Characterization of Mutants of *Salmonella typhimurium* by Counter-Current Distribution in an Aqueous Two-Polymer Phase System

**O. STENDAHL, K.-E. MAGNUSSON, C. TAGESSON, R. CUNNINGHAM, AND L. EDEBO**  
The Department of Medical Microbiology, The Medical School, Linköping, Sweden

Received for publication 28 December 1972

An aqueous, two-polymer phase system was employed in an attempt to separate and characterize a series of R mutants, derived from *Salmonella typhimurium* 395 MS, with differing lengths of lipopolysaccharide chains on their surfaces. R mutants with varying degrees of virulence and phagocytic resistance were unresolved in this system. However, the smooth MS bacteria were clearly separated from the rough cells and showed a high affinity for the polyethylene glycol-rich top phase. A uridine 5′-diphosphate-gal-4-epimeraseless mutant, phenotypically in R or S form depending on the growth medium, partitioned as the R mutants and S bacteria, respectively. These results demonstrate the great influence of long polysaccharide chains on the physicochemical properties of the cell surface.

Mixtures of aqueous solutions of two polymers, such as dextran and polyethylene glycol (PEG) may form a two-phase system, one phase rich in dextran, the other rich in PEG. Such systems are finding increasing application to biological problems as different particles are unevenly distributed between the phases, which can be used for separation of cells, cell organelles, viruses, and macromolecules (1–3). The sensitivity and utility of this technique are markedly improved when repeated partitions are performed in the manner of countercurrent distribution separation. Sequential partitioning allows the resolution of different erythrocytes (15), lymphocytes, and granulocytes (14). The method also provides information about the surface properties of the entities separated, since cellular chemical properties and surface charge influence partition properties (12).

The influence of the chemical composition of the bacterial cell surface on its overall physicochemical properties is largely unexamined, although such influences may profoundly affect the fate of the bacterial cell, particularly in its interaction with other cells. Such effects are, of course, technically difficult to detect in the complex situation of a host-bacterium interaction. This communication reports an attempt to simplify the problem by employing a well studied series of mutants derived from *Salmonella typhimurium* 395 MS. The mutants differ from their parent strain by possessing deficiencies in their lipopolysaccharide (LPS) synthesis (Fig. 1) (references 8, 9; A. A. Lindberg, Thesis, Karolinska Institutet, Stockholm, 1971). They differ with respect to virulence, immunogenicity (5), and phagocytic resistance (11), as well as polysaccharide chain length. These organisms have now been tested in a two-polymer aqueous phase system to assess the influence of their known differences on their distribution in the phases.

Earlier work showed a certain degree of correlation between the chemical composition of the LPS in these mutants and their ability to evade host defences. It was expected that a further correlation would appear in the present work: that the LPS on the cell surface would influence the two-phase separation, and provide a greater understanding of the role of LPS in modifying the cell surface properties of bacteria.

**MATERIALS AND METHODS**

**Bacteria.** The smooth, highly virulent (to mice) *S. typhimurium* 395 MS and the rough mutants derived from it have been described earlier (5, 8; A. A. Lindberg, Thesis, Karolinska Institutet, Stockholm, 1971).

**Cultivation.** All strains were kept at 4°C on agar slants before use. Their plaque patterns were checked at intervals. Bacteria were grown overnight in nutrient broth (Difco) at 37°C. Then, 1 ml of the initial culture was transferred into 9 ml of fresh medium and incubated for another 4 h on a rotary shaker (37°C). The bacteria were harvested by centrifugation (6,000 × g), washed twice in...
Fig. 1. Proposed structures of the LPS of S. typhimurium. Bottom, Relation between structure and chemotype (smooth, Ra-Rd), phage pattern (smooth, rough sensitive etc.), and the strains used in this study (S, RO-R10). A dotted line indicates that not all the molecules carry the indicated substituent. Abbreviations: OAc, O-acetyl-group; Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Glc, D-glucone; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycerod-manno-heptose; KDO, 2-keto-3-deoxyoctonate; Etn, ethanolamine. (For types of bonds, see Lindberg 1971.)

0.03 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.0, and kept in the cold until used. Before labeling, the bacteria were heat killed at 56 C for 1 h and washed twice in phosphate-buffered saline, pH 7.2 to 7.4. The uridine 5'-diphosphate (UDP)-gal-4-epimeraseless mutant was cultivated by the method of Lindberg et al. (10). Cells were grown for 4 h in galactose medium, harvested, and treated as above.

