Envelope Proteins in *Salmonella minnesota* Mutants

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SUMMARY

Envelope proteins of one smooth (S) strain and seven rough (R) mutants of *Salmonella minnesota* were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. All strains gave similar band patterns although some consistent differences were detected. A major polypeptide band at 54 k, which coincided with the flagellar component, was more prominent in S, Ra and Rb than in the Rc, Rd and Re chemotypes. The latter strains, however, showed more prominent bands at 48, 19 and 18 k. The stage of growth at which the cultures were harvested was also found to affect the band patterns, particularly in the 54 and 40 k regions. A closer examination of S, Ra and Re strains suggested that the levels of the major 40 and 37 k bands were slightly reduced in Re. It is concluded that the progressive loss of lipopolysaccharide components which occurs from the S chemotype through various degrees of roughness to Re is accompanied by a change in the envelope protein composition, apparently between Rb and Rc.

INTRODUCTION

Variation from 'smooth' to 'rough' in Gram-negative bacteria involves progressive loss of sugars in the lipopolysaccharide (LPS), with consequent changes in immunological specificity. This has been particularly thoroughly investigated in certain species of *Salmonella*, where strains have been isolated, with LPS, ranging from the fully smooth (S) type through varying degrees of roughness (Ra, Rb, Rc, Rd) to the extreme rough (Re) type, which contains only 2-keto-3-deoxyoctonic acid, lipid A and ethanolamine. The progressive attenuation of LPS in these series of mutants reflects the stepwise loss of enzymes involved in saccharide synthesis or assembly.

In contrast to this detailed knowledge of changes in LPS during smooth-to-rough variation, little has been published on possible changes in cell envelope proteins which may also occur during these mutations and which may thus affect the immunological specificity of the cells. The possibility of investigating such changes has been greatly facilitated by the introduction of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-p.a.g.e.) technique. Recently this method was used to show that phase variation in *Bordetella pertussis*, which may be analogous to smooth-to-rough variation in *Salmonella*, involved the loss of one, and possibly two, major proteins of the cell envelope (Parton & Wardlaw, 1975). In contrast, Morris (1973) found no changes in electrophoretic analyses of protein extracts of whole cells of smooth and rough *Brucella* strains. His results, however, were obtained by electrophoresis in the absence of SDS. Since starting this work, articles have appeared by Ames (1974), Ames, Spudich & Nikaido (1974) and Koplow & Goldfine (1974), the former group working along similar lines with *S. typhimurium* and the latter with *Escherichia coli*. Both groups have reported that rough mutants lack certain envelope proteins that are present in the smooth forms. *Salmonella minnesota* has a particularly extensive and well-characterized series of LPS mutants. The

envelope proteins of these strains, as determined by SDS-p.a.g.e., form the subject of this report. It will be shown that smooth-to-rough variation involves both gains and losses in envelope protein components.

METHODS

Bacterial strains. One smooth and seven rough strains of *S. minnesota* were obtained from Dr G. Schmidt of the Max Planck Institut für Immunobiologie, Freiburg, Germany. The strain designations and chemotypes are shown in Table 1. The chemical compositions of the corresponding lipopolysaccharides are reported by Lüderitz *et al.* (1971).

Media and growth. Stock cultures were grown on nutrient agar and, for larger scale growth, were inoculated into 1 l volumes of Nutrient Broth No. 2 (Oxoid), 2.5% (w/v), in 2 l flanged conical flasks. The flasks were shaken at 100 rev./min at 37 °C (or at 44 °C to inhibit production of flagella), usually for 16 h. Cells were collected by centrifugation at 4 °C, washed once in saline (0.85%, w/v, NaCl) and resedimented.

Envelope preparation. Packed cells were resuspended in saline at 4 °C and, after freezing, were broken by three passages through an LKB X-press. After thawing, the resulting suspension was diluted with saline and centrifuged at 100000 g for 30 min. The opaque pellet was dispersed in distilled water using a syringe with a 23 gauge needle and the suspension recentrifuged. This washing procedure was repeated, and the deposit was again suspended in water and then centrifuged in a bench centrifuge for 10 min to remove unbroken cells and debris. The supernatant was centrifuged at 100000 g for 1 h and the resulting envelope layer was removed, dispersed in a small volume of water and frozen until required.

