Genetic and Physiological Classification of Periplasm-Leaky Mutants of Salmonella typhimurium

R. A. WEIGAND* and L. I. ROTHFIELD
Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06032

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Mutants of Salmonella typhimurium that leaked periplasmic proteins were isolated. Four classes of mutants were identified by their increased sensitivity to dyes, detergents, or antibiotics. Conjugation studies indicated that representatives of two classes mapped in the proA-galE region of the Salmonella chromosome and two in the cys-argE region. According to their bacteriophage sensitivity pattern, all four of the mutant classes appear to retain the smooth lipopolysaccharide characteristic. One class of mutants has an abnormal cell envelope in which the outer membrane balloons away from the murein layer.

The periplasmic region of the cell envelope of gram-negative bacteria contains a number of proteins that do not escape into the medium under normal conditions, although they can be quantitatively released by treatment of whole cells by the cold osmotic shock procedure of Neu and Heppel (10).

To study the role of cell envelope components in preventing release of periplasmic proteins in normal cells, we previously developed techniques for the isolation of mutants that spontaneously leaked the periplasmic enzyme ribonuclease (RNase) 1 into the medium during normal growth (8).

In the present report we describe further genetic and physiologic studies of a large number of periplasmic-leaky mutants of Salmonella typhimurium.

MATERIALS AND METHODS

Organisms and media. The S. typhimurium strains used in this investigation are listed in Table 1. Cells were grown in proteose peptone-beef extract medium (PPBE) (17) or minimal medium (M9) (9). Solid media (PPBE agar and M9 agar) also contained 1.5% agar. PPBE-ribonucleic acid (RNA) plates contained 20 ml of PPBE agar with a 4-ml overlay of 1% yeast RNA (pH 7) (Sigma) in PPBE agar. Minimal media were supplemented with 0.4% glucose (Baker) and when appropriate: amino acids (Sigma), 20 \( \mu g/ml \); except for serine, which was 200 \( \mu g/ml \); nucleosides (Sigma), 5 \( \mu g/ml \); lipoid acid (Calbiochem), 0.2 \( \mu g/ml \); and streptomycin (Sigma), 200 \( \mu g/ml \).

Isolation of mutants. Cultures of S. typhimurium SA722 were grown to stationary phase in M9 medium containing serine, adonine, and thiamine. After the cells were pelleted and suspended in M9, they were treated with 4% ethyl methane sulfonate (Eastman Organic) for 20 min at 37 C. After the cells were washed three times with M9, survivors (28% survival) were immediately plated on PPBE and were cloned by streaking before further study. The resulting clones were screened for leakage of RNase by transferring them onto PPBE plates and onto PPBE-RNA plates. After 15 h at 37 C, the PPBE-RNA plates were flooded with 8 ml of 0.5 N HCl for 3 min. Mutants that leaked RNase were identified by the large halos around the colonies.

Conjugation technique. The donor strain was grown to midexponential phase in PPBE and then was mixed gently with a 10-fold excess of an exponential-phase culture of the F- recipient strain in PPBE. After 30 min to 3 h at 37 C, the mixture was diluted 10-fold with saline, followed by disruption of the mating pairs with a mechanical mating interrupter (9). The mixture was immediately spread on selective plates, and recombinants were scored after 72 h.

Mapping of RNase-leaky loci. Matings were performed as described above with the RNase-leaky mutants as donors and a series of auxotrophic F- strains (Table 1) as recipients. Matings were interrupted 30 to 60 min after the first appearance of recombinants for a selected marker, and 100 to 400 recombinants were isolated for each of the selected markers indicated in Table 1. Recombinants were cloned, and each clone was tested for RNase leakage on PPBE-RNA plates. For each selected marker, the ratio of RNase-leaky recombinant clones to total recombinant clones was determined, and the ratio was plotted against the genetic map position of the selected marker (Fig. 1). If the selected auxotrophic marker is transferred long before the lky mutation, very few of the recombinants would be expected to receive the lky mutation. If the selected marker is transferred after the lky mutation, about half of the recombinants should leak RNase. When the selected auxotrophic marker is very near the lky mutation, a high percentage of the recombinants are likely to leak RNase. Each lky mutation was assigned a map location corresponding to the point at which the ratio first reached its maximal value. For map positions of the selected markers, see reference 18.

