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MINIREVIEW

Antibiotic-Supersusceptible Mutants of *Escherichia coli* and *Salmonella typhimurium*

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The outer membrane (OM) of many gram-negative bacteria, such as strains of the family *Enterobacteriaceae*, acts as an effective permeability barrier against various noxious agents, including antibiotics (31, 32, 35, 36, 54). The diffusion of hydrophobic antibiotics through the enterobacterial OM is very restricted because of the lack of glycerophospholipid bilayers, the effective pathway for hydrophobic diffusion (33–35). In addition, large hydrophilic drugs are excluded; they cannot traverse the narrow porin pores, the channels for small hydrophilic nutrients (30–32). The structural integrity of the OM is attributable to its unique lipopolysaccharide (LPS) constituent; adjacent LPS molecules are apparently joined electrostatically by Mg^{2+}, Ca^{2+}, and polyamines to form a stable "roof of tiles" on the surface of the OM (25, 33, 36).

Mutants that have a defective OM permeability barrier function are useful in various fields of basic and applied research. The mutagenicity screening tests developed by Ames et al. (1) use LPS-defective deep rough mutants of *Salmonella typhimurium*; the OM of such strains is a less effective barrier against hydrophobic mutagens than is that of wild-type *S. typhimurium*. Many probes and drugs used in studies of bacterial cell physiology and biochemistry are hydrophobic and cannot penetrate the wild-type OM but cross the defective OM (34, 35, 54). In biotechnology, bioconversion processes and the production of recombinant proteins in *Escherichia coli* can greatly benefit from mutants that allow maximal diffusion of substrates and release of synthesized periplasmic proteins, respectively. Finally, the use of OM-defective mutants in antibiotic screening panels has led to the discovery of novel antibacterial substances that are active against gram-negative bacteria and inactive or much less active against gram-positive bacteria (12).

This review briefly summarizes the recent salient data on the OM-defective antibiotic-supersusceptible mutants of *E. coli* and *S. typhimurium*. Furthermore, the review compares the phenotypes of the mutants and shows that some of the very recently reported mutants carry the most supersusceptible phenotype.

THE *lpxA*, *firA*, AND *envA* MUTANTS

***lpxA2* mutant of *E. coli*.** As recently shown by Vuorio and Vaara (65), the conditionally lethal lipid A biosynthesis mutation *lpxA2* of *E. coli* (13) results, under growth-permitting conditions (28°C), in drastic antibiotic supersusceptibility. More specifically, the supersusceptibility is manifested to those classes of antibiotics (hydrophobic antibiotics and very large hydrophilic antibiotics) that are reasonably well excluded by the OM permeability barrier of the wild-type bacteria (65). As summarized in Table 1, the MICs of many hydrophobic antibiotics are 32- to 1,024-fold lower for the *lpxA2* strain than for the wild-type strain, and those of the large hydrophilic peptide antibiotics vancomycin and bacitracin are 32- and 256-fold lower, respectively. Comparisons with the other characterized OM permeability mutants (see below) indicate that the *lpxA2* mutant has the most antibiotic-supersusceptible phenotype. Accordingly, this mutant could be expected to be the most useful supersusceptible target strain in antibiotic screening panels.

lpxA encodes UDP-*N*-acetylglucosamine *O*-acyltransferase, the enzyme that catalyzes the first step of lipid A biosynthesis (41). The sequence of the mutant allele *lpxA2* has not been reported, but the mutant lacks detectable UDP-*N*-acetylglucosamine *O*-acyltransferase activity (13). At 42°C, the mutant produces lipid A at a 10-fold-lower rate than the control strain and finally dies (13). At the growth-permissive temperature (30°C), it produces only ca. 30% less lipid A than the control strain. Apparently, the mutation is not tight enough to arrest lipid A synthesis completely at 30°C. Perhaps supersusceptibility at the growth-permissive temperature is due to a relative lack of LPS in the OM. By analogy to the deep rough mutants, this lack could result in a compensatory increase in the glycerophospholipid content of the outer leaflet of the OM, allowing the generation of glycerophospholipid bilayer patches in the OM. Such phospholipid bilayers enable effective diffusion of hydrophobic solutes (33, 35). Evidence presented below may also suggest the contribution of some additional susceptibility-increasing mechanism, such as transient rupturing (and resealing) of the OM. Furthermore, whether glycerophospholipid bilayers really exist in the OM of the *lpxA2* mutant is not known. The non-OM-permeating probes that were used to detect glycerophospholipids in the outer leaflet of deep rough mutants (CNBr-activated dextran, phospholipase C [22]) are not applicable in the study of the *lpxA2* mutant, since its OM is expected to allow the entry of such probes into the periplasm (see below).

