutation as *aroA*(*serC*)*1123*::Tn*10*.

Thus insertion of Tn*10* in or near *aroA* can cause either Aro$^-$ or Aro$^-$ SerC$^-$ phenotype. We think this is because *aroA* is promoter distal to *serC* in a *serC aroA* operon.

Isolation and characterization of tetracycline-sensitive mutants of *aroA554*::Tn*10* strains. Two tetracycline-sensitive mutants of *aro-554*::Tn*10* strains of *S. typhimurium*, chosen for trial as live vaccines, had been shown not to revert to aromatic independence in tests able to detect reversion at 10^{-11} per bacterium per generation (21). These are strain SL3235, obtained by ampicillin selection in the presence of tetracycline from strain SL3218, an *aroA554*::Tn*10* derivative in the *S. typhimurium* FIRN line, and SL3261, obtained by selection on Bochner medium from strain SL1346, an *aroA554*::Tn*10* derivative in the WRAY line. The secondary mutations in these two strains did not cause any new nutritional requirement. Twenty additional tetracycline-sensitive mutants obtained by selection on Bochner medium (+DHB) from strain SL1346 (WRAY *aroA554*::Tn*10*) were investigated. Five of them, of independent origin, were of nutritional phenotype Aro$^-$ SerC$^-$. Of eight tested tetracycline-sensitive mutants without additional nutritional requirements, one reverted to Aro$^+$ at much higher frequency than its parent, indicating its origin by "nearly precise excision" of Tn*10* (16); the other seven did not revert to Aro$^+$ at detectable frequency. The two live-vaccine strains, the five Aro$^-$ SerC$^-$ mutants, and the seven nonreverting Aro$^-$ SerC$^+$ mutants were further investigated. Each was tested for gas production in glucose-phenol red broth as a test of function of gene *pfl*, which determines pyruvate formate lyase and lies close to *aroA* on the map (14, 17). Each of the strains was also crossed, as transductional recipient, to several *aroA* point mutants the order of whose mutation within *aroA* had been examined by Nishioka et al. (12). The two *aroA554*::Tn*10* parent strains, SL3218 in the FIRN line and SL1346 in the WRAY line, were similarly tested, for comparison.

The results are summarized in Table 2, in which the point mutations are shown in the order previously inferred (12); mutations whose relative order is not clear are indicated in footnote *d*. In crosses of the two *aroA554*::Tn*10* parent strains to the point mutants the yield of *aro*$^+$ recombinants was low, as expected, and lowest for donors with mutations in the central (*70*, *71*, *64*)–(*1*, *89*) part of the map. This suggests that the *aroA544* insertion is in or close to this segment.

The 14 tetracycline-sensitive mutants tested fell into six classes whose behavior and inferred genetic constitution are summarized in Fig. 1. Class "a," made up of strain SL3235 (the FIRN live-vaccine strain) and five of the nonreverting SerC$^+$ mutants, were Pfl$^+$ and gave *aro*$^+$ recombinants in crosses with all the point mutants at about the same frequencies as did the *aroA554*::Tn*10* parent strains. They probably

TABLE 2. Phenotypes and transductional analysis of *aroA554*::Tn*10* strains and their tetracycline-sensitive derivatives used as live vaccines; and additional tetracycline-sensitive mutants of strain SL1346[a]

