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Construction of Δ*aroA his* Δ*pur* Strains of *Salmonella typhi*

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***Salmonella typhi* strains with two deletion mutations, each causing an attenuating auxotrophy, have been constructed from strains Ty2 and CDC 10-80 for possible use as oral-route live vaccines. An *aroA*(*serC*)::Tn*10* transposon insertion was first transduced from a *Salmonella typhimurium* donor into each wild-type *S. typhi* strain. Transductants of the Aro⁻ SerC⁻ phenotype were treated with transducing phage grown on an *S. typhimurium* strain with an extensive deletion at *aroA*; selection for SerC⁺ yielded transductants, some of which were Δ*aroA*. A *his* mutation was next inserted into a Δ*aroA* strain in each line by two steps of transduction. Two deletions affecting de novo purine biosynthesis were used as second attenuating mutations: Δ*purHD343*, causing a requirement for hypoxanthine (or any other purine) and thiamine, and Δ*purA155*, causing an adenine requirement. The *purHD343* deletion was introduced into the Δ*aroA his* derivatives of each strain by cotransduction with *purH*::Tn*10*, and the *purA155* deletion was introduced into the CDC 10-80 Δ*aroA his* derivative by cotransduction with an adjacent silent Tn*10* insertion by selection for tetracycline resistance. Tetracycline-sensitive mutants of each of the three Δ*aroA his* Δ*pur* strains were isolated by selection for resistance to fusaric acid. The tetracycline-sensitive derivative of the CDC 10-80 Δ*aroA his* Δ*purA155* strain, designated 541Ty, and its Vi-negative mutant, 543Ty, constitute the candidate oral-route live-vaccine strains used in a recent volunteer trial (M. M. Levine, D. Herrington, J. R. Murphy, J. G. Morris, G. Losonsky, B. Tall, A. A. Lindberg, S. Stevenson, S. Baqar, M. F. Edwards, and B. A. D. Stocker, J. Clin. Invest. 79:885–902, 1987). Tetracycline-sensitive mutants of the Δ*aroA his* Δ*purHD* derivatives of strains Ty2 and CDC 10-80 may also be appropriate as live vaccines but have not been tested as such.**

Typhoid fever is still prevalent in various countries, with, at a rough estimate (D. Barua, World Health Organization, cited in reference 5), 12.5 million cases each year in the world exclusive of China. The killed vaccine now in use, given by injection, is moderately effective, but its unpleasant side effects have prevented its widespread use in civilian populations at risk (for a review of antityphoid vaccines, killed and live, see reference 12 and, for an update, references 5 and 10). In animal salmonelloses, attenuated strains of *Salmonella* species given as live vaccines by injection or by feeding are much more effective than killed vaccines given by injection. However, the testing of attenuated *Salmonella typhi* strains constructed for use as live vaccines is hampered by the failure of this organism to proliferate in nonprimate hosts except when given in highly artificial ways, such as by intraperitoneal injection into mice of very large inocula without adjuvant or of small inocula with hog gastric mucin. Despite this obstacle, several oral-route live-vaccine strains have been developed (12). *S. typhi* Ty21a, a *galE* Vi-negative mutant of strain Ty2, gave excellent protection in an initial field trial, but the results of extensive later trials, in Chile, have been less satisfactory (10). Thus, there is still a need for a strain of *S. typhi* retaining protective antigens but irreversibly attenuated for use as an oral-route live vaccine.

