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Construction of *Salmonella* Strains with Both Antigen O4 (of Group B) and Antigen O9 (of Group D)

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A *Salmonella* live vaccine causing both O4- and O9-specific immune responses would be of use, but no reported *Salmonella* serotype has both of these O antigens. Constructed *Salmonella typhimurium* strains with an *rfb* (O-antigen-specifying) gene cluster of type D in the chromosome and one of type B in an F′-*rfb*$^+$ factor, and those with the reverse combination reacted strongly with both anti-O4 (and anti-O5) and anti-O9 sera and, if they carried *recA*, could be maintained in this state by growth conditions selective for retention of the F′ factor. One of the two B.*rfb*$^+$ gene clusters of a (P22-lysogenic) *S. typhimurium* strain with a tandem duplication of a chromosomal segment including *hisD* and B.*rfb*$^+$ was replaced (by transduction) by a D.*rfb*$^+$ gene cluster; the resulting strain was O1$^+$ O4$^+$ O5$^+$ O9$^+$ and stable as such after being made *recA*. A stable O4$^+$ O9$^+$ derivative of a virulent *S. enteritidis* (O-group D) strain was made by transducing into it first the join point of an appropriate tandem duplication strain, together with the adjacent B.*rfb*$^+$ gene cluster, and then *srl*::Tn*10 recA*.

Salmonella serotypes in subgenus I (by convention, each given a species name) are grouped by O antigen, i.e., the antigenic character of the polysaccharide component of their surface lipopolysaccharide (LPS). Many species, including *Salmonella typhimurium*, fall into O-group B, with O-antigen factor 4; many others, including *S. typhi*, fall into group D1, with O-antigen 9. The O polysaccharides of group B and D1 strains are linear polymers of an oligosaccharide repeat unit, which in each of these O groups is a mannose α1-2 rhamnose α1-3 galactose trisaccharide with a dideoxyhexose branch on the mannose. In group B, this sugar is abequose (=dideoxygalactose); in group D1, it is tyvelose (=dideoxymannose). The basic O repeat units of group B and D1 strains are otherwise identical. In many group B strains, the abequose branch bears an acetyl group, determining O-factor 5; no corresponding factor occurs in group D1. Other O factors, which may be present or absent in strains of either group, reflect either glucosyl branch units on the galactose, for O-antigens 1 and 12_2; the nature of the link between repeat units, for O-antigen 27; or some unidentified modification of the O unit, for antigen 12_3.

All of the special genetic information needed to build the O-oligosaccharide unit of the group B or D1 kind is comprised in the *rfb* gene cluster, near the *his* (histidine biosynthesis) operon, at ca. 42′ on the *S. typhimurium* linkage map (10, 13). The complete *rfb* gene cluster of a group B *Salmonella* strain (which we designate B.*rfb*$^+$) may be replaced, by a transductional or conjugational cross, by the corresponding D.*rfb*$^+$ gene cluster of a group D1 strain, with consequent replacement of O-factor 4 (or 4,5) by O-factor 9; replacement of O9 by O4 can be similarly effected (10). O repeat units are synthesized and polymerized on an antigen carrier lipid before transfer to the LPS core. Polymerization of group B or D1 units, linking them α1,2, requires the function of gene *rfc*, located some distance from the *rfb* gene cluster; the *rfc*$^+$ allele of a group B strain can cause polymerization of O9 repeat units, as well as of O4 units, as can the *rfc*$^+$ allele of a group D1 strain.