Strain no. and description	Phenotype		No. of *aro*$^+$ transductants per 10^8 PFU with *aro* donor:											
	Pfl	SerC	*67*	*70*[d]	*71*[d]	*64*[d]	*554*[e]	*1*[f]	*89*[f]	*102*	*55*[g]	*46*[g]	*43*[g]	*aro*$^+$
aroA554::Tn*10* strains and live-vacine mutants[b]														
SL3218 (FIRN *aroA*::Tn*10*)	+	+	23	6	4	4	Tn*10*	18	8	21	55	57	38	5,000
SL3235 (FIRN live vaccine) class "a" CRR	+	+	8	3	3	1	IS*10*	17	9	19	56	31	59	6,100
SL1346 (WRAY *aroA*::Tn*10*)	+	+	11	6	3	4	Tn*10*	7	2	8	38	40	30	3,700
SL3261 (WRAY live vaccine) class "b" DEL	+	+	10	5	4	6	IS*10*	0	0	0	0	0	0	3,800
SL1346 Tc^s mutants[c]														
SL2416, class "a" CRR	+	+	6	2	4	2	IS*10*	2	0.5	0.8	6	2	1	3,500
SL2422, class "a" CRR	+	+	0.8	0.3	0.1	0.2	IS*10*	1	0.6	6	19	18	12	2,650
SL2412, class "c" CRR	+	+	0	0	0	0	IS*10*	0	0	0	0	0	0	2.5
SL2418, class "c" CRR	+	+	0	0	0	0	IS*10*	0	0	0	0	0	0	2.5
SL2411, class "d" INV	+	−	5	0.8	0.3	0.8	IS*10*	8	4	14	44	22	33	2,300
SL2421, class "d" INV	+	−	5	0.2	0	0	IS*10*	5	0.7	3	22	17	11	2,600
SL2409, class "e" DEL	+	−	0	0	0	0	IS*10*	2	0.8	2	24	22	21	3,400
SL2415, class "f" DEL	−	−	0	0	0	0	IS*10*	4	2	2	20	14	9	700
SL2417, class "f" DEL	−	−	0	0	0	0	IS*10*	2	2	9	27	10	22	1,500

[a] The segments inferred to be deleted or inverted in mutants of classes "a" through "f" are illustrated in Fig. 1.
[b] *aroA554*::Tn*10* strains and their tetracycline-sensitive mutants used as live vaccines.
[c] Additional tetracycline-sensitive mutants of strain SL1346.
[d,f,g] The relative order of the point mutations in each of these clusters is uncertain: *70*, *71*, and *64*; *1* and *89*; and *55*, *46*, and *43*.
[e] Inferred position of the Tn*10* insertion in *aroA554*::Tn*10* strains and of a residual IS*10* element in tetracycline-sensitive mutants of such strains.

have deletions or inversions extending from within Tn*10* into *aroA* but not far enough to overlap the nearest of the tested point mutations. (The data for the live-vaccine strain SL3235 and two of the other mutants of this class are included in Table 2).

Class "b" comprised only SL3261, the live-vaccine strain in the WRAY line. It was SerC$^+$ and Gas$^+$ and recombined at about the same frequency as its parent strain with the four point mutants inferred to be to the left of *aroA554*, but gave no *aro*$^+$ recombinants in crosses to the six point mutants inferred to be to the right of *aroA554*. This indicates a deletion extending from within *aroA554*::Tn*10* rightwards into and perhaps through *aroA*.

Class "c" consists of two mutants which retained *pfl* and *serC* functions and gave no *aro*$^+$ recombinants in crosses with the point *aroA* mutants. However, in control crosses with an *aroA*$^+$ donor they gave only ca. 0.1% of the number of recombinants obtained in a cross with an *aroA554*::Tn*10* recipient. As these two mutants gave normal yields of *his*$^+$ transductants, the low yield of *aro*$^+$ cannot result from any defect in phage adsorption or recombination capacity. We interpret this class as having deletions or inversions of chromosomal segments nearly as long as a P22 headful, so that transducing particles able to correct their defects are very uncommon; or they may have deleted or inverted segments longer than a headful length of DNA, therefore repairable only by the rare coincidental adsorption of two transducing particles of appropriate DNA content (18).