Analytic methods. Sensitivity to inhibitors was
TABLE 1. Genotypes of Salmonella typhimurium strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Selected markers*</th>
<th>Other markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA722ª</td>
<td>metA, trpB, hisF, iloA, pyrE</td>
<td>Hfr K10, serA, pur</td>
</tr>
<tr>
<td>SA571ª</td>
<td>galE, proA</td>
<td>xyl, strA, malA</td>
</tr>
<tr>
<td>SL761ª</td>
<td>gyrE, proA</td>
<td>purC, fla, iM, strA, fim, ile</td>
</tr>
<tr>
<td>SB100ª</td>
<td>tyrA, proA</td>
<td>purF, purC, iM, fla, strA, fim, ile, rha</td>
</tr>
<tr>
<td>SA1124ª</td>
<td>cysC,D deletion</td>
<td>metG</td>
</tr>
<tr>
<td>SB1301ª</td>
<td>cysl, lys</td>
<td>serA</td>
</tr>
<tr>
<td>SU277ª</td>
<td>tyr, argE</td>
<td>str</td>
</tr>
<tr>
<td>SA342ª</td>
<td>lip</td>
<td>ara</td>
</tr>
<tr>
<td>aroFª</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA225ª</td>
<td>purE</td>
<td></td>
</tr>
<tr>
<td>leuAª</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Auxotrophic markers that were used to select recombinants in the conjugation studies described in Fig. 1 and in the text. Gene symbols used are as defined by the donors of the strains and follow the nomenclature of Sanderson (18). All strains other than SA722 are F- strains.

Strain kindly provided by K. Sanderson of the University of Calgary.

Strain kindly provided by P. Hartman of The Johns Hopkins University.

assayed by colony-forming ability on inhibitor plates. The inhibitor plates contained 20 ml of PPBE agar plus one of the following formers: membrane blue (Baker), 100 µg/ml; chlorin red orange (Sigma), 100 µg/ml; rifampin (Sigma), 8 µg/ml; ethylenediamine-tetraacetate (EDTA), 1 mM; deoxycholate (Calbiochem), 10 mg/ml; sodium dodecyl sulfate (Sigma), 10 mg/ml. Clones were transferred from solid media onto the inhibitor plates, and growth was scored after 16 h at 37 C. Sensitivity to bacteriophages was measured by spot-testing bacteriophages on a freshly poured lawn of cells in enriched soft agar. Cyclic phosphodiesterase was assayed according to the procedure of Neu and Heppel (10) with the bis-(p-nitrophenyl)phosphate (Sigma) assay (0.2-ml volume). Acid hexose phosphatase activity was measured in an assay mixture (0.2 ml) containing 50 mM sodium acetate (pH 5.8), 5 mM EDTA, 0.5 mg of bovine serum albumin per ml and 5 mM [14C]glucose-6-phosphate (G-6-P) (1.8 x 10⁴ dpm/mM) (New England Nuclear). The reaction was terminated by heating at 100 C for 3 min. The liberated [14C]glucose was separated from [14C]G-6-P by a BioRad AG⁺1-X8 column in a Pasteur pipette by elution of the [14C]glucose with 3 ml of water and counting in a mixture of toluene, Liquifluor, and Biosolv (toluene: Liquifluor [New England Nuclear]:Biosolv [Beckman], 267:8:25). The RNase assay mixture (0.4 ml) contained 30 mM potassium phosphate (pH 7.0), 1.5 mM EDTA, and [14C]polyadenylic acid (0.68 mmol of phosphate/ml; 4 x 10⁶ dpm/mmol of phosphate) (Miles Laboratories). The reaction was terminated by adding 0.1 ml of 0.2% yeast RNA, 1 ml of cold 3% HClO₄, and chilling 15 min. The mixture was centrifuged at 10,000 x g for 10 min, and 1 ml of the supernatant was counted in the toluene, Liquifluor, and Biosolv mixture. Glucokinase was assayed in a mixture (0.2 ml) containing 100 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5), 10 mM adenosine 5'-triphosphate (Sigma), 5 mM MgCl₂, 2.5 mM NaF, and 0.5 mM d-[14C]glucose (40 mCi/mmol) (New England Nuclear). The reaction was stopped by heating at 100 C for 30 min; the products were applied to a BioRad AG⁺1-X8 column in a Pasteur pipette; and the column was washed successively with 4.5 ml of water, 6 ml of 5 mM HCl, and 3 ml of 50 mM HCl. The untreated glucose was eluted in the water wash, an undetermined radioactive contaminant was eluted in the 5 mM HCl wash, and the G-6-P was eluted in the 50 mM HCl wash. The [14C]G-6-P was counted in the toluene, Liquifluor, and Biosolv solution. G-6-P dehydrogenase was assayed in a mixture (1 ml) containing 87 mM glycylglycine (pH 8.0), 15 mM MgSO₄, 10 mM G-6-P (Sigma), and 0.4 mM nicotinamide adenine dinucleotide phosphate (Sigma). The reaction was started with the addition of nicotinamide adenine dinucleotide phosphate and initial rates were measured at 340 nm in a Gilford recording spectrophotometer. Ribose-binding proteins and histidine-binding proteins were assayed (2) by R. Aksamit and D. Koshland of the University of California at Berkeley, on cultures grown for 24 h at 30 C in VBC medium (20) containing 0.4% ribose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Neville (11) was carried out on isolated cell envelopes. The pellet of the 360,000 x g centrifugation, according to the procedure of Osborn et al. (16), was suspended and used as the source of cell envelope material.