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Aromatic-dependent strains of *S. typhimurium* and *S. dublin* used as live vaccines in mice or calves confer substantial protection against later challenge with virulent strains of the corresponding serotypes. There may also be, soon after vaccination, a degree of cross-protection, but later protection seems to be O4 or O9 specific. A *Salmonella* live-vaccine strain able to protect against both O4 and O9 strains might therefore be of use, for instance, for protection of calves against both *S. dublin* and *S. typhimurium* infections. However, no *Salmonella* serotype with both antigens O4 (or O4,5) and O9 has been reported among isolates from nature. We describe here the construction of such strains by bringing together B.*rfb*$^+$ and D.*rfb*$^+$ gene clusters in a single strain. In the accompanying report (18), we describe the composition of the LPS of stable O4$^+$ O9$^+$ strains and show that individual bacteria of such strains make LPS with both O4 and O9 reactivities and that at least some of their O-polysaccharide chains contain both kinds of repeat unit.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The starting and constructed strains used are listed in Table 1. Strain TR5225, an *S. typhimurium* LT2 derivative with a deletion of the promoter and proximal structural gene, *hisG*, of the *his* (histidine biosynthesis) operon and a tandem duplication extending from *hisD*, at ca. 42′, through *gnd*, the *rfb* cluster and *metG*, at ca. 44′, but not *purF*, at 47′ (1), received from John Roth (Department of Biology, University of Utah, Salt Lake City), had the expected requirement for histidine, or for histidinol at high concentrations, and was, as expected, O4$^+$ O5$^+$ O9$^-$. Contrary to expectation, it was also O1$^+$, indicating lysogeny for a converting phage, probably P22, since its ancestry includes a conjugational cross with strain HfrB2, which is P22 lysogenic. Strain TR5225 was used as the parent of an O4$^+$ O9$^+$ strain. However, as its lysogeny made TR5225 immune to phage P22 HT105/1 *int*, we could not use it as a transductional donor. Instead, we used TR5214, a similar but P22-sensitive tandem-duplication strain which, like TR5225, has *hisD* function dependent on a join point placing it adjacent to a new promoter.

TABLE 1. Bacterial strains

Strain no.	Relevant genotype[a]	Source or reference
Starting strains		
his-203	*S. typhimurium* LT2 Δ*hisOG203*	1
PL5[b]	*S. typhimurium* FIRN M7471 (ColE1) *leu cysI* D.*rfb*$^+$	9
SB526	*S. typhimurium* LT2 Δ*his*(*rfb*)*520*	12
SB1542	*E. coli* K-12 *his-3157*/F'S400 B.*rfb*$^+$ *gnd*$^+$ *his*$^+$	17
SB2887	Same as SB1542 but carries F'S401	17, 17a
TR4871	*S. typhimurium* LT2 *his-22 recA* cured of cryptic plasmid	John Roth
TR5214	Same as TR5225 (below) but different "pi" mutation	John Roth
TR5225	*S. typhimurium* LT2 Δ*hisOG203 strA* "pi-422" (=tandem duplication)	John Roth
TT3266	*S typhimurium* LT2 *his-712 srl-211*::Tn*5 recA1* cured of cryptic plasmid	John Roth
Constructed strains		
SL5232	Same as PL5 but *hisC8579*::Tn*10*	Transduction of *hisC8579*::Tn*10* into PL5
SL5233	*S. typhimurium* LT2 Δ*his*(*rfb*)*520*/F'S401 *his*$^+$ B.*rfb*$^+$	Conjugational cross of SB2887 with SB526
SL5234	*S. typhimurium* Δ*his*(*rfb*)*520*/F'S401 *hisD8557*::Tn*10* B.*rfb*$^+$	Transduction of *hisD8557*::Tn*10* into SL5233
SL5245	Same as PL5 but *hisC8579*::Tn*10*/F'S400 *his*$^+$ B.*rfb*$^+$	Conjugational cross of SB1542 with SL5232
SL5247	*S. typhimurium* LT2 Δ*his*(*rfb*)*520*/F'SF01 *his*$^+$ D.*rfb*$^+$	Transduction of *his*$^+$ D.*rfb*$^+$ into SL5234
SL5249	*S. typhimurium* LT2 Δ*his*(*rfb*)*520 argG1828*::Tn*10*/F'S401 *his*$^+$ D.*rfb*$^+$	Transduction of *argG1828*::Tn*10* into SL5247
SL5251	*S. typhimurium* LT2 *his-22 recA1* cured of cryptic plasmid/F'S401 *his*$^+$ D.*rfb*$^+$	Conjugational cross of SL5249 with TR4871
SL5287	Same as PL5 but *gnd*::Tn*10*	Transduction of *gnd*::Tn*10* into PL5
SL5303	*S typhimurium* LT2 Δ*his203 strA* "pi-422" (=tandem duplication) now *gnd*::Tn*10* D.*rfb*$^+$ (in one copy of the duplication)	Transduction of *gnd*::Tn*10* D.*rfb*$^+$ into TR5225
SL5313	Same as SL5303 but *srl-211*::Tn*5 recA1*	Transduction of *srl-211*::Tn*5 recA1* into SL5303
SL5340	Δ*hisOG203 gnd*::Tn*10*	Transduction of *gnd*::Tn*10* into *his-203*
SL5306	*S. enteritidis* NCT 5694 *metA*	NCTC 5694 by transduction and mutation
SL5341	*S. enteritidis metA gnd*::Tn*10* Δ*hisOG203*	Transduction of *gnd*::Tn*10 his-203* into SL5306
SL5352	*S. enteritidis metA* CRR[*gnd*::Tn10(Tc^s)] *his-203*	Mutation from SL5341
SL5374	*S. enteritidis metA his-203 gnd* "pi-422" (=tandem duplication) B.*rfb*$^+$ (in one copy of the duplicated segment)	Transduction of "pi-422" from TR5214 into SL5352
SL5396	Same as SL5374 but *srl-215*::Tn*5 recA1*	SL5374 by transduction