Viable counts were performed by spreading 0.1 ml onto eosin-methylene-blue agar and incubating overnight at 37 C. The colonies of the mutants used were macroscopically recognized.

Labeling with 51Cr and 125I. Heat-killed bacteria were labeled with 51Cr as previously described (11). For labeling with 125I, a modification of the method described by Biozzi et al. (4) was applied. In brief, to 109 to 1010 heat-killed bacteria suspended in 4 ml of saline, 0.05 ml of KI1 (18.5 mg of KI + 23.5 mg of KI1 per ml) and 1 mCi of carrier-free Na125I were added with constant stirring. The mixture was allowed to stand at room temperature for 3 h. After centrifugation, the cells were washed three times in cold saline and once in Tris buffer. Radioactivity in the last-wash supernatant fluid never exceeded 1% of that in the pellet.

Phase system preparation and countercurrent distribution. The aqueous phase system contained 4.40% (wt/wt) PEG 6000 (Union Carbide, N.Y.), 6.20% (wt/wt) dextran T500, lot 17 (Pharmacia Fine Chemicals, Uppsal, Sweden), and 0.03 M Tris buffer, pH 7.0 (1). It was prepared from stock solutions of 20% PEG and dextran in buffer and allowed to equilibrate at 4 C overnight.

An automatic thin-layer countercurrent distribution (CCD) apparatus (Incentive Research and Development AB, Stockholm, Sweden) with 120 cavities in the extraction train (2) was placed in a cold room (4 C) and used for all experiments. The charging volumes were chosen to give a moving interface. All but one of the cavities were charged with 0.8 ml of the bottom phase (dextran-rich) and 0.98 ml of the top phase (PEG-rich). The remaining chamber was loaded with 1.8 ml of the system containing bacteria (104 to 106 cells). After 115 transfers, each with a shaking time of 40 s and a settling time of 5 min, the phase system was dispersed by adding 1.8 ml of buffer to each cavity. Each chamber was then emptied into a plastic tube, and portions were removed for viable count determinations on eosin-methylene-blue agar. The numbers of heat-killed labeled bacteria were estimated with an auto-gamma scintillation counter with a NaI crystal (Packard Instrument Co., Downers Grove, Ill.). The activity was proportional to the bacterial concentration in each cavity. Charging with 0.55 ml of the bottom phase plus 0.70 ml of the top phase gave a similar distribution pattern. Thus, the system we primarily used (0.8 ml bottom phase, 0.98 ml top phase) behaved in the CCD apparatus as if the interface material was stationary.

The effective partition ratio (G) was calculated from the relationship G = vmax/(n - vmax), where vmax is the number of the cavity containing the peak of a distribution curve, and n is the number of transfers (13).

RESULTS

R mutants with varying degrees of virulence and phagocytosis resistance were the first tested. R4a (Ra) shows intermediate virulence for mice and is poorly phagocytosed by rabbit polymorphonuclear leukocytes. R10 (Rd) is nonvirulent for mice and is readily phagocytosed, even in the absence of antibodies. Fig. 2a shows the final distribution of these two mutants. No separation occurred when viable cells were used.
To eliminate the laborious and variable viable count, heat-killed cells were labeled with $^{51}$Cr and $^{125}$I radioisotopes. This also permitted the study of partitioning of the virulent smooth strain, MS. The distribution of the rough mutants was unaltered and coinciding, when cells were heat killed and labeled (Fig. 2b). All the R mutants of the 395 M series showed the same distribution. In contrast, smooth S. typhimurium 395 MS and its Rd mutant, MR10, separated extensively (Fig. 3), since the S bacteria showed a much higher affinity for the PEG-rich phase

**Fig. 2.** a, Countercurrent distribution (118 transfers) of a mixture of Salmonella typhimurium 395 MR4a (○) and R10 (○). Viable counts were obtained after dilution and plating onto eosin-methylene blue-agar, where R4a appeared smooth and R10 rough. b, Countercurrent distribution of Salmonella typhimurium 396 MR4a labeled with $^{125}$I (○), and R10 labeled with $^{51}$Cr (○). A total of $10^9$ cells was added to the system.

**Fig. 3.** Countercurrent distribution of the smooth, mouse-virulent S. typhimurium 395 MS, labeled with $^{51}$Cr (○), and R10 labeled with $^{125}$I (○).
(G = 11) than did MR10 (G = 0.2). On the other hand, R mutants with different amounts of LPS on their surface were unresolved in this phase system, even though they displayed differences in phagocytic resistance and virulence. In this respect, this two-phase system was less discriminative than the phagoeyte surface.

