# *SEMIROUGH STRAINS OF SALMONELLA*

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Rough mutants (or, more precisely, R forms as defined immunologically[1]) of *Salmonella* produce cell wall lipopolysaccharide (LPS) containing as monosaccharide units only glucose, galactose, heptose, and glucosamine,[2] and have none of the O-antigenic specificities of the parent strain. By contrast, smooth strains (S forms) have specific O antigens and produce LPS which in many O groups contain additional kinds of sugar,[3] in some instances proved to be determinants of O-antigenic factors.[4] It is believed[2, 5, 6] that R forms have defects in the synthesis of LPS, so that they synthesize incomplete LPS, corresponding to the central "core" of S-type LPS and totally lacking the "S-specific side-chain" in which the S-specific sugars,[7] if any, are located. Furthermore, Beckmann *et al.*[8] have found that there are two antigenically different kinds of R forms, $R_I$ and $R_{II}$; $R_{II}$ forms are believed to synthesize LPS lacking only the S-specific side-chain,[5, 6] whereas the LPS of $R_I$ strains is less complete than $R_{II}$ LPS and probably lacks one or more of the deeper monosaccharide units in addition to the S-specific side-chain.[5, 6] Salmonella T forms, once supposed intermediate between S and R forms,[9] produce LPS resembling that of R form in respect of sugar composition;[10] the biochemical basis of the smooth colonial morphology and saline stability which differentiate the T form from the R form is unknown.

We here report salmonella recombinants and mutants of a form intermediate between S and R both in biological properties and in LPS composition, and propose the hypothesis that in this form, which we call semirough or SR, only the innermost portion of the normal S-specific side-chain is attached to the $R_{II}$ core LPS.

TABLE 1

BACTERIAL STRAINS USED

| | Strain | Origin | Reference |
|---|---|---|---|
| SR recombinants | SL954, SH805 | from the cross, SL951 × SL952 | 12 |
| | SH835, SH834 | from the cross, SL951 × SH685 | 12 |
| SR mutants | SL428 | LT2 cured of a B phage, class C | 13 |
| | SL901 | P22-resistant, nonlysogenic segregant from SD14* (P22), class C | |
| SR-like mutant | SL733 | =St/22. P22-resistant, P22h- and P221-sensitive mutant of LT2, class D | 14 |
| S form parents | SL951 | *S. abony*, S form (O: 4, 5, 12), Hfr (=SW1391) | 11 |
| | SL952 | *S. montevideo*, S form (O: 6, 7), $F^-$ | 12 |
| | SH685 | *S. takoradi*, S form (O: 6, 8), $F^-$ | 12 |
| | LT2 | *S. typhimurium*, S form (O: 4, 5, 12) | 17 |
| R and T forms | TV119 | R form ($R_{II}$) from SD14 | 15 |
| | SL924 | *S. typhimurium*, T form. Nr. 771 of Kauffmann | 9 |
| | SL927 | *S. typhimurium*, T form. Nr. 997 of Kauffmann | |

* SD14 is a substrain of *S. typhimurium* LT2 with a number of genetic markers, but with the same O-antigenic formula as LT2 (cf. ref. 15).

Table 1 lists the strains used and their origins. These semirough strains were first encountered as recombinants in certain crosses between salmonella S strains of different O groups. An Hfr strain[11] of *S. abony* (group B; O-antigen, 4, 5, 12; S-specific sugars, mannose, rhamnose, and abequose) were crossed to an $F^-$ strain of *S. montevideo* (group $C_1$; O-antigen, 6, 7; S-specific sugar, mannose). When recombinants with the *his*$^+$ locus[11a] from the donor were selected, it was found that a few of them expressed the O-antigens 4,5,12 of the donor strain; but about two thirds of the *his*$^+$ recombinants differed serologically from either parent and formed a homogeneous group we term SR. Similar recombinants were obtained in crosses of a *S. abony* Hfr with a *S. takoradi* $F^-$ (group $C_2$; O-antigen,6,8; S-specific sugars, mannose, rhamnose, and abequose), and as a rare class, in crosses of a *S. montevideo* Hfr to a *S. typhimurium* $F^-$ (group B) strain. The detailed results of these crosses will be published elsewhere.[12]