[a] Other mutant characteristics of some strains, not relevant here, are not indicated.
[b] D.*rfb*$^+$ and, perhaps, *his*$^+$ of strain PL5 derive ultimately from a strain of *S. enteritidis* (of group D).

Phage P22 HT105/1 *int*, a "high transducing," integration-deficient derivative of phage P22 (14), was used for transduction. A set of rough-specific and smooth-specific phages (19) was used to determine the phage sensitivity pattern, and hence the smooth or rough character, of strains. Sensitivity to a male-specific phage, M13, was considered proof of the presence of an F' factor in a strain.

Media. Oxoid blood agar base, code CM55, and Oxoid nutrient broth no. 2, code CM67, were used as rich media. The defined medium used was that of Davis and Mingioli (4), with glycerol (5 ml/liter) and trisodium citrate (0.4 g/liter) as the energy source. Required amino acids were added when appropriate. For tests of HisD phenotype, i.e., ability of histidine auxotrophs to utilize histidinol as the sole source of histidine, defined medium was supplemented with L-histidinol at ca. 500 μg/ml; lower concentrations allow only very slow growth of HisD$^+$ His$^-$ strains, presumably because of inefficient uptake.

Genetic methods. Histidine-independent or tetracycline-resistant transductants were obtained by the drop-on-lawn method (8); plates of selective agar (defined medium without histidine, or of CM55 agar with tetracycline at 25 μg/ml) were flood inoculated with the recipient strain (a broth culture for selection of tetracycline-resistant transductants or a saline suspension when selection was for the His$^+$ or HisD$^+$ phenotype), and ca. 0.01-ml drops of the transducing phage lysate, at various dilutions, were added after the bacterial inoculum had dried. Because of the expected phenotypic delay, kanamycin-resistant transductants were obtained by mixing the phage and the recipient strain in broth and diluting and incubating them to allow some generations of growth before plating on CM55 agar with kanamycin at 25 μg/ml. Transductant clones were purified by single-colony reisolation from selective medium. Conjugational crosses were done by plate mating (cross-streaking). *recA* character was determined by testing UV sensitivity and, for strains derived from a P22-lysogenic descendant of strain LT2 (which is lysogenic for phage Fels2), by testing broth cultures for the presence of either of these phages.

For construction of an F' factor carrying the D.*rfb*$^+$ gene cluster, plasmid F's400 of Voll (17), which includes *his*$^+$ and B.*rfb*$^+$ from *S. typhimurium*, was transferred by conjugation from *Escherichia coli* SB1542 into strain SB526, which is *S. typhimurium* LT2 Δ*his*(*rfb*)*520*, a deletion removing the whole *his* operon and all testable genes of the *rfb* cluster (12). A His$^+$ transconjugant, SL5233, smooth and therefore able to absorb phage P22, was made *hisD8557*::Tn*10* by transduction, with selection for tetracycline resistance. A transductant, SL5234, with the inferred genotype Δ*his*(*rfb*)*520*/F's400 B.*rfb*$^+$ *hisD8557*::Tn*10*, was treated with a phage lysate of strain PL5, an LT2 derivative with a D.*rfb*$^+$ gene cluster, originally from *S. enteritidis* (9). Of 48 His$^+$ transductant