We describe here the construction of strains of *S. typhi* made nonvirulent by the introduction of complete and nonreverting mutational blocks in biosynthesis paths, causing a requirement for metabolites not available (or not available at sufficient concentrations) in host tissues. Nearly 40 years ago, Bacon and his colleagues (1) reported that induced auxotrophic mutants of *S. typhi* of three classes were of reduced virulence (in mice): those mutants requiring a purine or a purine plus thiamine, those responding to aspartic acid, and one mutant requiring *p*-aminobenzoic acid. The discovery of transposons that cause antibiotic resistance has made it relatively easy to transfer insertion mutations causing the requirements described above into appropriate strains of *Salmonella* species. In *Escherichia coli* and its relatives, a block in the aromatic biosynthesis (*aro*) path causes a requirement for aromatic metabolites, including two which are unavailable (at least in sufficient concentrations) in mammalian tissues: *p*-aminobenzoic acid, as precursor of folic acid, and 2,3-dihydroxybenzoic acid (DHB), as precursor of the iron-capturing compound enterobactin (enterochelin). Transposon-generated deletion or deletion-inversion mutations of gene *aroA* caused a virtually complete loss of virulence in *Salmonella typhimurium* (7), and strains of *S. typhimurium* and *Salmonella dublin* with such *aroA* mutations have given promising results as live vaccine, oral or parenteral, in mice, calves (7, 16, 19, 20), and sheep (15). We have made two Vi-positive strains of *S. typhi* nonvirulent, as tested in a mouse model, by transducing into each of them a deletion (previously characterized in *S. typhimurium*) of much of gene *aroA*. The *S. typhi* strains made *aroA* were then given mutation *hisG46*, causing a histidine requirement, to facilitate the recognition of coincidental infection rather than reversion as the cause should a person develop typhoid fever soon after taking live vaccine.

We thought it advisable to introduce a second attenuating mutation to the Δ*aroA hisG* strains as a further guarantee of safety. Mutation to a purine requirement causes reduced virulence both in *S. typhi* (1) and in other species (14). We therefore wished to transduce a known deletion in the de novo purine biosynthesis path from *S. typhimurium* into our Δ*aroA his* strains of *S. typhi*. The first 10 steps of the pathway convert phosphoribosyl pyrophosphate to IMP; IMP is then converted to GMP by two steps involving genes

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guaB and *guaA*or to AMP by two steps involving genes *purA* and *purB*. The purine requirement of mutants blocked before IMP formation is satisfied by hypoxanthine or by hypoxanthine plus thiamine for some classes. Blocks preventing conversion of IMP to GMP cause a requirement for guanine, and blocks preventing its conversion to AMP cause a requirement for adenine. Our first choice of a *pur* deletion mutation to be transferred to *S. typhi* was *purHD343*, a deletion of part of two genes in the *purJHD* operon which causes a loss of activity of three enzymes needed for conversion of phosphoribosyl pyrophosphate to IMP. In our experiments and in those of R. Brown and B. A. D. Stocker (unpublished data), blocks causing a purine requirement satisfied by hypoxanthine or hypoxanthine plus thiamine seemed to cause a satisfactory attenuation of *S. typhi*. However, as discussed below, the results of experiments (14) on the attenuating effects of different *pur* and *gua* mutations on the virulence in mice of a Vi-positive *S. dublin* strain suggested that a block preventing conversion of IMP to AMP, causing an adenine requirement, would cause more complete attenuation than a block in IMP formation or in its conversion to GMP. A deletion within gene *purA* was therefore transferred into one of our *aroA his* strains of *S. typhi*. Tests of the virulence in mice of some of our constructed strains are described elsewhere (M. F. Edwards, Ph.D. thesis, Stanford University, Stanford, Calif., 1985; M. F. Edwards and B. A. D. Stocker, manuscript in preparation). We and our colleagues (11) report the results of volunteer trials of our Vi-positive Δ*aroA his* Δ*purA* candidate live-vaccine strain 541Ty, and of its Vi-negative derivative, 543Ty.

MATERIALS AND METHODS

Bacterial strains. The constructed *S. typhi* strains (Table 1) are derivatives of *S. typhi* Ty2 or *S. typhi* CDC 10-80. *S. typhi* Ty2, received as WR4014 from L. S. Baron (Walter Reed Army Institute of Research, Washington, D.C.), is a Vi-positive xylose-fermenting strain of phage type E1. *S. typhi* CDC 10-80 is a recent clinical isolate of phage type A which differs from *S. typhi* Ty2 in its failure to ferment xylose and by the less complete masking of its O antigen by Vi antigen. Both strains grew slowly on simple defined medium supplemented with tryptophan and cystine. The *S. typhimurium* strains used as transductional donors (Table 2) are derivatives of strain LT2; those with transposon Tn*10* insertions were received from J. R. Roth (Department of Biology, University of Utah). Strains were stored on Dorset egg slants at room temperature or as glycerol suspensions at −70°C.