Preparation of flagella. Cells were grown, collected and washed as described above, then suspended in 50 ml cold saline. Flagella were removed by blending in a MSE homogenizer at full speed for 2 min with the flask cooled in an ice-water bath. The sheared cells were removed by centrifugation at 10000 g for 30 min at 4 °C then resuspended in saline and both the supernatant (containing sheared flagella) and the cell suspension were centrifuged again. Sheared cells were used for envelope preparation as described above. Flagella were either concentrated by dialysis against polyethylene glycol 4000 (BDH) or were collected by centrifugation at 100000 g for 1 h.

Electrophoresis. Slab gel electrophoresis with a discontinuous SDS buffer system was based on the methods of Laemmli (1970) as modified by Ames *et al.* (1974). Separating and stacking gels contained 10 and 5% (w/v) acrylamide, respectively. Both the gels and the electrode buffer contained 0.1% (w/v) SDS. The total dimensions of the slabs were approximately 8.0 × 7.0 × 0.3 cm, the separating gels being 6.0 × 7.0 × 0.3 cm.

Envelope samples were adjusted to contain 2 mg protein/ml as determined by the method of Herbert, Phipps & Strange (1971). To solubilize the envelopes, a 0.5 ml sample was added to 0.5 ml of 0.125 M-tris-HCl buffer pH 6.8, containing 4% (w/v) SDS, 10% (v/v) β -mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue, and the mixture was heated to 100 °C for 5 min except where stated. The volume of sample mixture normally applied to the gels was between 25 and 50 μ l.

Electrophoresis was done in a Uniscil slab gel electrophoresis unit (Universal Scientific Ltd, London) at a constant current of 15 mA/gel for approximately 2 h. Staining and destaining was by the method of Weber & Osborn (1969) except that the gels were destained by soaking in several changes of the destaining solution.

Densitometer traces of stained gels were obtained using a Joyce Loebel u.v. scanner. The instrument was modified to have an effective scanning aperture of 0.05 mm and was fitted with a 280 nm interference filter.

Table 1. *Nomenclature and characteristics of S. minnesota strains*

Strain number	Synonym	Chemo-type	Flagella*	Pili*	Slide agglutination with 0.3% auramine	Inhibition† (mm diam)		
						Erythro-mycin	Crystal violet	Sodium deoxy-cholate
SF1111	<i>S. minnesota</i> (smooth)	S	+++	++	—	< 6	< 6	< 6
SF1112	R60	Ra	+++	—	+	< 6	< 6	< 6
SF1127	R345	Rb	+++	+	+	< 6	< 6	< 6
SF1119	R5	Rc	+	±	+	9	10	< 6
SF1121	R7	Rd ₁	+	—	+	11	10	< 6
SF1287	RZ ₂	Rd ₁	±	—	+	10	10	8
SF1117	R3	Rd ₂	+	±	+	13	14	10
SF1167	R595	Re	+	—	+	17	16	17

* Presence of flagella and pili was determined by electron microscopy of negatively-stained preparations: + + +, abundant; + +, common; +, few; ±, very few; —, none observed.

† Inhibition was tested on nutrient agar using paper discs 6 mm in diameter containing 10 µg erythromycin, 10 µg crystal violet or 2 mg sodium deoxycholate.

For molecular weight determinations, the following marker proteins were run under identical conditions: bovine serum albumin (mol. wt 67k; Sigma); yeast hexokinase (45k; Sigma); bovine pancreatic chymotrypsinogen (25.7k; Miles-Seravac, Maidenhead, Berkshire); trypsin (23k; Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex); sperm whale myoglobin (17.2k; Koch-Light); and horse-heart cytochrome *c* (11.7k; Koch-Light). Each protein band is referred to by the estimated molecular weight obtained by interpolation on the scale provided by the marker proteins.

Electron microscopy. Cells were fixed for negative staining with formaldehyde (final concn. 4%, w/v), centrifuged and resuspended in distilled water. Suspensions were mixed with an equal volume of 2% (w/v) phosphotungstic acid or 2% (w/v) ammonium molybdate and dried on Formvar-coated copper grids.