All the hydrophobic antibiotics shown in Table 1 are present largely in charged forms. Rifampin is a dipolar ionic molecule (a zwitterion), whereas the others are anionic or cationic. They have notably high apparent partition coefficients in octanol-phosphate buffer (novobiocin, 48.8; rifampin, 16.0; erythromycin, 0.79 to 0.89; clindamycin, 0.70 [69]). The *lpxA2* mutant appears to be equally supersusceptible to rifampin and erythromycin (increase in susceptibility to both antibiotics, ca. 64- to 512-fold). On the other hand, Plésiat and Nikaido (40) recently showed that an uncharged hydrophobic 3-oxosteroid probe molecule diffused significantly more quickly through the OMs of wild-type enteric bacteria than did its monoanionic derivative (difference in permeability coefficients [P], 15- to 17-fold). Furthermore,

TABLE 1. Susceptibility of *E. coli* SM101 (*lpxA2*), CDH23-213 (*firA*), LS583 (*envA1*), and D21f2 (*rfa*; LPS chemotype Re) to various antibiotics[a]

Antibiotic	Susceptibility index[b] for a mutant defective in:			
	lpxA	*firA*	*envA*	*rfa*
Rifampin	64–≥512	128–≥512	64	64
Rifabutin	≥512	≥512		64
Fusidic acid	≥128	256	≥64	≥64
Roxithromycin	1,024	128		64
Erythromycin	64–512	128	≥128	32–64
Clarithromycin	512	128		64
Azithromycin	64	64		64
Novobiocin	64	≥256	4	64
Mupirocin	32	32–64		32
Clindamycin	32	16		4
Bacitracin	≥256	≥32	≥32	≥4
Vancomycin	32	4	≥16	2
Benzylpenicillin	16	4		
Dicloxacillin	≥8			≥8
Nalidixic acid		4	8	
Cefuroxime	8			1
Cefoxitin	2		2	0.5
Cefotaxime	2			0.5
Gentamicin	2			1
Kanamycin		2	2	
Fosfomycin		1	1	
Cycloserine		1	1	

[a] Data are from references 52, 56–59, 64, 65, and 69.
[b] Approximate ratio between the MIC for the wild-type parent and that for the corresponding mutant.

even though both probes penetrated the OM of the deep rough Re mutant with a ca. 20-fold-increased efficiency, the P of the charged molecule remained ca. 20-fold lower than that of the uncharged one (40). Therefore, it could be expected that uncharged hydrophobic antibiotics have lower MICs for enteric bacteria (and their supersusceptible mutants) than do their charged analogs.

The *lpxA2* mutant differs from the deep rough mutants in being notably susceptible to vancomycin and bacitracin (Table 1) (65). Both are large peptide antibiotics (molecular weights, 1,449 and 1,411, respectively) and hydrophilic (partition coefficients in octanol-phosphate buffer, 0.004 and 0.09, respectively [69]). The OM of the *lpxA2* mutant leaks periplasmic macromolecules at 28°C (66), probably through transient ruptures. Therefore, it is not surprising that it allows the entry of peptide antibiotics as well. In concert, it could be expected that the OM of the *lpxA2* mutant is permeable to other large molecules, too.

With regard to the monoanionic β-lactams, which are hydrophilic or relatively hydrophilic and penetrate the wild-type OM through porin pores, the MICs of cefoxitin and cefotaxime are only twofold lower for the *lpxA2* mutant than for the wild type (Table 1). These two β-lactams have relatively high P through the OmpF channel of *E. coli* (370 and 180 nm/s, respectively [32]). On the other hand, the MIC of cefuroxime (P, 50 nm/s [30]) is 8-fold lower for the *lpxA2* mutant than for the wild type, and the MIC of benzylpenicillin (P, 20 nm/s [32]) is 16-fold lower (Table 1). These data indicate that both of these β-lactams traverse the OM of the *lpxA2* mutant mainly via a non-porin pathway (putative phospholipid bilayer patches and/or ruptures [see above]).