In their cultural and serological properties (Table 2), the SR recombinants were

TABLE 2

BIOLOGICAL PROPERTIES OF SR FORMS AS COMPARED WITH S AND R FORMS

| | SR form | S form | R form |
|---|---|---|---|
| Appearance of colonies on solid media* | Smooth | Smooth | Rough |
| Growth in liquid media* | Some deposit<br>Supernatant turbid | No deposit<br>Homogeneous growth | Heavy deposit<br>Supernatant clear |
| Sensitivity to phage P22 | Resistant | Sensitive (group B) | Resistant |
| Presence of S-antigen† | | | |
| O-4 | + | +++ (group B) | — |
| O-5 | + or − | +++ (group B) | — |
| O-6, 7 | — | +++ (group $C_1$) | — |
| Anti-S(4, 5, 12) antibody precipitated by LPS ($\mu$g)‡ | 9 | 58 | <2 |

* Media used include Difco nutrient agar as solid medium, and Difco nutrient broth (with 0.5% NaCl added) as liquid medium.

† Tested by slide agglutination of intact cells in factor antisera diluted with 0.2% NaCl. Also tested by several other methods. See text for details.

‡ Figures represent the amount of antibody protein precipitated by LPS containing 10 m$\mu$moles of heptose after 48 hr at 0°C. The antigen/antibody ratio during incubation was well in the region of antibody excess. LPS from strains SH805, LT2, and TV119 were used in this experiment.

intermediate between S and R forms, giving smooth-looking colonies but growing with deposit in broth and being unstable in saline suspensions. This instability made it difficult to determine the presence of O-antigen factors. However, the presence of O-4, indicated by slide agglutination, has been confirmed by the ability of one SR recombinant to evoke anti-O-4 antibody in the rabbit; SR recombinants also absorbed anti-O-4 serum but their absorbing capacity was poor, which suggests that they have much less factor O-4 than S forms of group B salmonella. O-5 was tested by absorption of monofactor anti-O-5 serum by SR bacteria, and by quantitative complement fixation test[16] using the same serum and SR LPS; some SR recombinants had no activity, and others had weak O-5 activity. The presence of O-12 was uncertain; the failure of SR strains to adsorb phage P22 suggests its absence.[17] LPS from several SR strains did not fix complement when incubated with anti-RII antibody (Fig. 1). LPS from an SR recombinant precipitated much less antibody from an anti-4,5,12 serum than did a 4,5,12 LPS. No O-6 or O-7 specificity was detected in any SR recombinant.

The sugar composition of the LPS of the SR recombinants and of their S parent strains, and, for comparison, of R and T variants of group B salmonella, is recorded

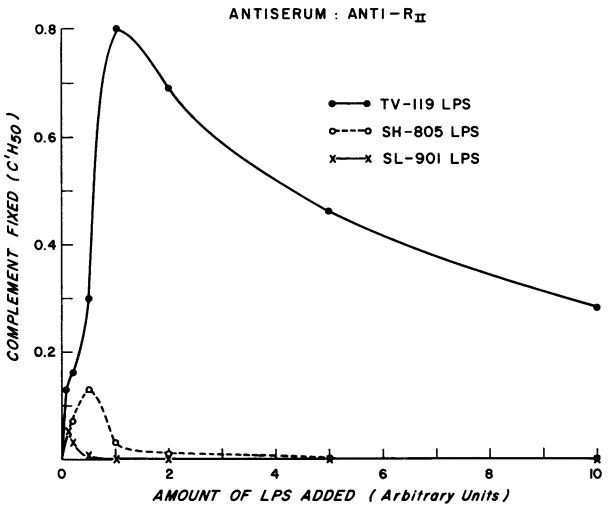


FIG. 1.—System for complement fixation[16] contained, in a total volume of 1.2 ml, 0.1 ml of anti-RII serum (diluted 1:500), antigen, and 0.2 ml of guinea pig complement (diluted 1:250). After overnight incubation at 0°C, 0.2 ml of sensitized sheep red blood cells (= $10^7$ cells) were added, and hemolysis was measured after 1 hr at 37°C. LPS prepared by phenol extraction (see legend to Table 3) was used as antigen. One unit of LPS is the amount which contains 0.13 mμmole of heptose. LPS and SH835 gave results similar to SH805 and SL901, and LPS from two other RII mutants gave results similar to TV119.

in Table 3. The quantitative results for sugars are expressed as moles per two moles of heptose, because heptose is present only in the "backbone" of LPS[18] and because there is probably one side-chain per two heptose units.[19] LPS preparations were also analyzed qualitatively by thin layer chromatography on cellulose after hydrolysis with $N$ $H_2SO_4$. The analyses of the S, R, and T forms agreed with earlier reports.[2, 3, 6] No mannose, rhamnose, or abequose was detected in the LPS of the R and T forms. The LPS of the group $C_1$ S form parent contained much mannose but