RESULTS

Preliminary characterization of strains

The smooth strain and the seven rough mutants of *S. minnesota* were subjected to a variety of tests as shown in Table 1. Electron microscopic examination for flagella and pili was made because of their possible contributions to the proteins in the envelope fraction. In the 16 h cultures used for envelope preparations, flagella were abundant on the S, Ra and Rb strains. They were also detected on all the other strains but in much smaller numbers. Pili were only abundant on a proportion of cells of the smooth strain but were also observed, in small numbers, in Rb, Rc and Rd₂. None were detected on Ra, Rd₁ or Re. It was thus apparent that smooth-to-rough variation in *S. minnesota* was accompanied by changes in these appendages in addition to changes in the LPS.

The slide agglutination and growth inhibition tests were used to differentiate between the S form and the various R mutants (Lüderitz *et al.* 1971; Ames *et al.* 1974), mainly as a check on the authenticity of the strains. Results of the slide agglutination test with 0.3% (w/v) auramine agree with those of Schmidt *et al.* (1969*a*). Our finding that sensitivity to erythromycin, crystal violet and sodium deoxycholate increases from Ra to Re agrees closely with the results of systematic studies performed on these strains by Schmidt, Schlecht & Westphal (1969*b*) and Schlecht & Westphal (1970).

Fig. 1

Fig. 1. Envelope protein profiles of the various chemotypes of *S. minnesota* from 16 h cultures. Molecular weights (k-daltons) are given on the left.

Fig. 2

Fig. 2. Envelope proteins obtained from unsheared (column 1) and sheared (column 3) cells of the S strain run with isolated flagella (column 2).

Comparison of envelope protein profiles

In the initial part of this investigation, SDS-p.a.g.e. was done by the method previously used for *Bordetella pertussis* envelopes (Parton & Wardlaw, 1975). However, since the publication of the paper by Ames *et al.* (1974) on *S. typhimurium*, the system used by these authors has been employed and has given much better resolution of the protein bands.

Fig. 1 shows the envelope protein profiles of all eight chemotypes of *S. minnesota* from 16 h cultures. Envelopes were prepared from cultures grown on the same occasion and treated under identical conditions. The band patterns of the S, Ra and Rb envelopes were very similar although the S envelopes appeared to contain smaller amounts of certain high molecular weight (> 70k, i.e. 70 k-dalton) bands (arrowed) which were present in all the rough strains. The band patterns of the remaining Rc to Re chemotypes were almost indistinguishable from each other but showed consistent differences from S, Ra and Rb.

S, Ra and Rb envelopes possessed a major band at 54k which was much diminished in the other strains. The latter, however, possessed bands at 48k and at about 18k which were more prominent than in the S, Ra and Rb strains. Some variation in the relative densities of the major bands at 40 and 37k was also noted. In S, Ra and Rb, and possibly Re, the 37k

band was the major band, whereas in the other strains the two bands were of approximately equal density. It was found, however, that the level of the 40k component varied significantly in S envelopes prepared on different occasions.

Identification of flagellin band

Because the strains differed markedly in their content of flagella, experiments were done to see if any of the apparent differences in envelope proteins were due to differences in this component. Flagella obtained from the S strain by shearing were run in parallel with envelopes from both sheared and unsheared cells (Fig. 2). The concentration of isolated flagella was adjusted, as closely as possible, to match that of flagella attached to unsheared cells. The flagellar protein band was found to coincide with the prominent 54k band in S, Ra and Rb envelopes. These strains were the only ones containing large numbers of flagella (see Table 1). There was no apparent difference between the band patterns of the envelopes from sheared and unsheared cells even though the former no longer possessed significant numbers of flagella when examined by electron microscopy. In addition, when envelopes from sheared cells of the S, Rd₂ and Re strains were compared, they showed the same band differences as envelopes from unsheared cells. Flagella prepared from the S strain on several different occasions and from the Rb and Re strains were indistinguishable and gave only a single band at 54k.

Because the envelopes from sheared and unsheared cells showed no apparent difference in the 54k region, additional experiments were made to determine whether flagella accounted for the major part of the 54k band or whether they simply gave a band coincident with a major 54k component of the envelope. Envelopes were prepared from cells grown at 44 °C to inhibit production of flagella (Kerridge, 1961). Growth of *S. minnesota* at 44 °C was found to inhibit production of flagella as determined by electron microscopy. Again there was no apparent difference from bacteria grown at 37 °C in the 54k region, though minor bands in other regions of the gel did change. Thus the difference between the abundantly flagellate strains S, Ra and Rb and the other, sparsely flagellate, strains could not definitely be attributed to differences in numbers of flagella.