Media. Blood agar base (code CM55; Oxoid Ltd.) and nutrient broth 2 (code CM67; Oxoid) were used as nutrient media; for growth of aromatic-dependent strains, they were supplemented with DHB at ca. 1 μg/ml, and for selection of tetracycline-resistant transductants these media were supplemented with tetracycline at 20 μg/ml. The defined medium of Davis and Mingioli (3) was used, with 5 ml of glycerol or 5 g of glucose per liter, as the energy source and with tryptophan and cystine in all cases to satisfy the requirements of wild-type *S. typhi*. Other supplements were added as appropriate, usually by spreading 1 drop (ca. 0.05 ml) of a stock solution on a plate immediately before inoculation; the stock solutions of amino acids and purines were 1 or 0.5% (wt/vol), and those of vitamins, *p*-aminobenzoic acid, and DHB were 0.1% (wt/vol). Tetracycline-sensitive mutants of Tn*10*-carrying strains were selected on a medium which contains autoclaved chlortetracycline with fusaric acid (13); this was supplemented with DHB to allow enterobactin synthesis by aromatic-dependent strains (8).

TABLE 1. Constructed *S. typhi* strains

Mutant characters[a]	Strain number of derivative from line:	
	Ty2	CDC 10-80
aroA(*serC*)*1121*::Tn*10*	510Ty	511Ty
aroA.DEL407[b]	514Ty	515Ty
Δ*aroA hisD8557*::Tn*10*	520Ty	521Ty
Δ*aroA hisG46*	522Ty	523Ty
Δ*aroA metA900*::Tn*10*	516Ty	517Ty
Δ*aroA his purD1734*::Tn*10*	524Ty	525Ty
Δ*aroA his* $purD^{+}_{Stm}$[c]	528Ty	
Δ*aroA his purH887*::Tn*10* Δ*purHD343*	616Ty	611Ty
Δ*aroA his* Δ*purHD343* CRR...[*purH887*::Tn*10* (Tc^{s})]	620Ty	619Ty
his Δ*purHD343* CRR...[*purH887*::Tn*10* (Tc^{s})]	634Ty	633Ty
Δ*aroA his zbj-908*::Tn*10* Δ*purA155*		531Ty
Δ*aroA his* Δ*purA155* CRR475[*zbj-908*::Tn*10* (Tc^{s})]		541Ty
Δ*aroA his* Δ*purA155* CRR475[*zbj-908*::Tn*10* (Tc^{s})], Vi negative		543Ty

[a] Allele symbols abbreviated after first mention. CRR..., Tn*10*-generated complex rearrangement mutation causing new phenotypic character indicated in parentheses.
[b] See Results for explanation.
[c] $purD^{+}$ gene of *S. typhimurium* origin.

Transduction. An *int* (integration-negative) derivative of a high-transducing phage P22 variant, P22 HT105/1 (18), was used to transduce genes from *S. typhimurium* donors either to *S. typhi* recipients or to *S. typhimurium* recipients to construct strains to be used as donors. Lysates, usually of ca. 10^{10} PFU/ml, were used to evoke transductants by the drop-on-lawn method (9); the selective medium was either blood agar base with tetracycline or defined medium with appropriate supplements. Putative transductant clones were purified by single-colony reisolation on the selective media before characterization. Vi and O9 antigens were tested for