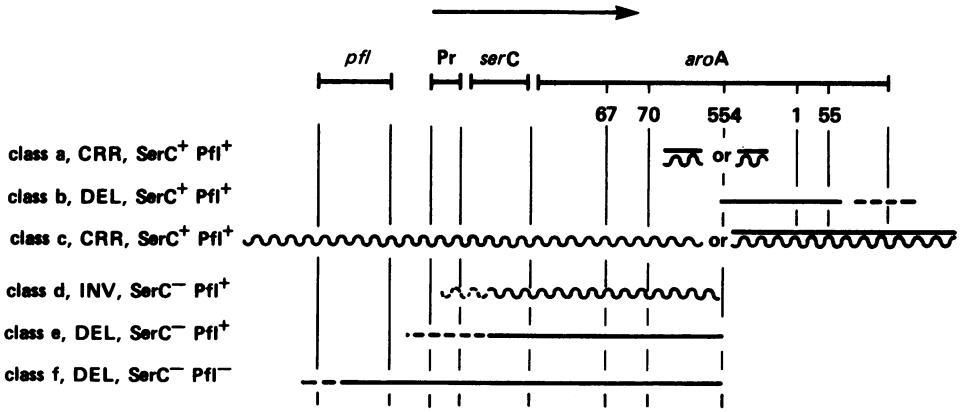

FIG. 1. Inferred deletions or inversions in tetracycline-sensitive, nonreverting mutants of *aroA554*:Tn*10* strains (distances not to scale). (——) Deleted segment. (∿∿∿) Inverted segment. (▭▭▭) Segment either deleted or inverted. Interrupted lines indicate possible extension of deleted or inverted segment. The parent strains have Tn*10* inserted at *aroA544*. Both the deletion and the inversion mutants are inferred to have a residual copy of IS*10* at position *aroA554*, and the inversion mutants are inferred to have a second IS*10* at the other end of the inverted segment. The point mutation sites shown are representatives of those closest to and farthest from *aroA544* on each side.

(Transductional crosses to point-mutant recipients would have shown whether the defects were deletions or inversions. However, the WRAY strain, though susceptible to P22-mediated transduction and not lysogenic for any P22-like phage, does not support growth of phage P22 or related general transducing phages, so derivatives in this line could not be tested as donors).

Class "d" comprised two mutants which were Pfl$^+$ SerC$^-$ and able to recombine with point mutants with sites to the right of *aroA554* and with several of those to its left. Since they gave *aro*$^+$ in crosses with point mutants on the left, their loss of *serC* function cannot result from a deletion leftwards from *aroA554* into or through *serC*; it must therefore result from inversion of a segment with a left endpoint within *serC* or the promoter region of the *serC aroA* operon.

Class "e" consisted of a single Pfl$^+$ SerC$^-$ mutant giving no *aro*$^+$ recombinants in crosses to the point mutants with affected sites to the left of *aroA554*; this is as expected for a strain with a deletion from *aroA* leftwards to an endpoint either within *serC* or in the promoter of the *serC aroA* operon or beyond it, but not so far as to involve gene *pfl*.

Class "f" comprised two Pfl$^-$ SerC$^-$ mutants giving no *aro*$^+$ recombinants with point mutants to the left of *aroA554*; we attribute them to deletions extending leftwards from *aroA554* through *serC* and the *serC aroA* promoter and into or through *pfl*.

Tn*10* insertions near *aroA*. P22 lysates of pools of LT2 sublines made tetracycline resistant by random Tn*10* insertions (9) were screened for ability to evoke *aro*$^+$ tetracycline-resistant transductants from an *aroA* recipient. Four independent Tn*10* insertions at sites cotransducible with *aroA*, labeled *zbj-903* through *zbj-906*, did not cause auxotrophy or obvious alteration in phenotype other than tetracycline resistance. Each was crossed by transduction to recipients with *aroA554*::Tn*10*-derived deletions: SL3261 (rightward deletion) and SL2415 (leftward deletion extending through *serC* into or through *pfl*). Transductants selected as tetracycline resistant were scored for *aro*. The results (Table 3) suggest that each of the silent Tn*10* insertions is to the left of *aroA*, close to the left end of the *aroA serC pfl* leftward deletion, but with at least *zbj-904* and *zbj-906* not overlapped by the deletion.