Pili did not appear to be removed from the S strain by the shearing procedure and consequently no visible band corresponding to pili was obtained in the flagellar preparation. Only the S and Rb cells in these particular cultures were found to possess many pili and the envelope profiles of these strains could not readily be differentiated from that of the non-piliated Ra strain. It was presumed, therefore, that these appendages did not influence the envelope band patterns either because they were present in too small amounts or because the pili band coincided with a more prominent envelope band.

Effect of growth phase

Schnaitman (1974b) has shown that the outer membrane protein profiles of *E. coli* strains and other enteric bacteria could be significantly affected by the stage of growth at which the cultures were harvested. In the present work, comparison of *S. minnesota* envelope proteins was initially carried out using cells routinely grown overnight for 16 h. To determine whether some of the differences between the strains were due to differences in growth rates, envelopes were prepared from various strains harvested either at the same stage of exponential growth or several hours after the end of the exponential phase.

Figure 3 shows the growth curves of the S, Ra and Re strains and the times at which the cultures were harvested. The bacteria were initially grown overnight at 37 °C on nutrient agar plates and then inoculated into 100 ml amounts of nutrient broth. After incubation for

Fig. 3. Extinction changes in cultures of the S (●), Ra (○), and Re (△) strains grown in nutrient broth. Envelopes were prepared from cells harvested at the points indicated.

4 h, these cultures were adjusted to the same extinction values and 7 ml of each were used to inoculate 700 ml of nutrient broth. The S and Ra strains grew at approximately the same rate for about 4 h but the final turbidity of the S culture was greater than that of the Ra strain. The Re strain grew more slowly, but after 7 h the turbidity of the culture had equalled that of the Ra strain. Cultures of each strain were harvested in the exponential phase (e.p.), when they had reached an E_{540} of 1.0. Cultures grown in parallel were harvested after 9½ h when exponential growth had ceased, though some increase in extinction was still taking place. These cultures were thus termed post-exponential phase (p.e.p.).

Densitometer traces of the envelope protein profiles of the S strain from e.p. and p.e.p. cultures and from the 16 h culture grown previously are shown in Fig. 4. The profiles show major differences in two regions. The 54k band was found to increase in intensity from e.p. to p.e.p. envelopes. This change was paralleled by an increase in the number of flagella as seen by electron microscopy and presumably reflects an increase in the amount of flagellin in the envelope fraction. The 40k band, which was a major band in both the e.p. and p.e.p. envelopes, was greatly decreased in the 16 h preparation. In addition, the 16 h profile showed an increase in a band at 45k.

The preparations from the Ra strain showed a similar change in the 54k band. Both Ra and Re strains showed a slight loss of the 40k band in the 16 h preparation but this was not as marked as in the S strain. Otherwise, all three preparations were very similar in both the Ra and Re strains.

When e.p. or p.e.p. envelope profiles of the S, Ra and Re strains were compared, similar differences were observed. Densitometer traces of the p.e.p. envelopes are shown in Fig. 5. The changes were similar to those seen in the envelopes from 16 h cultures (Fig. 1). The major 54k band in S and Ra envelopes was much diminished in Re but the latter strain possessed increased amounts of the 48k band and two bands at about 18 and 19k. The major 40 and 37k bands showed the same relative densities in all three strains, but the Re strain appeared to contain slightly less of both of these bands. Also, high molecular weight (> 70k) bands in S were present in increased amounts in the Ra and Re form. It should be noted that the resolution of these bands in the envelope profile of the S strain varied in different gels but

Fig. 4

Fig. 5

Fig. 4. Densitometer traces of *S. minnesota* S envelope proteins prepared from cells harvested in exponential phase (e.p.) or post-exponential phase (p.e.p.) and compared with a sample from a 16 h culture prepared on a previous occasion. The molecular weights of prominent bands (k-daltons) are indicated; they were obtained as described in Methods.

Fig. 5. Densitometer traces of the envelope proteins prepared from p.e.p. cells of the S, Ra and Re chemotypes.

could be improved if the concentration of SDS in the upper electrode buffer and in the stacking gel was increased from 0.1 to 0.2% (w/v). The differences between the smooth form and the rough mutants in this region may therefore be more apparent than real.

