The Lipid A Biosynthesis Mutation *lpxA2* of *Escherichia coli* Results in Drastic Antibiotic Supersusceptibility

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The conditionally lethal *lpxA2* mutant of *Escherichia coli*, which lacks detectable UDP-N-acetylglucosamine acyltransferase activity and which produces greatly reduced amounts of lipid A after a shift to 42°C (S. Galloway and C. R. H. Raetz, J. Biol. Chem. 265:6394–6402, 1990), was found to be, at conditions which promote normal growth, remarkably susceptible to a number of antibiotics. The MICs of hydrophobic antibiotics, such as rifampin, erythromycin, clindamycin, and fusidic acid, were 32- to >128-fold lower for the *lpxA2* strain than for the parent type strain, and those of the peptide antibiotics vancomycin and bacitracin were 32- and 256-fold lower, respectively. Furthermore, the *lpxA2* strain was found to be sensitive to hypoosmotic conditions. Comparisons with the other characterized outer membrane permeability mutants, such as the heptose-deficient strains of *E. coli* and *Salmonella typhimurium*, the *acrA* and *abs* mutants of *E. coli*, and the *ssc-1* and class SS-B mutants of *S. typhimurium*, indicated that the *lpxA2* mutant had characterizedly the most antibiotic-supersusceptible phenotype. These findings advocate the possible use of the *lpxA2* strain as a tool in various fields of basic and applied bacterial research in which the impermeability of the outer membrane currently poses problems.

The poor activities of numerous antibacterial agents against gram-negative bacteria, such as strains of the family *Enterobacteriaceae* and the genus *Pseudomonas*, are due to the poor permeation of those agents through the outer membrane (OM) of such bacteria (15–18). In fact, acting as a permeability barrier towards noxious agents has been implicated as one of the main functions of the OM (18).

The lipopolysaccharide (LPS) constituent of the OM is crucially involved in the permeability barrier function. Bacterial mutants which produce defective, heptose-deficient LPS consisting only of the lipid A-2-keto-3-deoxyoctulosonic acid (KDO) portion have lost part of the barrier function (15, 18, 28). However, there are other, less-characterized mutants which appear to be even more susceptible to hydrophobic and certain other antibiotics than the heptose-deficient mutants (10, 31).

The lipid A-KDO region is apparently essential for the viability of the cell. Three types of characterized lipid A-KDO mutants exist (20). All are temperature sensitive, are conditionally lethal and, at non-growth-permissive temperatures, have even more profound defects in LPS synthesis than do the heptose-deficient mutants. These are the mutants defective in KDO synthesis or transfer, the *lpxB* mutants, which have defective lipid A synthase, and the *lpxA* mutants. *lpxA* mutant SM101, characterized by Galloway and Raetz (8), is defective in the first step of LPS biosynthesis and lacks all detectable UDP-N-acetylglucosamine acyltransferase activity. It produces significantly reduced amounts of lipid A after a shift to 42°C and quickly dies at that temperature (8).

Thus far, no reports have been published on the OM permeability barrier function of the temperature-sensitive LPS mutants at growth-permissive temperatures. In this paper, we report that *lpxA* mutant SM101 is extremely susceptible to hydrophobic antibiotics and two large peptide antibiotics, vancomycin and bacitracin, at conditions (28°C) which still enable growth comparable to that of the *lpxA* parent. Our studies indicate that the *lpxA* mutant is significantly more susceptible to antibiotics than the heptose-deficient and other OM permeability barrier mutants and advocate the possible use of the *lpxA* mutant as a supersusceptible target strain in screening studies in which new antibacterial or mutagenic compounds are being tested.

MATERIALS AND METHODS

**Bacterial strains.** *lpxA2* strain SM101 [*E. coli* K-12 *lpxA2 thr-1 leuB6 hisG4 rpsL136 eda50 thi-1 lacYI Δ(gal-att) ara-14 syl-5 mtl-1 tsx-78 rfdD1] as well as its *lpxA* mutant strain, SM105, have been described by Galloway and Raetz (8). *E. coli* JM109 [*E. coli* K-12 recA1 endoA1 gyrA96 thi hsdR17 supE44 relA1 Δlac-pro F′ (traD36 proAB lacIq) M15] is a commonly used cloning host strain (14). D212f(4) is a rough LPS chemotype Re mutant of D21 (*E. coli* K-12 *trp pro his strA tsx ampA1).

*Salmonella typhimurium* LT2 strain SH5014 (LPS chemotype Rb) is a typical and well-characterized representative of rough *S. typhimurium* and was widely used in our previous susceptibility studies (27, 29, 31, 34). SH7616 is its antibiotic-supersusceptible class SS-B mutant (29, 31), and SH7622 is its antibiotic-supersusceptible *ssc-1* mutant (10, 29). SL1102 (39) is an *S. typhimurium* strain with mutation *rfaE*343 (causing LPS chemotype Re).