None of the tested Bochner derivatives of *zbj-903* (87 isolates) or *zbj-904*::Tn*10* (18 isolates) was auxotrophic. Mutants affected in *aroA* or *serC* function were therefore sought by Bochner selection followed by ampicillin enrichment for auxotrophy (see Materials and Methods). Table 4 records the Aro, Ser, and Pfl (gas production) phenotypes of clones of survivors of ampicillin killing of pooled Bochner-resistant mutants of *zbj-903*, *zbj-904*, and *zbj-905*::Tn*10* strains. The results were qualitatively similar in all the experiments, except that no gas-negative mutants were isolated from the *zbj-905*::Tn*10* strain. (Given the order *pfl-serC-aroA* and that the four *zbj* insertion sites are to the left of *aroA* [Table 3], the isolation of gas-negative mutants from *zbj-903*::Tn*10* and *zbj-904*::Tn*10* but not from *zbj-905*::Tn*10* strains suggests the gene order [*zbj-903 zbj-904*]–*pfl*–*zbj-905*–*serC*–*aroA*.)

TABLE 3. Proportion of tetracycline-resistant transductants acquiring donor *aroA*$^+$ in crosses of *zbj*::Tn*10* donors to recipients with leftward or rightward deletions derived from *aroA554*::Tn*10*

Recipient	No. of *aroA*$^+$ transductants/no. of transductants in cross with donor:			
	zbj-903	*zbj-904*	*zbj-905*	*zbj-906*
SL2415, leftward deletion	29/29	26/28	28/28	28/30
SL3261, rightward deletion	40/48	19/30	28/30	13/24

TABLE 4. New phenotypic traits of mutants isolated from *zbj*::Tn*10* strains by Bochner selection followed by ampicillin enrichment for (new) auxotrophic character

Phenotypic class (new characters)[a]	No.[b] among clones derived from parent[c]:				
	SL2438, *zbj-903*	SL2444, *zbj-903*	SL2439, *zbj-904*	SL2445, *zbj-904*	SL2447, *zbj-905*
None	4	8	14[d]	3	8[d]
Gas$^-$	0	1		0	
Aro$^-$ SerC$^-$ Gas$^-$	13	1	1	5	0
Aro$^-$	1	2	1	3	1
Aro$^-$ SerC$^-$, pale	10	8	0	8	6

[a] Gas production was tested in glucose-phenol red broth with Durham tube. "Pale" indicates pale yellow-brown color after overnight incubation, attributed to decolorization of the acid form (yellow) of the phenol red indicator.

[b] Because of the way selection was applied, some phenotypically similar clones in any one experiment may be members of a single mutant clone.

[c] Parent strain SL2447 is LT2 *aroA102*, made *aro*$^+$ *zbj-905*::Tn*10* by transduction, and therefore prototrophic. The other parent strains are from strain SL1027 (which is Met$^-$ Trp$^-$, etc., see Table 1) made *zbj*::Tn*10* by transduction.

[d] These clones, not having any new nutritional requirements, were not tested for gas production.

All the Bochner selection-ampicillin enrichment experiments except that involving the *zbj-905*::Tn*10* parent gave some Aro$^-$ SerC$^-$ Pfl$^-$ clones. These may be rightward deletions from *zbj-903* or *-904* through *pfl* and *serC* into or through *aroA*, or through *pfl* into the promoter or proximal gene, *serC*, of the *serC aroA* operon. Three such clones were crossed, as recipients, to the *aroA* point mutants (Table 5). One of them, SL2456, gave no wild-type recombinants with any of the point mutants, as expected if it has a deletion extending from *zbj-903* through *pfl*, *serC*, and *aroA*. The other two, SL2440 and SL2462, derived from *zbj-904* parents, gave wild-type recombinants with all the point mutants. We think they have deletions rightward from *zbj-904* through *pfl* into the *serC aroA* operon and with a right endpoint either in this operon's promoter or proximal structure gene, *serC*, or in the left extremity of *aroA*; a deletion with its right end at any of these points would cause loss of *aroA* function, even though the structural gene, or most of it, would be intact.