colonies tested, 46 were O4$^+$ O5$^+$ O9$^-$, like the recipient, and two were O4$^-$ O5$^-$ O9$^+$ and tetracycline sensitive, we infer because of replacement of B.*rfb*$^+$ *hisD*::Tn*10* in F′ by D.*rfb*$^+$ *his*$^+$. One of the two, SL5247, made *argG*::Tn*10* by transduction (to allow contraselection when used as a conjugational donor), was designated SL5429.

Serological methods. In tests at Stanford, strains were tested by slide and tube agglutinations with commercial (Difco or BBL) *Salmonella* O sera for factors O1, O4, O5, and O9. In Stockholm, rabbit O1, O4, O5, and O9 factor sera, prepared as described by Kauffmann (7), were from the National Salmonella Reference Laboratory at the National Bacteriological Laboratory.

RESULTS

F′ strains with the O4$^+$ O5$^+$ O9$^+$ phenotype. The F′ factor F′s400 of Voll (17) carries the wild-type *his*$^+$ and B.*rfb*$^+$ gene clusters of *S. typhimurium*. This F′ factor, housed in an auxotrophic *E. coli* host, was transferred by a plate conjugational cross from SB1545 to an *S. typhimurium* recipient, SL5233, which has a wild-type D.*rfb*$^+$ chromosomal gene cluster, originally derived from an *S. enteritidis* O9,12 strain (9), and is histidine requiring because of *hisC*::Tn*10*. Of 12 His$^+$ Thr$^+$ Pro$^+$ transconjugant clones tested by slide agglutination, 5 were O4$^+$ O9$^-$, 4 were O4$^-$ O9$^+$, and 3 were O4$^+$ O9$^+$. A double-reacting clone, SL5245, was tetracycline resistant and histidine independent (therefore, it was inferred to have *hisC8579*::Tn*10* in the chromosomal copy and *hisC*$^+$ in the F′ copy of the *his* operon) and sensitive to male-specific phage M13, the phenotype expected for a strain with no alteration in the chromosome and with the complete F′ factor from the donor. The O4$^+$ O9$^-$ and O4$^-$ O9$^+$ transconjugants presumably resulted from replacement of *his*::Tn*10* in the recipient chromosome by *his*$^+$ from F′ by crossovers at points such that chromosomal D.*rfb*$^+$ was replaced by B.*rfb*$^+$ from the F′ factor in five clones but not in the other four.

The above-described transconjugant clones demonstrated coexpression of chromosomal D.*rfb*$^+$ with an F′-borne B.*rfb*$^+$ gene cluster. For tests of the reverse situation, we used F′s401 (17), which we found to include B.*rfb* from its *S. typhimurium* parent; we constructed (see Materials and Methods) a derivative with its *his*$^+$-B.*rfb*$^+$ region replaced by *his*$^+$-D.*rfb*$^+$, originally from *S. enteritidis*. The F′ *his*-D.*rfb*$^+$ plasmid, designated F′s401-D1, was transferred by conjugation to strain TR4871, a *his recA* derivative of *S. typhimurium* LT2. All of several purified transconjugant clones gave strong positive slide agglutination with anti-O4 (and anti-O5) and also anti-O9 sera and retained these reactivities on subculture on medium without histidine to ensure retention of the F′ *his*$^+$ factor. However, these clones, and their parent TR4871, although smooth by the criteria of phage sensitivity pattern, agglutinability by anti-O sera, and stability in 4% NaCl in tube agglutination tests, showed a pattern of deposit suggestive of a partly rough character and so were unsuitable for analysis of chemical composition of LPS, etc.