TABLE 3

QUANTITATIVE ANALYSIS OF SUGARS IN LPS

| Classification | | Molar Ratio of Sugars* | | | | Presence of mannose† |
|---|---|---|---|---|---|---|
| | | Heptose | Hexose | Rhamnose | Abequose | |
| SR recombinants | SL954 | 2.0 | 2.7 | 0.3 | 0.2 | + |
| | SH805 | 2.0 | 4.6 | 0.7 | 0.6 | + |
| | SH835 | 2.0 | 2.7 | 0.4 | 0.4 | + |
| | SH834 | 2.0 | 3.0 | 0.4 | 0.5 | + |
| SR mutants | SL428 | 2.0 | 3.2 | 0.5 | 0.4 | + |
| | SL901 | 2.0 | 3.2 | 0.5 | 0.5 | + |
| SR-like mutant | SL733 | 2.0 | 2.8 | 0.3 | 0.2 | + |
| S forms (O: 4, 5, 12) | SL951 | 2.0 | 11.4 | 3.9 | 2.7 | +++ |
| | LT2 | 2.0 | 7.5 | 2.4 | 2.6 | +++ |
| S form (O: 6, 7) | SL952 | 2.0 | 15.1 | 0.0 | 0.0 | +++ |
| R form | TV119 | 2.0 | 1.7 | 0.0 | 0.0 | — |
| T forms | SL924 | 2.0 | 5.7 | 0.0 | 0.0 | — |
| | SL927 | 2.0 | 3.0 | 0.0 | 0.0 | — |

* Heptose arbitrarily taken as 2.0. See text.

† Based on the results of thin-layer chromatography after acid hydrolysis.

LPS was prepared by phenol extraction[20] and purified by repeated ultracentrifugation until almost all nucleic acids were removed. Heptose was assayed by a modified cysteine-sulfuric acid reaction,[19] D-glycero-L-manno-heptose as standard; hexose by anthrone reaction,[21] D-glucose as standard; rhamnose by cysteine-sulfuric acid reaction.[22] Abequose was assayed, after hydrolysis of LPS for 30 min at 100°C with $N$ $H_2SO_4$, by thiobarbituric acid reaction[23] modified to eliminate interference from 2-keto-3-deoxyoctonic acid; difference between optical densities at 532 mμ and at 560 mμ used for calculation. In anthrone reaction, D-galactose and D-mannose produced only 60% and 55%, respectively, of the color produced by the equivalent amount of D-glucose.

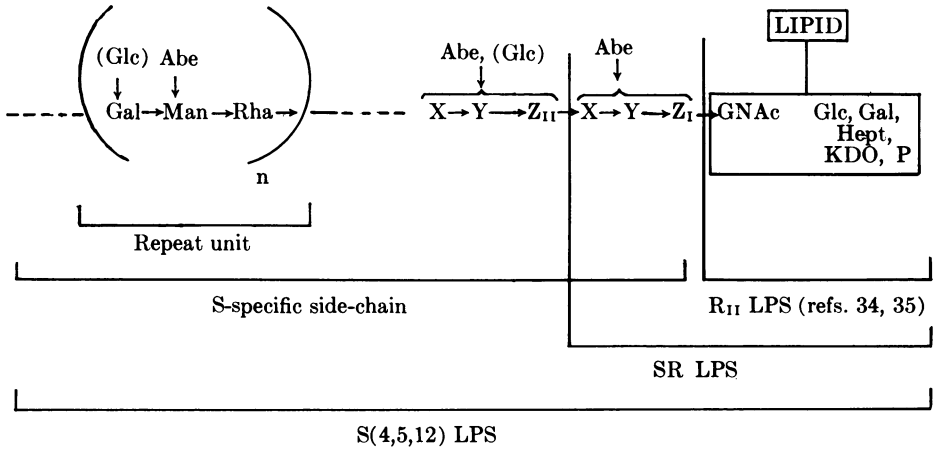


FIG. 2.—Hypothetical structure of LPS from several variants of group B salmonella Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; Glc, D-glucose; GNAc, N-acetyl-D-glucosamine; Hept, L-glycero-D-mannoheptose, KDO, 2-keto-3-deoxyoctonic acid.[24] The structure shown here is based on the studies from several different laboratories. The sugar sequence in the S-specific side-chain is mainly based on the studies of Staub and associates (reviewed in ref. 26) and also on the idea first proposed by Robbins and Uchida[25] that side-chains of LPS from some salmonella strains consist of many repeat units. X, Y, or Z each represents one of the sugars in the galactosyl-mannosyl-rhamnose repeat unit. Our *in vitro* incorporation studies[33] suggest that X, Y, and Z correspond to mannose, rhamnose, and galactose, respectively. Although there is no chemical evidence yet that the S-specific side-chain is linked to N-acetylglucosamine of the "core," such a linkage is suggested by the fact that $R_{II}$ immunological specificity is well masked in S-form LPS.