At least one Aro$^-$ SerC$^+$ Pfl$^+$ clone was obtained from each of the three *zbj*::Tn*10* parents tested (Table 4). We attribute such clones to inversion of a segment extending from the *zbj*::Tn*10* site through *serC* to an endpoint within *aroA*; gene *serC* (and *pfl*, for mutants of *zbj-903* or *zbj-904*::Tn*10* parents), with promoter intact, would be expected to function normally despite inversion. One of these clones, SL2441, derived from a *zbj-904*::Tn*10* parent, was crossed to the *aroA* point mutants as donors; the low yield of *aro*$^+$ recombinants (Table 5) is as expected for a recipient with an inversion of the postulated type (8).

Mutants of nutritional character Aro$^-$ SerC$^-$ but producing gas in glucose-phenol red broth were obtained from *zbj-903*, *-904*, and *-905* parents (Table 4). All 32 isolates of this type (not necessarily all of independent origin) differed from the 34 tetracycline-sensitive isolates found to have other combinations of Aro, SerC, and gas production character in that the glucose-phenol red broth cultures after overnight incubation were a pale brown color, instead of bright yellow, apparently because of decolorization of the acid form of phenol red. The cause of the "pale" phenotype

TABLE 5. Phenotypes and transductional analysis of auxotrophic tetracycline-sensitive mutants of *zbj*::Tn*10* strains

Strain no.	Parent Tn*10* site	Phenotype			No. of *aro*$^+$ or *aro*$^+$ *serC*$^+$ transductants per 10^8 PFU with *aro* donor:										
		Pfl	SerC	Aro	*67*	*70*[a]	*71*[a]	*64*[a]	*1*[b]	*89*[b]	102	*55*[c]	*46*[c]	*43*[c]	*aro*$^+$
SL2456	*zbj-903*	−	−	−	0	0	0	0	0	0	0	0	0	0	2,800
SL2440	*zbj-904*	−	−	−	6	11	8	7	3	4	13	29	16	12	2,200
SL2462	*zbj-904*	−	−	−	41	10	8	9	7	9	21	22	ND[d]	22	3,300
SL2441	*zbj-904*	+	+	−	2	0.8	0	0.5	0.5	0.5	11	10	2	3	2,700
SL2454	*zbj-903*	+	−	−	144	32	55	53	76	42	46	94	ND	59	6,700

[a,b,c] The relative order of the point mutations in each of these clusters is uncertain: *70*, *71*, and *64*; *1* and *89*; and *55*, *46*, and *43*.
[d] ND, Not done.

is not known, but we feel reasonably sure that this class are *pfl*$^+$. We infer them to have inversions of segments extending from their *zbj*::Tn*10* insertions to a right endpoint in the promoter of the *serC aroA* operon or in *serC*. Several of this class, including SL2454 (Table 5), when crossed to the *aroA* point mutants as donors gave *aro*$^+$ recombinants with all of them, - as expected if they have the postulated type of inversion.