To obtain a derivative of *S. typhimurium* LT2 of stable O4$^+$ O9$^+$ character, we made use of TR5225, a strain with a tandem duplication of a segment of the chromosome which includes the *rfb* gene cluster (Table 1 and Materials and Methods). This strain is of the class obtainable as partial revertants of a histidine auxotroph, LT2 Δ*his-203*, with a deletion of the promoter and first structural gene, *hisG*, of the histidine biosynthesis operon but an intact copy of *hisD*, which specifies the enzyme for the last step in the pathway, conversion of histidinol to histidine. The illegitimate crossover generating the duplication joins *hisD*$^+$, through the join point, to a new promoter with the appropriate orientation; this restores *hisD* function and allows growth with histidinol as the source of histidine. Loss of the duplication by crossover between homologous regions necessarily removes the join point and abolishes *hisD* function; strains can therefore be maintained with their duplication intact by growth with histidinol as the histidine source. The duplicated segment in TR5225 includes the *rfb* cluster; to change one of the two copies of B.*rfb*$^+$ of TR5225 to D.*rfb*$^+$, it was treated with a lysate of SL5287, a constructed *gnd*::Tn*10* D.*rfb*$^+$ LT2 derivative (Table 1). Only 1 of the 32 tetracycline-resistant transductants tested was O4$^+$ O9$^+$; this clone, SL 5303, required histidine or histidinol, as expected. We infer that in this transductant one of the two chromosomal copies of *gnd*$^+$ and one of the two B.*rfb*$^+$ gene clusters were replaced by *gnd*::Tn*10* and D.*rfb*$^+$ from the donor.

Elimination of tandem duplications requires the function of gene *recA*. We used phage grown on TT3266 (=LT2 *recA srl*::Tn*5*) to evoke kanamycin-resistant transductants from SL5303. Of 18 such transductants, 1 was found to be *recA*, as shown by its UV sensitivity and failure to liberate either phage Fels2 or P22, carried by it and its ancestor, TR5225. *recA* transductant SL5313 was, as expected, stably O4$^+$ O9$^+$; all of 16 colonies isolated after passage in broth for ca. 65 generations were O4$^+$ O9$^+$ and tetracycline resistant and required histidine or histidinol, like the original strain. By contrast, only 5 of 16 colonies from a similarly passaged culture of an *srl*::Tn*5 recA*$^+$ sister transductant were O4$^+$ O9$^+$ and tetracycline resistant and required histidine or histidinol; the other 11 were all O4$^-$ O9$^+$ and unable to use histidinol as a histidine source, and 7 of them were tetracycline sensitive.

We next constructed an O4$^+$ O9$^+$ version of a fully virulent (for mice) strain of *S. enteritidis*, SL5306, which is strain NCTC 5694 given a stable *metA* mutation not affecting virulence and of O9 antigenic character. This strain was made a Δ*his-203* mutant (deletion of the promoter of the *his* operon and *hisG* gene but not *hisD*) by cotransduction with *gnd*::Tn*10* from strain SL5340 (Table 1). A Δ*his-203 gnd*::Tn*10* transductant with unaltered O antigen was designated SL5341; a tetracycline-sensitive mutant isolated from it by Bochner selection (2) was designated SL5352. To introduce a tandem duplication including the *rfb* gene cluster into SL5352, it was treated with a lysate of strain TR5214; this latter strain has a tandem duplication like that of strain TR5225, described above. TR5214 thus has two copies of a chromosomal segment including B.*rfb* and with one of its two copies of *hisD*$^+$ adjacent, across the illegitimate join point, to an effective promoter. In a cross with TR5214 as the donor and a Δ*his-203* recipient, selection for the ability to use histidinol as a source of histidine (that is, for the HisD$^+$ phenotype) yields transductants which have incorporated the join point of the duplication and, in consequence, have a tandem duplication of the same extent as that of the donor, TR5214 (see reference 1 for a discussion of the mechanism). One such HisD$^+$ transductant found to react with anti-O4 and anti-O9 sera was inferred to have the donor B.*rfb*$^+$ gene cluster in one copy of the duplicated segment. This transductant, SL5374, was O5$^-$ by slide agglutination, as expected, since *S. enteritidis* lacks a functional *oaf* gene for addition of acetyl to the abequose of B-type repeating units, and O1$^-$ because it was not lysogenic for phage P22 or other O1-determining phage. Transductant SL5374 was inferred to

have B.*rfb*$^+$ in one copy of the duplicated segment and D.*rfbB*$^+$ in the other. To prevent elimination of its duplication, strain SL5374 was made *recA* by cotransduction with *srl*::Tn*5*, with selection for kanamycin resistance, to produce strain SL5396.