Effect of temperature of solubilization

Previous work has shown that the temperature of solubilization may affect the band pattern of envelope proteins in SDS-p.a.g.e. (Schnaitman, 1973; Ames *et al.* 1974; Parton & Wardlaw, 1975). In an attempt to resolve further possible differences between the envelope proteins of the different chemotypes, envelopes which had been solubilized at 37 °C for 30 min were compared with those solubilized at the usual 100 °C for 5 min. Figure 6 shows the band patterns of the S and Re chemotypes, obtained from 16 h cultures, after solubilization at the two temperatures.

A comparison of the S and Re preparations solubilized under the usual conditions (Fig. 6, columns 2 and 3 respectively) reveals the same differences as described earlier. Both preparations showed similar changes in their band patterns when solubilization was done at 37 °C

Fig. 6. Envelope protein profiles of S and Re chemotypes obtained from 16 h cultures. Solubilization was at 100 °C for 5 min or at 37 °C for 30 min.

for 30 min (columns 1 and 4). The most striking change was that the major bands in the 100 °C profile (37 and 40k) were much reduced in the 37 °C profile. Another band at 21 k was also significantly reduced. On the other hand, three bands (50, 38.5 and 28k) appeared to be more prominent in the 37 °C profile. In addition, the 48 and 18k bands, present only in the Rc to Re chemotypes, appeared to be somewhat diminished in the 37 °C profile.

These differences are presumably due to differences in susceptibility of the proteins to solubilization, since the typical 100 °C profile could be obtained when a sample already solubilized at 37 °C was heated to 100 °C. Thus, although solubilization temperature affected the envelope protein profiles, similar changes occurred in the smooth and rough strains and no further differences in protein composition could be demonstrated in this way.

DISCUSSION

Salmonella minnesota resembles other Gram-negative bacteria whose envelopes have been examined by SDS-p.a.g.e. in showing a complex pattern of at least 40 polypeptide bands. This presumably reflects the minimum number of different polypeptides in the envelope since each band may contain two or more polypeptide species which happen to have the

same mobility. The finding of Schnaitman (1974*a*) that the major outer membrane band of *E. coli* obtained by SDS-p.a.g.e. at neutral pH is composed of four distinct polypeptide groups, supports this interpretation. This must be borne in mind when interpreting changes in the density of a band. For example, a reduction in density could be due to a partial loss of a single component or to the complete loss of one polypeptide group from a mixture.

Schnaitman (1974*b*) has shown that the relative levels of the major outer membrane proteins of *E. coli*, *Salmonella* and *Shigella* strains depend on the stage of growth at which cultures are harvested. Changes in envelope protein composition have also been shown in *E. coli* cells in which DNA synthesis or cell division was altered (Inouye & Pardee, 1970; Siccaldi *et al.* 1971; Siccaldi, Lazdunski & Shapiro, 1972). In the present study, the envelope protein profiles of exponential-phase cells were compared with those of cells in which exponential growth had ceased, though because a complex medium was used some growth was still taking place. No major differences were seen except one in the 54 k region which appeared to be due to a change in flagellar content. Envelope profiles of older (16 h) cultures, however, particularly in the smooth strain, showed a decrease in the major 40 k band. It is therefore essential when comparing the envelope proteins of different strains to ensure that all strains are at the same stage of growth. The variations in the level of the 40 k band could represent a true difference between the various chemotypes but are more likely due to differences in the state of growth of the cultures.

Recent studies by Ames *et al.* (1974) have shown that certain major envelope polypeptides of *S. typhimurium* were lost or diminished in extreme rough (Rd₂ and Re) mutants derived from this strain. They also showed that the polypeptides were major components of the outer membrane portion of the envelope. Similar findings were made by Koplow & Goldfine (1974) when they examined extreme rough mutants of *E. coli*. The present observations on *S. minnesota* indicate that although changes in envelope protein composition do occur during smooth-to-rough variation, these changes are not as marked as those in *S. typhimurium* and *E. coli* and are not all deletion changes.