DISCUSSION

The identification of two mutants with deletions leftwards from *aroA554*::Tn*10* through *serC* and into or through *pfl* (class "f," Table 2 and Fig. 1), and of another with a deletion extending into or through *serC* but not affecting *pfl* (class "e," Table 2 and Fig. 1), showed the gene order *pfl-serC-aroA*, as in *E. coli* (1, 3). The properties of all the tetracycline-sensitive mutants isolated from *aroA554*::Tn*10* strains (Table 2) or from *zbj*::Tn*10* strains (Table 5) can be accounted for by deletions or inversions extending for various distances to one side or the other from the site of their Tn*10* insertions. One result at least seemed to indicate that *serC* and *aroA* made up an operon, with *serC* being promoter proximal. Strain SL2454, a tetracycline-sensitive mutant of a *zbj-903*::Tn*10* parent, was Pfl$^+$ SerC$^-$ Aro$^-$ (Table 5). If, as inferred, *pfl* lies between *zbj-903* and *serC*, the Pfl$^+$ character of this mutant indicates inversion of a segment extending from *zbj-903*, not its deletion. Such an inversion would inactivate only the gene or operon disrupted by its right endpoint, and thus only *serC* or only *aroA*, if these two genes were in separate operons. An inversion with its right end in the promoter or proximal structural gene of a *serC aroA* operon would account for the loss of both *aroA* and *serC* functions and also for the high yield of *aro*$^+$ recombinants in crosses of SL2454 with the *aroA* point mutants (Table 5).

The recognition of two Tn*10* insertions causing SerC$^-$ Aro$^-$ phenotype, taken with the existence of other Tn*10* insertions at *aroA* causing only Aro$^-$ phenotype, gave unequivocal evidence that *serC* and *aroA* are in the same operon, with *serC* nearest the promoter. Luca Comai and his colleagues (Calgene, Inc., Davis, Calif.), independently of our investigation, have cloned an *aroA*-containing DNA segment from *S. typhimurium* LT2 (4); David Stalker (personal communication) has now by base-sequencing detected an open reading frame promoter proximal to the *aroA* structural gene and has recently confirmed our interpretation that this open reading frame is gene *serC*. The functional significance of the grouping of *serC* and *aroA* in a single operon is not known. However, one may note that serine and DHB are both precursors of the bacterial iron-binding compound enterochelin (=enterobactin). Iron starvation is known to cause depression of several genes for proteins concerned in iron acquisition and also modifications in the structure of several tRNA species, including two tRNA^{ser} species (11). Grouping of one gene of the common aromatic biosynthesis pathway and one gene concerned in serine biosynthesis into a single operon may permit their coregulation according to the iron status of the bacterium.

One objective of our work was to find the nature of the secondary mutations causing inability to revert in two tetracycline-sensitive derivatives of *aroA554*::Tn*10* strains chosen for investigation as live vaccines (7, 21). Transductional crosses showed one of them, strain SL3261, to have a deletion extending from the transposon through all tested point mutations to its "right" (Table 2, Fig. 1). The other live vaccine strain, SL3235, recombined with all the tested *aroA* point mutants, as did five nonreverting but Pfl$^+$ SerC$^+$ mutants of SL1346 (class "a" in Table 2 and Fig. 1). We think it likely that these strains have deletions or inversions extending from within Tn*10* to the left or the right into *aroA* but not as far as the nearest point mutation. However, some tetracycline-sensitive mutants of *his*::Tn*10* strains were reported to have genetic lesions of unknown nature, apparently not extending outside the transposon, yet causing reduction in rate of reversion to *his*$^+$, in some of them by as much as 100-fold (8). Even though live-vaccine strain SL3235 gave no aromatic-independent mutants in tests that had been shown by reconstruction to be able to detect reversion at a rate of 10^{-11} per bacterium per generation, we cannot exclude the possibility that this strain's secondary mutation is entirely within Tn*10*, yet causes 1,000-fold or greater reduction in rate of reversion to *aro*$^+$. For this reason, in later construction of candidate oral-route live-vaccine strains of *Salmonella typhi* (M. F. Edwards and B. A. D. Stocker, unpublished data), nonreverting *aroA* derivatives have been obtained not by selection of tetracycline-sensitive mutants of strains made *aroA554*::Tn*10* by transduction but instead by first transducing a transposon insertion causing Aro$^-$ SerC$^-$ phenotype into a wild-type strain, then replacing it with *serC*$^+$ Δ*aroA* by transduction, with selection for serine-pyridoxine independence.

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