TABLE 2. *S. typhimurium* strains used as transductional donors

Strain[a]	Genotype	Origin or reference
his-46	*hisG46*	
pur-155	Δ*purA155*[b]	J. S. Gots
pur-310[c]	Δ*purG310*	J. S. Gots
pur-343	Δ*purHD343*	6; J. S. Gots
SL2961	Δ*purHD343 metA900*::Tn*10*	This work
SL2976	Δ*purHD343 purH877*::Tn*10*	This work
SL5236	*aroA*.DEL407 *galE*[d]	8; this work
TT47	*hisD8557*::Tn*10*	J. R. Roth
TT256	*metA900*::Tn*10*	J. R. Roth
TT310	*purD1734*::Tn*10*	J. R. Roth
TT418	*glyA540*::Tn*10*	J. R. Roth
TT472	*aroA*(*serC*)*1121*::Tn*10*	8
TT1455	*aroA554*::Tn*10*	7; J. R. Roth

[a] All strains are derivatives of *S. typhimurium* LT2.
[b] For evidence indicating that mutation *pur-155* is a deletion at *purA*, see Bailen, Ph.D. thesis.
[c] In reference 22 this strain is described as *ath-10*.
[d] Mutation *aroA*.DEL407, obtained by Tn*10*-generated deletion in an *aroA554*::Tn*10* strain (7, 8), was transduced by phage P1 from a *galE* mutant of strain SL3261 into an LT2 *galE* recipient.

by slide agglutination with sera from Difco Laboratories or BBL Microbiology Systems.

RESULTS

Introduction of nonreverting *aroA* defects into *S. typhi* strains. Most but not all of the tetracycline-resistant clones evoked from the two wild-type *S. typhi* strains by phage P22 HT grown on *S. typhimurium* TT1455 (LT2 *aroA554*::Tn*10*) were aromatic dependent. These and other aromatic-dependent *S. typhi* strains on occasion grew poorly or irregularly on complex media unless the media were supplemented with DHB. Gene *serC*, for biosynthesis of serine and pyridoxine, is in the same operon as *aroA* and is operator proximal to it (8). The identification of *S. typhimurium* mutants with Tn*10* insertions in *serC* or perhaps in the promoter of the *serC-aroA* operon (8) enabled us to transfer a previously characterized (8, 19) Tn*10*-generated deletion of the distal part of gene *aroA* of *S. typhimurium* into the *S. typhi* wild-type strains by two steps. The deletion used, *aroA*.DEL407, was shown by genetic evidence to extend through several nonidentical point *aroA* mutations (8) and is now known, from negative-probe tests (M.K. Halula and B. A. D. Stocker, unpublished observations), to have removed at least the ca. 700 C-terminal base pairs of *aroA*. Phage P22 HT grown on TT474 [LT2 *aroA*(*serC*)*1121*::Tn*10*] evoked tetracycline-resistant colonies (ca. 10^{-7}/PFU from Ty2 and ca. 10^{-6}/PFU from CDC 10-80); tiny colonies, inferred to be abortive transductants to tetracycline resistance, were obtained from CDC 10-80 but not from Ty2. Representative transductants, designated 510Ty in line Ty2 and 511Ty in line CDC 10-80, were treated with phage grown on SL5236 (LT2 *aroA*.DEL407 *galE*) on plates selective for the ability to grow without serine or pyridoxine. About 30% of the SerC$^+$ clones obtained were aromatic dependent but tetracycline sensitive and were inferred to have resulted from replacement of the *serC*::Tn*10* *aroA*$^+$ segment of the recipient chromosome by *serC*$^+$ *aroA*.DEL407 of the donor. Representative transductants were numbered 514Ty (Ty2 line) and 515Ty (CDC 10-80 line).