Stable *S. typhimurium* O1$^+$ O4$^+$ O^5 O9$^+$ tandem-duplication strain SL5313 and stable *S. enteritidis* O4$^+$ O9$^+$ tandem duplication strain SL5396 were used for chemical and serological analyses of LPS character as described in the accompanying report (18). Agglutination tests with O-factor sera prepared at the National Bacteriological Laboratory, Stockholm, confirmed their serological character, as stated above.

DISCUSSION

We obtained bacterial clones containing both a D.*rfb*$^+$ gene cluster and a B.*rfb*$^+$ gene cluster by two methods: introduction of a second *rfb* gene cluster as part of an F′ *his* plasmid and construction of tandem-duplication strains of *S. typhimurium* and *S. enteritidis* with different *rfb* clusters in the two copies of the repeated segment. So far as could be judged from slide agglutination reaction, factor O4 (and O5) was expressed as strongly as factor O9; in F′ *his rfb*$^+$ strains, the location of a particular *rfb* gene cluster, in the chromosome or the F′ factor, did not affect its apparent degree of expression. For more precise estimates, see the results of chemical determination of the abequose versus tyvelose content of the O polysaccharide of the two stable double-reacting strains described in the accompanying report (18).

Recently, a physical map of the whole *rfb* cluster of *S. typhimurium* was reported (3) and the genes for the last steps in the biosynthesis of the precursors of the dideoxyhexose branches of the O repeat unit of salmonellae of O-groups B, D, and A have been cloned and sequenced (15, 16, 20). In *S. typhimurium*, gene *rfbJ* specifies the enzyme which converts CDP-4-keto-2,3-dideoxy-D-glucose to CDP-abequose. Gene *rfbS*, at the corresponding position in *S. typhi* (O-group D), specifies the enzyme for conversion of the same substrate to CDP-paratose, which in *S. typhi* (but not in *S. paratyphi* A) is converted to CDP-tyvelose by the product of a functional adjacent gene, *rfbE*. In the course of that work, strains carrying appropriate combinations of chromosomal and plasmid-borne *rfb* genes were noted to express both factors O4 and O9 (as did our partial diploids) or both O4 and O2.

In view of the relative ease of construction of strains with both factors O4 and O9, it is noteworthy that no such strains have been reported among natural isolates. However, *S. zuerich*, of O-group D_3 and O-antigenic formula 1,9,12, (46),27, is of interest because this O-antigen formula suggests that its O units are of two sorts with respect to the trisaccharide backbone: some with an α-mannosyl unit (substituted at carbon 3 with tyvelose) and others with, instead, a β-mannosyl unit similarly substituted (11). The mannosyl unit forms the nonreducing end of the physiological repeating unit or unit of biosynthesis, and it would therefore be expected that the enzymes for synthesis of the mannose unit would be part of an *rfb* gene cluster. If this is the case, then one may speculate that *S. zuerich* (and other group D3 species) has two different *rfb* gene clusters, both of which are expressed. Furthermore, the results of coprecipitation tests suggested that in one fraction of the O polysaccharide of this organism, O27 and O46 specificities reside in the same molecule, indicating the presence of both α-mannosyl- and β-mannosyl-containing repeat units in a single chain. Genetic analysis of this serotype might produce results of interest.

Attenuated strains of various pathogenic bacterial species, used as live vaccines, in many instances give much better protection against experimental challenge or natural exposure than does use of killed bacterial vaccines of the same strain, as recently shown for *S. typhimurium* in mice, calves, and sheep. Such protection is believed to be, in large part, O antigen specific. An efficient method for producing nonvirulent auxotrophic derivatives, unaltered in antigenic character and effective as live vaccines, is now available for salmonellae of O-group B or D (5, 6). We plan to prepare and test as a live vaccine a strain stable in the O4$^+$ O9$^+$ state with an auxotrophic defect causing loss of virulence to see whether it can provide protection against challenge by both mouse-virulent *S. typhimurium* of O-group B and *S. enteritidis* of O-group D_1.

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