Electron microscopy. Electron microscopy was carried out as described previously (21).

RESULTS

Mutant isolation and mapping. One hundred mutants that leaked RNase were independently isolated as described above. Twenty of the mutants that showed the most marked leakage were selected for further study.

Since we have been unsuccessful in developing a positive selection technique, the mutants responsible for RNase leakage were roughly mapped by conjugation with the isolated mutants acting as chromosome donors and well-marked F- strains as recipients; RNase leakage was scored as an unselected marker. The loci responsible for RNase leakage fell in two widely separated regions of the S. typhimurium genetic map. Mutants that mapped in the proA-galE region were defined as lkyCD and those mapping in the cysl-argE region as lkyAB (Fig. 1). As described below, differences in phenotypes among the mutants suggested that each region might be further subdivided. Precise definition of the map locations was complicated by the relatively poor
efficiency of gene transfer of late markers, which made it difficult to obtain large numbers of recombinants.

**Leakage of periplasmic versus intracellular proteins.** Quantitation of excretion of several periplasmic and intracellular proteins was obtained from studies in liquid medium. The proteins that were examined included five periplasmic proteins (RNase, acid hexose phosphatase, cyclic phosphodiesterase, ribose-binding protein, and histidine-binding protein) and two intracellular proteins (G-6-P dehydrogenase and glucokinase). Data from mutants representing the four RNase-leaky phenotypes (lkyA, lkyB, lkyC, lkyD; see below) are compared with the parental strain (SA722) in Table 2. Although the extent of leakage varied, all mutants excreted significant amounts of RNase, acid hexose phosphatase, and cyclic phosphodiesterase (Table 2). Excretion of binding proteins was not directly correlated with RNase leakage. Minimal excretion of ribose- or histidine-binding proteins was observed in the two lkyAB mutants, whereas the lkyCD mutants excreted large amounts of both proteins. With one exception (R67) the leaky mutants did not release significant amounts of the two intracellular proteins that were tested (Table 2).

**Correlation of phenotypes and genotypes.** Inhibitor studies indicated that the lkyAB and lkyCD groups could be further subdivided. Of mutants mapping in the lkyCD region, two subgroups could be distinguished based on sensitivity to rifampin (Table 3). Representatives of one subgroup (lkyC) typified by R60 were indistinguishable from the wild type in their sensitivity to rifampin, whereas lkyD mutants showed considerably increased sensitivity to this agent. Both subgroups were sensitive to several detergents.