Introduction of a *his* (histidine requirement) marker mutation. Next, a *his* allele causing a histidine requirement satisfied by histidinol (at high concentration) was introduced by two steps as a marker character. Each *S. typhi* strain given the *aroA* deletion was made *hisD8557*::Tn*10* by transduction from *S. typhimurium* TT47, with selection for tetracycline resistance; the tetracycline-resistant clones tested had a histidine requirement not satisfied by histidinol (because gene *hisD* specifies histidinol dehydrogenase, which is required for the last step in histidine biosynthesis). Representative *hisD*::Tn*10* clones 520Ty (Ty2 line) and 521Ty (CDC 10-80 line) were treated with a lysate of LT2 *hisG46*, a strain with a missense mutation in gene *hisG*, which is immediately adjacent to *hisD*. Selection was made on defined medium supplemented with cystine, tryptophan, an aromatic pool, and histidinol (ca. 200 μg/ml, since a high concentration is required for growth of *his* strains which are *hisD*$^+$, presumably because of inefficient uptake of histidinol). Some transductants were *his*$^+$, but others were of the kind we sought, i.e., unable to grow unless provided with either histidine or histidinol and tetracycline sensitive. Representative transductants of inferred genotype *S. typhi* *aroA*.DEL407 *hisG46* were numbered 522Ty (Ty2 line) and 523Ty (CDC 10-80 line).

Introduction of a *pur* mutation causing an early block in purine biosynthesis. We next wished to introduce a nonleaky nonreverting defect in purine biosynthesis into the two Δ*aroA his S. typhi* strains as a second attenuating mutation. Selection for tetracycline resistance after application of lysates of appropriate *S. typhimurium* donor strains yielded transductants with Tn*10* insertions in genes *purD*, *purG*, *purH*, or *purC* in each of the two *S. typhi* lines (data not shown). One of several tetracycline-sensitive mutants isolated from strain 524Ty, a *purD1734*::Tn*10* transductant from the Ty2 line, did not revert at a detectable frequency and gave no *pur*$^+$ recombinants in transductional crosses with point *purD* mutants of *S. typhimurium* as donors, indicating that the mutant probably originated from an extensive transposon-generated deletion or deletion-inversion at *purD*. However, the absence of a genetic fine-structure or restriction map of this gene prevented a rigorous test of its origin, so we discontinued work with this line and instead looked for selectable characters in *S. typhimurium* determined at loci cotransducible with *pur* genes in which deletion mutations had been identified. Mutation *purG310* (formerly called *ath-10*) in *S. typhimurium* LT2 in transductional crosses behaved as an extensive deletion (22). Phage grown on strain *purG310* evoked Gly$^+$ transductants from 527Ty, a *glyA546*::Tn*10* derivative in the *S. typhi* CDC 10-80 line; however, none of the transductants tested was Pur$^-$, despite the closeness of these *pur* and *gly* genes on the linkage map (17).

Mutation *purHD343* in *S. typhimurium* is a well-characterized (6) deletion of adjacent parts of genes *purH* and *purD* in the *purJHD* operon, situated at 89 min (17), close to *metA* on one side and *thiA* on the other side. About one-third of the tetracycline-resistant clones evoked from an *S. typhimurium* recipient by phage grown on a constructed Δ*purHD343* *metA900*::Tn*10* strain, SL2961, were purine exacting, but no such cotransduction was observed when the recipient was instead *S. typhi* of either line. Transductants of character *metA900*::Tn*10* in the two *S. typhi* lines were treated with phage grown on *S. typhimurium* Δ*purHD343*; none of the ca. 70 Met$^+$ clones tested from each cross had the purine requirement of the donor. We also selected tetracycline-resistant transductants in a cross of an *S. typhimurium* Δ*pur* *HD343* *metA*::Tn*10* donor, SL2961, to an *S. typhi* recipient, 528Ty, which had previously been given, by two successive transductions, gene *purD*$^+$ (and, presumably, adjacent genes) of *S. typhimurium* origin; however, none of the transductants obtained was Pur$^-$. Attempts to introduce Δ*purHD343* by cotransduction with *thiA*::Tn*10* were likewise unsuccessful.