**Antibiotic susceptibility determinations.** The MICs of the antibiotics were measured in L broth (32) as described previously (31). The inoculum was L agar-grown cells, and the inoculum size was 10⁶ cells per ml. Bacitracin (65,000 U/mg) was from Sigma Chemical Co., St. Louis, Mo. The sources of other antibiotics were as described previously (31).

**Measurement of the effect of osmolality on growth.** The effect of osmolality was studied in medium L1/5 (1:4 mixture [vol/vol] of L broth and deionized water) in the presence of increasing concentrations of a solute (NaCl or sucrose). The assays were done by using microdilution plates. The inocu-
TABLE 1. MICs of various antibiotics for lpxA2 mutant strain SM101 of E. coli, its parent type control strain SM105, and deep rough heptose-deficient E. coli D21f2

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml) for:</th>
<th>Parent type control strain SM105</th>
<th>lpxA2 mutant strain SM101</th>
<th>Heptope-deficient strain D21f2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>16</td>
<td>0.25</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Clindamycin</td>
<td>64</td>
<td>2</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>≥128</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Novobiocin</td>
<td>256</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Rifampin</td>
<td>2</td>
<td>0.03</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>64</td>
<td>4</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td>≥1,024</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.03</td>
<td>0.015</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.5</td>
<td>0.125</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>4</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

a As determined at 28°C.

b NA, not applicable.

RESULTS

SM101 (lpxA2) is known to be thermosensitive. It quickly dies at 42°C but grows indistinguishably from the wild type at 30°C (8). Furthermore, it produces almost normal levels of lipids A at 30°C, while at 42°C there is an approximately 10-fold inhibition of the lipid A synthesis rate relative to the rate of glycerophospholipid synthesis (8). In our experiments, SM101 grew like its parent did at 28°C, somewhat more slowly at 30°C, and not at all at 37°C. Therefore, we performed all the antibiotic susceptibility assays and other tests at 28°C.

We first tested the susceptibility of SM101 to a set of hydrophobic antibiotics (fusidic acid, erythromycin, clindamycin, novobiocin, and rifampin). All these are believed to traverse the OM through the hydrophobic pathway of diffusion (15-18, 34). This pathway is practically closed in wild-type strains but is open in certain OM mutants, such as the deep rough heptose-deficient LPS mutants (see the references listed above). SM101 turned out to be very susceptible to all these antibiotics. Their MICs for SM101 were 32- to >128-fold lower than those determined for parent type control strain SM105 and, furthermore, comparable to those for heptose-deficient E. coli D21f2 (Table 1).

Among the beta-lactam antibiotics tested, the most hydrophobic ones (dicloxacillin, benzylpenicillin, and cefuroxime) had MICs remarkably (>8-fold) lower than SM101 than for SM105 (Table 1). The MIC of the hydrophilic beta-lactam imipenem was only slightly lower for SM101 than for SM105, and cefotaxime (another hydrophilic beta-lactam) was equally active against SM101 and SM105. These hydrophilic beta-lactams penetrate the OM through the porin pathway. Furthermore, SM101 did not display any remarkable increase in susceptibility to gentamicin and chloramphenicol. These antibiotics also penetrate the OM without the aid of the hydrophobic pathway.

Thus, the highest sensitization ratios (i.e., the ratios between the MIC for the wild-type strain and that for SM101) were obtained with the hydrophobic antibiotics, as in the case of the heptose-deficient mutants of E. coli (Table 1) (17, 28, 30) and S. typhimurium (28, 30, 34). As evident from literature data, these sensitization ratios for SM101 are generally (i) much higher than those for E. coli DC2 (=abs) (5, 9) and acrA (6, 28) mutants and (ii) comparable to those for the ssc-1 (10) and class SS-B (31) mutants of S. typhimurium.

We next proceeded to study the susceptibility of SM101 to the large peptide antibiotics vancomycin and bacitracin. Both are too large to penetrate the OM through porin channels. The only examples of notably vancomycin-susceptible mutants of enterobacteria described in the literature are the thermosensitive ssc-1 mutant of S. typhimurium (10) and E. coli 16 (24). However, the latter mutant has not yet been characterized in molecular terms. With regard to bacitracin, no remarkably bacitracin-susceptible enterobacterial mutants have thus far been described. Interestingly, we found that SM101 was extremely susceptible to both vancomycin and bacitracin (Table 2). The MICs for SM101 were almost as low as those for susceptible gram-positive bacteria such as staphylococci (3). Table 2 also shows that SM101 was clearly more susceptible to both peptides than the ssc-1 mutant (at 28°C) and that the SS-B mutant was resistant.