Electron microscopy provided further support for a difference between lkyC and lkyD mutants based on altered morphology of the lkyD class. The morphological defect in four lkyD mutants consisted of a defect in invagination of the outer membrane during formation of the division septum. This frequently resulted in ballooning out of the outer membrane from the
periplasmic proteins in extracellular culture fluid\textsuperscript{a}

<table>
<thead>
<tr>
<th>Strain</th>
<th>Periplasm</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNase\textsuperscript{a}</td>
<td>AHP\textsuperscript{a}</td>
</tr>
<tr>
<td>SA722</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td>R67 (\textit{lkyA})</td>
<td>4.12</td>
<td>1.49</td>
</tr>
<tr>
<td>R104 (\textit{lkyB})</td>
<td>3.8</td>
<td>6.15</td>
</tr>
<tr>
<td>R60 (\textit{lkyC})</td>
<td>1.78</td>
<td>0.99</td>
</tr>
<tr>
<td>R71 (\textit{lkyD})</td>
<td>26.3</td>
<td>43.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Units are expressed as extracellular activity as percentage of total activity. Abbreviations: AHP, acid hexose phosphatase; CPD, cyclic phosphodiesterase; RBP, ribose-binding protein; HBP, histidine-binding protein; G-6-P DH, glucose-6-phosphate dehydrogenase; GK, glucokinase; ND, not determined.

\textsuperscript{b} Each strain was grown for 3 h at 37 C in PPBE and centrifuged at 12,000 \times g for 10 min. The pellet was suspended in fresh PPBE and broken in a French pressure cell at 15,000 lb/in\textsuperscript{2}. Assays were performed on the broken-cell suspension (cellular activity) and on the 12,000 \times g supernatant of the original culture (extracellular activity). Total activity was the sum of cellular and extracellular activities.

\textsuperscript{c} Cells and supernatant were prepared as described (see text), and cells were subjected to osmotic shock (10). The shock fluid and the supernatant of the original culture were each concentrated by vacuum dialysis and diluted against 150 mM NaCl-10 mM sodium phosphate buffer, pH 6.0, and assays were performed as described. Total activity was defined as the sum of the activity in the shock fluid and in the culture supernatant (extracellular activity).

### Table 3. Sensitivity to inhibitors\textsuperscript{a}

<table>
<thead>
<tr>
<th>Strain</th>
<th>AO MB</th>
<th>DOC</th>
<th>SDS</th>
<th>EDTA</th>
<th>RIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA722</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>R67 (\textit{lkyA})</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>R104 (\textit{lkyB})</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>R60 (\textit{lkyC})</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>R71 (\textit{lkyD})</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Sensitivity (S) or resistance (R) to inhibitors was determined by colony-forming ability on inhibitor plates as described. Abbreviations: AO, acridine orange; MB, methylene blue; DOC, deoxycholate; SDS, sodium dodecyl sulfate; RIF, rifampin.

The septal region, with formation of a large bleb on the surface of the cell (Fig. 2). When 28 other independently isolated RNase-leaky mutants were screened for rifampin sensitivity, seven were rifampin sensitive. Electron microscopy revealed similar outer-membrane abnormalities in three of these. No obvious morphologic abnormalities were observed in the \textit{lkyAB} or \textit{lkyC} mutants examined.

The basis of the relationship between rifampin sensitivity, bleb formation, and RNase excretion has not yet been clarified. Three spontaneous revertants from rifampin sensitivity to rifampin resistance were found to have reverted simultaneously to the wild-type phenotype with regard to bleb formation, RNase excretion, and sensitivity to deoxycholate and sodium dodecyl sulfate, so that these four characteristics may be due to the same primary defect. On the other hand, a large number of additional revertants to rifampin resistance did not simultaneously lose the other characteristics of the \textit{lkyD} mutants. Since rifampin resistance can result from a variety of causes, this result is not too surprising. Nevertheless, we believe that further genetic and biochemical studies will be required to firmly establish a common cause for the several phenotypic characteristics of the \textit{lkyD} mutants.