Transfer of *purHD343* to the two *S. typhi aroA his* strains was finally achieved by cotransduction with a Tn*10* insertion in gene *purH* itself. The four available *S. typhimurium* strains with Tn*10* or Tn*5* inserts in the *purJHD* operon were crossed to the Δ*purHD343* recipient strain; some *pur*$^+$ recombinants were obtained with *purH877*::Tn*10* as donor. The recovery of *pur*$^+$ recombinants showed that the site of the insertion was not overlapped by the *purHD* deletion. Of 13 transductants obtained in the same cross by selection for tetracycline resistance, the 11 which could revert to *pur*$^+$ were inferred to have incorporated a segment of donor chromosome corresponding to the *purHD* deletion of the recipient, in addition to the segment with the Tn*10* insertion. One of the two nonreverting tetracycline-resistant transductants, SL2976, when used as donor gave no *pur*$^+$ recombinants in crosses with either Δ*purHD343* or *purH877*::Tn*10*, indicating genotype Δ*purHD343* *purH577*::Tn*10*. (The behavior in crosses of the other nonreverting transductant indicated that it arose by transposition of Tn*10* to a chromo-

somal site other than *purH*.) Phage grown on SL2976 (inferred to have both the Tn*10* insertion and the *purHD* deletion) evoked tetracycline-resistant transductants from the Δ*aroA hisG* derivatives in the two *S. typhi* lines. Transductant clones requiring a purine (and vitamin B_1) in addition to the parental requirements were numbered 616Ty (Ty2 line) and 611Ty (CDC 10-80 line); their tetracycline-sensitive mutants, 620Ty and 619Ty, respectively, were considered appropriate as candidate live-vaccine strains. An *aro*$^+$ transductant was isolated from each of these strains to allow tests of the reduction of virulence in mice caused by the mutations affecting the *purJHD* operon in the absence of the *aroA* defect.

Introduction of a purine mutation causing an adenine requirement. In three *Salmonella* strains virulent for mice (one Vi-positive *S. dublin* and two *S. typhimurium* strains), blocks in the conversion of IMP to AMP, causing a requirement for adenine, caused a more complete loss of virulence than did blocks preventing conversion of phosphoribosyl pyrophosphate to IMP, causing a purine requirement satisfied by provision of hypoxanthine or any other purine (14). As the deletion mutation Δ*purHD343*, when introduced into the two *S. typhi* strains, blocks only IMP synthesis, we decided to introduce a nonleaky nonreverting *purA* or *purB* mutation, blocking conversion of IMP to AMP, into our two strains of *S. typhi* made Δ*aroA hisG*. Strain LT2 *purA155*, isolated in the laboratory of J. S. Gots (Department of Microbiology, University of Pennsylvania School of Medicine), has a deletion within gene *purA*, as shown by the failure of all mutagens tested to evoke revertants and by the failure of the strain to give *pur*$^+$ recombinants in transductional crosses in either direction with several *purA* point mutants which do yield *pur*$^+$ when they recombine with each other (M. Bailen, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1964). A silent Tn*10* insertion, *zbj-908*::Tn*10*, at a locus cotransducible with *purA*$^+$ had been identified previously by screening pools of Tn*10* insertion mutants (S. Chung and B. A. D. Stocker, unpublished observations). Phage grown on an *S. typhimurium* strain of genotype Δ*purA155 zbj-908*::Tn*10* was used to evoke tetracycline-resistant clones from the two *S. typhi* Δ*aroA his* strains, 522Ty and 523Ty. None were obtained in several attempts with the Ty2 line, but three of five clones isolated from the CDC 10-80 line recipient, 523Ty, required adenine. One of them, 531Ty, was used for isolation of tetracycline-sensitive mutants. One such mutant, 541Ty, unaltered from its parent, 531Ty, in nutritional character and antigenic constitution, was used as a parent of Vi-negative mutants by selection for resistance to Vi phages. Strain 541Ty, which is *S. typhi* CDC 10-80 Δ*aroA hisG46* Δ*purA155* CRR475 [*zbj-908*::Tn*10* (Tc^s)] (Table 1, footnote *a*), and its Vi-negative mutant, 543Ty, constitute the candidate oral-route live-vaccine strains recently tested in volunteers (11).