One drastically more defective E. coli OM mutant category (ompA lpp) not only is antibiotic supersusceptible but also requires a higher ionic strength (i.e., a higher concentration of electrolytes) for optimal growth than the wild-type strains (26). As evident from Fig. 1A, the growth of SM101 was affected by electrolytes. For optimal growth, increased concentrations of NaCl were needed. The stimulatory effect of NaCl on the growth of SM101 was probably due to osmotic protection, since the effect was also demonstrated with sucrose (300 mM; Fig. 1B). In this respect, SM101 clearly differed from the ompA lpp strain, for which sucrose was not able to replace electrolytes. The results in Fig. 1A are from an experiment in which growth was measured after incubation for 24 h. A similar clear-cut difference between SM101 and SM105 was observed in experiments with shorter incubation times (10, 14, and 18 h; data not shown).

DISCUSSION

As reviewed above, no reports have been published on the OM permeability barrier function of the lipid A biosynthesis mutants. This paper shows that the lpxA2 mutant strain of E. coli is extremely susceptible to a number of antibiotics (hydrophobic antibiotics and large peptide antibiotics) against which the OM normally is a very effective barrier. Our results clearly indicate that the lpxA2 mutant is signifi-
deficient mutants have been regarded as susceptible to bacitracin and vancomycin, a closer look at these data (15, 24, 30) as well as the results in Table 2 in the present paper indicates that the heptose-deficient mutants display a low-grade susceptibility increase only, whereas the lpxA2 mutant is almost as susceptible as the susceptible gram-positive bacteria. Furthermore, the class SS-B mutant and the acrA mutant are as resistant to both peptides as are their parents (6, 31) and as the abs (DC2) mutant is to vancomycin (24) (the susceptibility of DC2 to bacitracin has not been published).

Even though it is well known that the biochemical defect in the lpxA2 strain is the lack of detectable UDP-N-acetylgalactosamine acetyltransferase activity (the enzyme required in the first step of lipid A biosynthesis), the mechanism by which supersusceptibility is mediated is not known. At the growth-permissive temperature (30°C), the lpxA2 strain produced almost normal amounts of lipid A, despite the enzyme defect, which is manifested at that temperature, too (8). As suggested by Galloway and Raetz (8), the lpxA2 mutation is probably not "tight" enough to arrest lipid A biosynthesis completely at 30°C. The supersusceptibility at the growth-permissive temperature could be due to a relative lack of LPS in the OM and, in analogy to the deep rough mutants (16, 17, 25), a resultant compensatory increase in the glycero-phospholipids of the outer leaflet of the OM, allowing the generation of glycerophospholipid bilayers in the OM. These act as channels for the diffusion of hydrophobic compounds. However, the drastic susceptibility pattern, clearly different from that in the heptose-deficient mutants, and the sensitivity to hypoosmotic conditions (Fig. 1) suggest the contribution of some additional mechanism.

Only a small number of hydrophobic drugs and peptide antibiotics were used in this study. However, most likely the lpxA2 strain is also susceptible to numerous other such compounds which are active against gram-positive bacteria and many or certain gram-negative bacteria but against which the normal enterobacterial OM apparently acts as an effective permeability barrier. These agents include such currently therapeutically used antibiotics and chemotherapeutic agents as mupirocin (37), the antifungal imidazoles (11), numerous antimetabolites, mutagens, and carcinogens (2, 7, 13, 17), various polyelectrolytes (1, 7, 38, 40), hundreds of peptide antibiotics (1, 7, 13, 19, 35, 40), phenols and polycyclic hydrocarbons (2, 22), long-chain saturated (23) and polyunsaturated (12) fatty acids, and parabens (21). Many of those compounds have already been shown to be more or less active against the deep rough mutants.

It is clear that an extremely supersusceptible strain such as the lpxA2 mutant might be an instrumental tool in studies of bacterial cellular physiology and biochemistry as well as in biotechnology, both fields in which the OM permeability barrier currently poses problems. The use of supersusceptible strains could replace the use of bacteria permeabilized by chelators such as EDTA (13) or hexametaphosphate (32), polymyxin B nonapeptide (1, 33, 34, 40), or deacylpolymyxins (36). Bioconversion processes, mutagenicity studies, and the production of recombinant proteins in E. coli would greatly benefit from strains which have OMs that are maximally permeable (to substrates, hydrophobic mutagens, and synthesized periplasmic proteins, respectively). In those fields, the lpxA2 mutant would indeed prove useful because it differs from many other permeability mutants in being extremely susceptible to peptide antibiotics as well. Similarly, the lpxA2 mutant could be a useful addition to the

![Graph A](image1)

**FIG. 1.** Dependency of the growth of the lpxA2 mutant on the osmolarity of the growth medium. lpxA2 strain SM101 (○) and control strain SM105 (●) were grown at 28°C in medium L1/5 (see Materials and Methods) in the presence of increasing concentrations of NaCl (A) or sucrose (B) for 24 h, after which growth was quantified. Means ± standard deviations of three independent determinations are shown. OD, optical density.

![Graph B](image2)
bacterial strain panels used in the screening of antibiotic substances.

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