***firA* (*omsA*, *ssc*) mutants of *E. coli* and *S. typhimurium*.** *S. typhimurium* SH7622 is known to be very susceptible to a wide variety of hydrophobic antibiotics and dyes as well as to detergents (50). In 1990, Hirvas et al. (18) identified and sequenced the gene mutated in this strain, named the *ssc* gene, and showed that it encodes a 36-kDa protein. Furthermore, they presented evidence that *ssc* is analogous to the *firA* locus of *E. coli* (18, 19). *firA* had previously been thought to be involved in transcription, since its mutant alleles partially suppress the rifampin-resistant phenotype of the *E. coli rpoB* strain, which possesses rifampin-resistant RNA polymerase (26, 27). Sequencing of *firA* of *E. coli* revealed that it is 88% identical to *ssc* of *S. typhimurium* and that the corresponding proteins are 96% identical (10). Quite recently, Vuorio and Vaara (64) reported that another antibiotic-supersusceptible mutant, the so-called *omsA* mutant strain CDH23-213 of *E. coli* (52), resembles SH7622 phenotypically and that *omsA* is allelic to *firA*. To date, *omsA* of CDH23-213 and *ssc-1* of SH7622 are the best-characterized *firA* alleles known. Both encode FirA with one amino acid alteration (Ser-271→Asn in CDH23-213 and Val-291→Met in SH7622) (19, 64).

CDH23-213 is very susceptible to hydrophobic antibiotics as well as to vancomycin and bacitracin (Table 1) but displays no increased susceptibility to the very small hydrophilic antibiotics fosfomycin and cycloserine, which diffuse through porin channels. It is highly supersusceptible to antibiotics even at temperatures as low as 28°C (64). At 37°C, it leaks periplasmic proteins but grows well (52). At 42°C, it dies and lyses (52). SH7622, on the other hand, is clearly less antibiotic susceptible at a low temperature (33°C) than at 37 or 39°C (19). Furthermore, it does not leak its periplasmic contents at 37°C (64). Such leakage takes place, however, at the lethal temperature (42°C) (64). Accordingly, CDH23-213 possesses a somewhat more defective *firA* allele than does SH7622. Another very defective allele appears to be that which encodes FirA with Gly-289→Lys (21).

The function of *firA* is not known. In sequence comparisons, it shows the highest level of homology to *lpxA* (63). This fact is evident on both the nucleotide and the protein levels. The FirA and LpxA proteins share two long regions of distinct homology (homology, defined as equivalent amino acids, 43%) and a peculiar hexapeptide repeat theme (63). This theme also exists in several acetyltransferases of bacteria (63; see also references 11 and 55). Furthermore, *firA* is located on the chromosome 560 bp upstream of *lpxA* and immediately downstream of *ompH* (*hlpA*, encoding a 16-kDa cationic protein found in isolated OMs [17, 20, 24, 60]) (10, 18, 19). These three genes may be cotranscribed. The phenotype of the most defective *firA* mutant (CDH23-213) is strikingly similar to that of *lpxA2* mutant SM101 (see above). Like SM101, CDH23-213 produces greatly decreased amounts of LPS; its rate of lipid A synthesis relative to that of phospholipid synthesis at 42°C is less than 20% of that of the control strain (at 37°C, it is ca. 50%) (15). Furthermore, LPS produced by *firA* mutants at 42°C is modified to contain palmitic acid (14, 15), as are the LPS precursors manufactured when lipid A biosynthesis is blocked by other mechanisms (41). Taken together, these results suggest that FirA is involved in lipid A biosynthesis and that it has a function resembling that of LpxA (i.e., an acyltransferase function). At least six to eight of the enzymes directly participating in lipid A biosynthesis still await molecular characterization (41); these include those [UDP-3-*O*-(3-hydroxymyristoyl)-acetylglucosamine deacetylase and UDP-3-*O*-(3-hydroxymyristoyl)-glucosamine *N*-acyltransferase] that catalyze the steps immediately following that catalyzed by the LpxA protein.