no rhamnose or abequose; its high content of hexose suggests that O-6,7 LPS has long S-specific side-chains containing much hexose. The LPS of the S form parent of group B contained much mannose, rhamnose, and abequose; if, as is generally believed, the S-specific side-chain in group B is made up of "repeat units,"[25] each containing one mole each of mannose, rhamnose, galactose, and abequose (and perhaps also one glucose)[26] (cf. Fig. 2), the average number of "repeat units" per chain was not less than three or four. The LPS of the SR recombinants contained mannose, rhamnose, and abequose, but in very much smaller amounts than the LPS of S form of group B. If the assumed repeat unit is present in SR LPS, the average number of repeat units per two heptose units, or per side chain, is only 0.3–0.7;[27] one quarter to one thirteenth the average number in S form LPS.

The side-chains of the SR LPS might be of uniform length and composition; or there might be a few S-specific side-chains of normal length and many R "stubs," totally lacking any S-specific side-chains. Since such "stubs" would presumably react with anti-$R_{II}$ antibody, the absence of $R_{II}$ specificity in SR LPS (Fig. 1) is strong evidence against the latter notion. $R_I$ forms, which have no defect in the synthesis of S-specific side-chain itself, are known to synthesize a soluble hapten containing all the S-specific sugars.[8] Such a hapten was not found in the extracts of SR recombinants, a finding consistent with the assumption that they have a defect preventing them from completing the synthesis of a normal S-specific side-chain.

Perhaps the SR recombinants lack one or more enzymes which in S-form salmonella of group B are concerned with the elongation of the 4,12 side-chain[27a] after the attachment to the R core of the most proximal units of the side-chain—the small

amount of mannose, rhamnose, and abequose in the SR LPS representing these proximal units.[28] Our genetic interpretation of the appearance of SR recombinants in crosses of group B and group C salmonella is that the group C parent lacks the genes for several of the enzymes needed for the synthesis of 4,12 side-chain, including the gene(s) for its elongation; that in the group B parent all the genes missing in group C parent, *except a gene or genes for elongation*, are closely linked to *his;* and that in consequence many of the group C recipients acquiring *his*$^+$ from the group B donor acquire also all the genes missing in the group C parent except one or more unlinked elongation genes, and at the same time lose all or some of the genes they had for the synthesis of group C O-antigens.[29]

If absence of the "4,12 elongation enzyme(s)" results in the SR phenotype instead of the S phenotype of group B, then mutation in the structural gene(s) for these enzyme(s) should produce the SR phenotype in S strains of group B. Most mutants of *S. typhimurium* strain LT2 selected for resistance to phage P22 were R mutants, of classes *rouA* and *rouB*,[15] which synthesize $R_I$- and $R_{II}$-type LPS,[8] respectively. A minority, however, lacked some features of the R phenotype. The serological properties of these mutants have not yet been fully investigated. Their pattern of resistance to a series of phages[30] divide them into two classes. Mutants of class C, typified by strain SL428, received from Dr. N. D. Zinder as a "part-rough" mutant, resembled the SR recombinants in phage resistance pattern, cultural characters, and sugar composition of LPS (Table 3). We suppose them to be SR mutants, resulting from mutational loss of the same elongation enzyme(s) as are missing in the SR recombinants. In two SR mutants of this class the site of mutation mapped between *gal* and *try*, i.e., remote from *his*,[31] a finding consistent with our interpretation of group B by group C crosses discussed already. The other group of mutants, class D, including the P22-resistant mutant st/22 of Yamamoto and Anderson,[14] differ from SR recombinants and the class C mutants in phage resistance pattern[30] and in some cultural characters, although the composition of LPS from one of them resembled that of SR LPS (Table 3). In two mutants of class D, the site of mutation was near *str*, i.e., remote from both *his* and from the *gal–try* region.[31] In contrast to the LPS of SR recombinants and class C mutants, their LPS reacted, in complement fixation test, with anti-$R_{II}$ serum almost as strongly as an $R_{II}$ LPS did. Possibly their LPS has many R "stubs," and side-chains which may be of normal length but are fewer in number than in S-form LPS.