One difficulty encountered in interpreting the changes in band patterns was due to differences in numbers of flagella in the various strains. The band produced by isolated flagella was found to coincide with the major 54 k band present in the abundantly flagellate S, Ra and Rb strains. The other, sparsely flagellate, strains showed only a minor band in this region. However, envelopes from sheared cells of the S strain or from cells grown at 44 °C to inhibit flagellar formation still showed a heavy band at 54 k, even though the cells did not contain significant numbers of flagella. The shearing procedure could possibly have left flagellar fragments still attached to the envelope though it is unlikely that this would account for the major part of the band. Similarly, if growth at 44 °C inhibited assembly of flagella rather than synthesis of flagellin, some of this constituent would probably be present in the envelope. These results were surprising, for differences in the 54 k band could be seen in the e.p. and p.e.p. envelopes where the difference in the numbers of flagella was much less marked. One explanation which fits these observations is that only a minor part of the flagellin or 54 k component is present actually in flagella and the rest is normally contained in the envelope, though no further evidence for this is available.

Ames *et al.* (1974) showed that their strains of *S. typhimurium* contained two types of flagella, namely phase 1 and phase 2. The phase 1 and 2 flagellins gave two heavy bands in the envelope profile at 50 and 57 k which were clearly absent from the envelopes of the non-flagellate extreme rough strains. In the previous paper (Ames, 1974) the relative amounts of these two bands were seen to vary (depending on the phase of the flagella) in whole cell profiles of otherwise isogenic strains.

In addition to the loss of the 54k band in the Rc to Re chemotypes, the Re strain appeared to contain slightly less of the major 40 and 37k bands. This change may have been common to all strains from Rc to Re, since their envelope protein profiles from 16 h cultures were very similar. The 40 and 37k bands possibly corresponded to the major outer membrane proteins of *S. typhimurium* (Ames *et al.* 1974) since both groups of proteins are solubilized at 100 °C but not at 37 °C. There was, however, no marked loss of these proteins in the extreme rough (Re) strain of *S. minnesota*, as observed in the corresponding strains of both *S. typhimurium* and *E. coli*.

In addition to the losses of protein material in Rc and Rd, certain bands appeared to be more prominent in these strains. In particular, a band at 48k in Rc to Re strains was not clearly defined in the S, Ra and Rb strains. Koplow & Goldfine (1974) found that their heptose-deficient strain of *E. coli* contained a band at 38k which was not present in the parent strain. This band appeared to be a component of the cytoplasmic membrane.

Ames *et al.* (1974) and Koplow & Goldfine (1974) showed that the protein changes in *S. typhimurium* and *E. coli* took place in the outer membrane, though they could also be seen in envelope or even whole-cell protein profiles. It therefore seems unlikely that the apparent lack of differences in *S. minnesota* was due to the different method of envelope preparation or that the differences were obscured by the cytoplasmic membrane proteins.

Ames *et al.* (1974) and Koplow & Goldfine (1974) have suggested that the loss of outer membrane proteins and the alterations in the LPS of the rough mutants result from a single mutation and that this could indicate a close association between these components. Both groups of workers have isolated revertants from the extreme rough strains which have regained the wild-type LPS and have also regained normal levels of the proteins. It was suggested that the proteins might need LPS components in order to be incorporated into the membrane, or vice versa. If so, the study of a series of chemotypes as in *S. minnesota* might help to determine how this association occurs. In the present investigation, the main change in envelope proteins occurred between Rb and Rc where changes in the composition of the pentasaccharide region of the basal core take place (Lüderitz *et al.* 1971).

Finally, the finding that the temperature of solubilization of the envelope proteins can markedly affect band patterns has been widely reported. The bands which show changes with temperature are also the ones which are lost in smooth-to-rough variation in *S. typhimurium* (Ames *et al.* 1974) and *E. coli* (Koplow & Goldfine, 1974) and in phase variation in *B. pertussis* (Parton & Wardlaw, 1975). In *S. minnesota* the 37 and 40k bands which are not present in the 37 °C profiles, are also slightly diminished in the Re forms. Possible reasons for the unusual behaviour of the proteins with these solubilization temperatures have been suggested (Schnaitman, 1973; Ames *et al.* 1974). This behaviour might in some way reflect certain properties and therefore certain functions (such as the interaction with LPS discussed above) which are no longer essential in the envelopes of the rough strains.

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