Mutants mapping in the \textit{lkyAB} region also appeared to fall into more than one phenotypic class. Most \textit{lkyD} mutants, typified by R67, were indistinguishable from the parental strain in their resistance to a variety of agents (Table 3) and are termed \textit{lkyA}. In contrast, a single RNase-leaky mutant that mapped in the \textit{lkyAB} region, R104, showed increased sensitivity to detergents and to EDTA. Conjugal studies indicated that the mutation in R104, tentatively termed \textit{lkyB}, was located at an approximate genetic map position of 102 min, compared to a map position of 90 min for the \textit{lkyA} locus. These genetic and phenotypic differences suggest that the mutation in R104 may be present in a gene separate from the other mutants mapping in the \textit{lkyAB} region. Several spontaneous revertants of this strain (R104) were isolated that had lost the abnormal sensitivity to EDTA. Three of these were found to have simultaneously reverted to the wild-type pattern of resistance to deoxycholate and sodium dodecyl sulfate and no longer leaked RNase into the medium. The distribution of phenotypes among 32 mutants that leaked large amounts of RNase was: \textit{lkyA}, 63%; \textit{lkyB}, 9%; \textit{lkyC}, 3%; \textit{lkyD}, 25%.

Generation times in PPBE medium were 25

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\textsuperscript{a} Units are expressed as extracellular activity as percentage of total activity. Abbreviations: AHP, acid hexose phosphatase; CPD, cyclic phosphodiesterase; RBP, ribose-binding protein; HBP, histidine-binding protein; G-6-P DH, glucose-6-phosphate dehydrogenase; GK, glucokinase; ND, not determined.
to 30 min for the parental strain and for mutant strains R60, R67, and R104. The doubling time for strain R71 was 35 min. Generation times in M9 medium supplemented with glucose, serine, adenine, and thiamine were 60 to 70 min for all five strains.

None of the present mutants appeared to contain altered lipopolysaccharides, as judged by their unchanged pattern of sensitivity to bacteriophages, which are highly specific for the structure of the carbohydrate chains of lipopolysaccharide (6, 22). The parental and four periplasmic-leaky strains were all resistant to bacteriophages C21, 6SR, and P1 and were sensitive to bacteriophages P22, Felix O and 9NA (6, 22). This pattern of sensitivity indicates that these strains have complete polysaccharide chains.

None of the major protein bands that were visible by sodium dodecyl sulfate-gel electrophoresis were absent from the cell envelopes of the mutants, although there were some differences in relative amounts of the different bands.

DISCUSSION

It seems clear that mutations in any one of several genes can affect the barrier function that prevents loss of periplasmic enzymes from normal cells. The present study identifies at least two (lkyAB and lkyCD) and probably four separate classes of mutants that share the periplasmic-leaky phenotype.

In addition, a mutation in the rfaF locus also results in leakage of periplasmic proteins (7). In the latter case, the mutation results in synthesis of lipopolysaccharides with incomplete polysaccharide chains, and it seems reasonable to conclude that the altered structure of this major outer-membrane component is directly or indirectly responsible for the observed defect in barrier function of the outer membrane in the rfaF mutant.

There was no apparent defect in lipopolysaccharide structure in the present mutants, so that the leakage of periplasmic proteins and the increased sensitivity to detergents and rifampin are likely to result from defects in other structural components of the cell envelope. Thus far, these components have not been identified.

Several other workers have described mutants of Escherichia coli that leak enzymes or are hypersensitive to drugs (3, 4, 19, 23). Olden and Wilson (15) have reported on mutants that leak β-galactosidase and alkaline phosphatase. Egan and Russell (5) described temperature-sensitive, osmotic-remedial mutants that both leak RNase and show hypersensitivity to inhibitors. Normark et al. (12–14) have reported mutants that are presumably envelope mutants and that show hypersensitivity to inhibitors. Thus, a large number of enteric bacterial mutants have been described that may have in common an increased membrane permeability. The relationship of the many pleiotropic pheno-
types of these seemingly similar mutants awaits further investigation.

The only mutants discussed in the present article that were morphologically abnormal were the lkyD group, in which there was a defect in invagination of the outer membrane during formation of the division septum. The leakage of periplasmic proteins in these mutants could be due to a generalized defect in the integrity of the cell envelope or outer membrane over the entire surface of the cell. On the other hand, the release of periplasmic proteins might be the result of the mechanical rupture of outer membrane that occurs when the blebs break off from the cell, a phenomenon that was suggested by the appearance of blebs free in the medium seen by phase contrast, Nomarski differential interference contrast, and electron microscopy. A more detailed description of this interesting class of mutants will be published separately.

ACKNOWLEDGMENTS

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LITERATURE CITED


LEAKAGE OF PERIPLASMIC PROTEINS

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