No enzyme defect has yet been established in any of the SR strains. However, we can easily envisage one sort of enzyme which will be needed for the elongation of the 4,12 side-chain. Recent studies[32, 33] suggest that the most proximal part of the S-specific side-chain contains the same sequence of sugars as the remainder, attached to N-acetylglucosamine, the terminal sugar of the R "stub" of $R_{II}$ LPS. If the S-specific side-chain of group B salmonella consists of a repeat unit, probably of the structure shown in Figure 2, and if the sugar units are added one by one, then one enzyme will be needed to attach the most proximal sugar of the S-specific side-chain ($Z_I$ of Fig. 2), be it rhamnose, galactose, or mannose, to the N-acetylglucosamine, and presumably another enzyme will be required to add the second ($Z_{II}$ of Fig. 2) and subsequent units of this sugar, since they must now be attached not to N-acetylglucosamine but to the appropriate sugar of the galactosyl-mannosyl-rhamnose repeat unit. Lack of the latter enzyme would result in the addition of only one repeat

unit to each terminal N-acetylglucosamine and would account for the quantitative sugar analyses of LPS from SR strains.

So far as we are aware, the presence of the salmonella SR form as here defined has not previously been established. Robbins and Uchida[36] recently reported that a mutant form of the phage $\epsilon^{15}$ converted *S. anatum* to a form with an LPS containing abnormally few S-specific determinant groups. But, unlike the LPS of SR forms, this LPS apparently contains two or more repeat units per two heptose units. Finally, it should be stressed that the explanation discussed in this paper is still tentative and many more genetic, immunological, and biochemical studies are necessary. Such studies are in progress.

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[11a] The following symbols for genetic loci were used: *his*, histidine biosynthesis; *try*, tryptophan biosynthesis; *gal*, galactose fermentation; *str*, streptomycin resistance.

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[27] The assay used for heptose had a tendency to overestimation when applied to the whole LPS. Therefore, these ratios may actually be much closer to 1.0.

[27a] 4,12 side-chain is the side-chain with the immunological specificity of O-4,12, such as the one in LPS of S form salmonella of group B.

[28] It is unlikely that the short side-chains of SR LPS result from slow synthesis of the precursors of LPS, because crude extracts of SR recombinants synthesized all the sugar nucleotides tested for as rapidly as did an extract of a 4,5,12 S-form.

[29] Similarly we can assume that the gene for O–5, which presumably controls the formation of galactose acetylase (cf. ref. 26), is not included in the gene cluster closely linked to *his*. SR recombinants which have received this gene perhaps acetylate the galactose unit in the S-specific side-chain, and show a small amount of O–5 specificity; those which have not do not show any O–5 activity (Table 2).

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## *ISOLATION OF A SOLUBLE TRANSPLANTATION ANTIGEN**

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The immunological hypothesis of graft rejection is founded upon the second-set phenomenon:[1] recipient animals which have already rejected grafts reject subsequent donor grafts in accelerated fashion. According to this hypothesis, "transplantation antigens" are defined as donor-specific substances which are "released" by the graft and thereby sensitize the recipient. Operationally, the activity of tissue extracts as transplantation antigens may be demonstrated by their ability to induce more or less donor-specific sensitization such that subsequent transplants are rejected in accelerated fashion.[2]

Chemical analyses of extracts capable of inducing skin sensitization have suggested that their antigenicity depended upon, or was associated with, desoxyribonucleoproteins,[2] mucoproteins,[3] and/or lipoproteins.[4] Recently, five independent groups of investigators[4–8] have demonstrated that materials with the bulk properties of lipoproteins function as transplantation antigens in skin-sensitizing,[5] enhancing,[6] and serologic[4–8] assays. These preparations (*a*) were water-insoluble, (*b*) contained approximately equal amounts of lipide and protein and only small amounts of carbohydrate, (*c*) were inactivated by organic solvents, and (*d*) failed to move on electrophoresis at several pH's. Solubilized derivatives of these extracts[6, 8] retained serologic activity but had lost their sensitizing activity.

A soluble transplantation antigen was prepared from water-lysed tumor cells by Manson *et al.*[9] This antigen was of low potency; it could immunize against donor strain tumor or tail-skin grafts but not against body-skin grafts.