Whole cells are known to collect at the interface of some two-phase systems (3) at least to some extent. In our experiments with MS and R10, there seemed to be a partition mainly between the top phase and the interface for MS and between the interface and the bottom phase for the R mutants (Table 1).

The influence of the amount of complete O antigen on the cell surface was shown more specifically in the following way. One batch of the UDP-gal-4-epimeraseless mutant, R9, was grown in medium supplemented with galactose and one in medium without galactose. The synthesis of complete LPS-chains occurred only in the presence of galactose (11). Figure 4 exemplifies the results obtained. R9 grown in galactose separated efficiently from R9 cells grown without galactose.

**DISCUSSION**

The survival of enterobacteria in host tissues depends, in general, on the ability to evade phagocytosis. Complete LPS on the surface of smooth *Salmonella typhimurium* is known to increase its resistance to phagocytosis by rabbit polymorphonuclear leukocytes (11) and macrophages (6), and its virulence for mice. The virulence of some *Escherichia coli* strains and of *S. typhi* is thought to be increased by acid polysaccharide surface antigens, the K and Vi antigens, respectively. In spite of these observations, however, a strict correlation between surface structure and virulence is not established, even in the case of the LPS of *S. typhimurium*. Furthermore, the functional significance of cell wall LPS in promoting virulence and conveying resistance to phagocytosis remains obscure. Great differences in pathogenicity exist, even among bacteria with identical polysaccharide antigenic chains. For example, *S. typhimurium* and *S. paratyphi* B share the same O antigens 1, 4, 5, 12, but the first is particularly pathogenic for mice, the other for man. Such complexity calls for new approaches to the study of host-parasite interaction.

The experiments reported here represent an attempt to correlate physicochemical properties of bacteria with their tendency to interact with phagocytes. The complexity of the experimental situation was reduced by capitalizing on the fact that phagocytosis of R mutants of *S. typhimurium* occurs at significant rates without conspicuous need for serum factors (11). It seemed reasonable, in the light of the relative simplicity of the system used, to expect that large differences in physicochemical properties within the series of mutants of *S. typhimurium* could be discerned.

**TABLE 1. Quantities of labeled MS and MR10 bacteria found in the top phase, interface, and bottom phase after separation in one step**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Radioactivity (%) in:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top phase²</td>
<td>Interface</td>
</tr>
<tr>
<td>MS</td>
<td>78</td>
<td>18</td>
</tr>
<tr>
<td>MR10</td>
<td>4</td>
<td>26</td>
</tr>
</tbody>
</table>

* The two-phase system used was comprised of 2.5 ml of PEG-rich phase and 2.0 ml of dextran-rich phase.
² PEG-rich phase.
³ Dextran-rich phase.

![Figure 4](http://iai.asm.org/)

**Fig. 4. Countercurrent distribution of a UDP-gal-4-epimeraseless mutant (R9) grown for 4 h in medium with glucose (○) and glucose plus galactose (●). The cells were labeled with ¹³¹I and ⁵¹Cr, respectively.**
All R mutants tested (R0-R10; R9 grown without galactose) showed a similar partition pattern, whereas the parent S bacteria showed a much higher affinity for the PEG-rich phase. The results obtained with the R9 mutants grown with and without galactose confirmed that the separation examined in this study was due to the presence or absence of long polysaccharide chains in the cell wall and was not likely due to other differences which may exist between mutants. The sole known difference in the case of R9 grown with or without galactose is the presence of long polysaccharide chains in the phenotype grown with galactose which promptly moved this phenotype from the dextran phase into the PEG-rich phase.

In spite of the relative simplicity and artificiality of this system, our working hypothesis was only partially verified. We predicted a separation of S and R bacteria, but expected the R cells to concentrate in the PEG-rich phase, not the S form. We hypothesized that, since the LPS of R bacteria is more hydrophobic (7), these cells should prefer the PEG-rich phase, since PEG is higher up the hydrophobic ladder than dextran (1). However, the hydrophobic part of the LPS, i.e., the lipid portion, may be buried in the interior of the outer envelope such that only the saccharide portion is exposed. Alternatively, other cell surface properties such as charge may overshadow the hydrophilic and hydrophobic affinities (12). Such charged groups, which do not necessarily belong to the LPS, may be more or less exposed in R and S bacteria.

ACKNOWLEDGMENTS

This work was supported by grant B72-40X-2183-06B from the Swedish Medical Research Council. The skilled technical assistance of Kerstin Hagersten is gratefully acknowledged.

LITERATURE CITED