DISCUSSION

Our objective was to construct from wild-type *S. typhi* a strain or strains appropriate for use as live vaccine. The desiderata for such a strain were as follows: (i) two deletion mutations, at loci well separated on the linkage map, each causing auxotrophy and thus nonvirulence; (ii) a marker character; (iii) unaltered antigenic character; and (iv) sensitivity to all antibiotics active on typical strains of *S. typhi*. We achieved our objective by the isolation from both Ty2 and CDC 10-80 of derivatives with a deletion at *aroA* (at 19 min), mutation *hisG46*, and a deletion in the *purJHD* operon (at 89 min) and by isolation of a CDC 10-80 derivative with the same *aroA* and *his* mutations and a deletion in gene *purA* (at 96 min). All three attenuating mutations had been characterized as deletions by genetic analysis in *S. typhimurium*; the nature of the *aroA* mutation has been confirmed in some of the live-vaccine strains by the failure of digests of chromosomal DNA of the strains to bind a ^{32}P-labeled probe comprising the ca. 700 C-terminal base pairs of gene *aroA*$^+$ of *S. typhimurium* (Halula and Stocker, unpublished observations). The marker allele, *hisG46*, is a reasonably stable missense mutation not likely to affect virulence or behavior in host tissues. No change in antigenic character would be expected to result from the alleles transduced into the live-vaccine strains or from the transposon-generated mutations which restored sensitivity to tetracycline to the strains made *purHD* or *purA* by cotransduction with adjacent Tn*10* insertions. (The two-step procedure used to introduce *aroA* and *hisG46*, in which step 2 restores tetracycline sensitivity, could not be used to introduce the *pur* alleles.) The only known alteration in antigenic character was the deliberate loss of Vi antigen in strain 543Ty.

The poor or irregular growth of the *aroA* strains of *S. typhi* sometimes seen on rich media not supplemented with DHB contrasts with the normal growth of *aroA* strains of *S. typhimurium* on such media and suggests that *S. typhi* is more dependent on the enterobactin method for iron acquisition than is *S. typhimurium*. Adenine-dependent *Salmonella* species grow poorly on tryptic soy agar, apparently because this medium contains insufficient adenine or adenosine (2). With the qualifications mentioned above, our live-vaccine strains of *S. typhi* grew well on all rich media tested, unlike *S. typhi* Ty21a, a live-vaccine strain thought to have unidentified mutations affecting nutritional character and rate of growth which were induced by the mutagen exposure used to obtain its *galE* mutation.

The extent of attenuation caused by the *aroA* and *purHD* mutations introduced into the Ty2 and CDC 10-80 strains has been tested by intraperitoneal injection into mice, with hog gastric mucin as an adjuvant; each mutation by itself caused a complete loss of virulence (Edwards, Ph.D. thesis; Edwards and Stocker, in preparation). The *aroA his purA* derivative of strain CDC 10-80, 541Ty, and its Vi-negative mutant, 543Ty, caused no adverse effects in volunteers who drank up to 2×10^{10} CFU preceded by sodium bicarbonate (11), and nearly all these subjects gave evidence of a cellular immune response, though little or no humoral antibody response was shown. Although adenine requirement caused more complete attenuation in *Salmonella* species virulent for mice than did requirements satisfied by any purine (14), there is now evidence (21) that in *S. typhimurium* the addition of a *purA* mutation to an *aroA* strain reduces its live-vaccine efficacy, probably because the mutation reduces the survival of the bacteria in the mouse tissues. Thus the *aroA his purHD* derivatives of strains Ty2 and CDC 10-80, if tested in human volunteers, might prove more effective than the previously tested derivatives 541Ty and 543Ty.

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ADDENDUM

Since the completion of our work, a stable *aroA* mutation in *S. typhi* Ty2, obtained from an *aroA554*::Tn*10* transduc-

tant by selection for tetracycline sensitivity, has been described (4).

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