***envA1* mutant of *E. coli*.** The biochemical defect in the

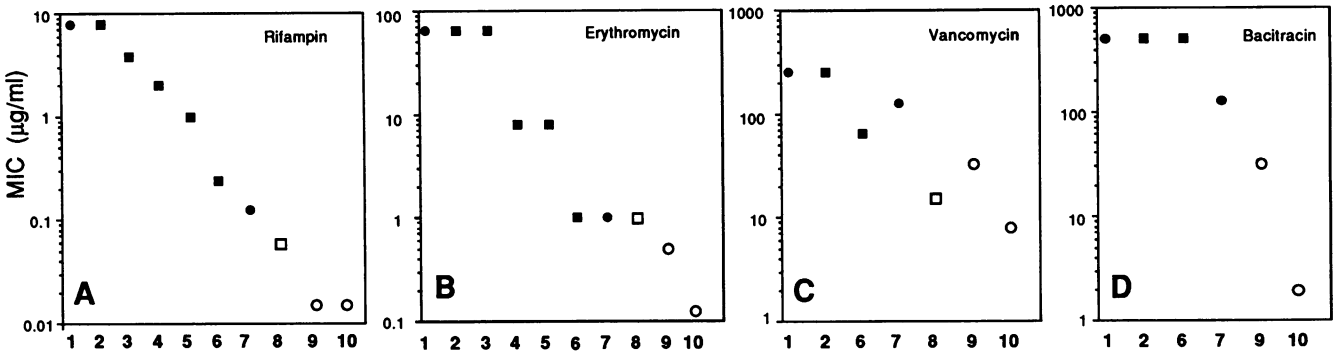

FIG. 1. MICs of rifampin (A), erythromycin (B), vancomycin (C), and bacitracin (D) for *E. coli* (circles) and *S. typhimurium* (squares) mutants defective in the synthesis of LPS core oligosaccharide (closed symbols) as well as those defective in *lpxA* or *firA* (open symbols), as determined in Luria broth at 28°C (SM101 and CDH23-213), 39°C (SH7622), or 37°C (other strains) (19, 57–59, 64, 65). Strains: 1, SM105 (*E. coli* K-12 wild-type control); 2, SH5014 (LPS chemotype Rb_2); 3, SL761 (Rc); 4, SL1032 (Rd_1); 5, SL1181 (Rd_2); 6, SL1102 (Re); 7, D21f2 (Re); 8, SH7622 (*firA*); 9, CDH23-213 (*firA*); 10, SM101 (*lpxA*).

envA1 mutant still awaits discovery (49, 69). The mutant produces decreased amounts of LPS (25 to 30% reduction) and, in this sense, bears some resemblance to the *lpxA2* and *firA* mutants described above. Like those mutants, it is supersusceptible not only to hydrophobic antibiotics but also to peptide antibiotics (Table 1) (69) and leaks its periplasmic enzymes (69). However, while the *lpxA1* and *firA* mutants are morphologically indistinguishable from their parents (50, 52), the *envA1* mutant grows in chains and is unable to form a complete invagination of the OM during cell division (3, 9). Furthermore, while *firA* and *lpxA* are located at 4 min on the *E. coli* chromosome (7, 10), *envA* resides at 2 min, immediately downstream of a murein synthesis-cell division cluster including *murC*, *ddl*, *ftsQ*, *ftsA*, and *ftsZ*, and is apparently cotranscribed with these genes (2). The *envA*-encoded 34-kDa protein does not show notable homology to other known *E. coli* proteins; the mutant protein has a single alteration (His-19→Tyr) (2). Deletion of *envA* is lethal (2).

Both the *envA1* mutant and the *firA* mutants have a relative lack of phosphatidylglycerol (3, 15). Since phosphatidylglycerol is a substrate in the synthesis of membrane-derived oligosaccharide (MDO) and since these mutants probably lose MDO as they lose their other periplasmic constituents, this lack has been suggested (15) to reflect an increased rate of synthesis of MDO.

MUTANTS DEFECTIVE IN THE INNER CORE REGION OF LPS

The *E. coli* and *S. typhimurium* mutants that elaborate the most truncated LPS core, i.e., deep rough LPS of the Re type (no heptoses; two 3-deoxy-D-manno-octulosonate residues only), have been extensively characterized (for reviews, see references 34 and 35) and can be regarded as the golden standard of antibiotic-supersusceptible mutants. While they are notably supersusceptible to hydrophobic antibiotics (Table 1) (29, 34, 35, 42, 49, 51), recent studies that have determined the MICs of a large set of hydrophobic antibiotics for both the Re-type mutants and the *lpxA2* and *firA* mutants indicate that the latter mutants are more susceptible than the Re-type mutants (56–58, 65). This fact is also shown in Fig. 1. With regard to vancomycin and bacitracin, the Re-type mutants display a low-grade susceptibility increase only (29, 46, 51, 65), whereas the *lpxA2* mutant is almost as susceptible as the susceptible gram-positive bacteria (Fig. 1; compare with MICs in reference 68). Other deep rough mutants, i.e., those elaborating the gradually less truncated LPS types Rd_2, Rd_1, and RP^- (16, 35, 42), are less susceptible than the Re type mutants (Fig. 1) (35, 42, 61). The Rc type displays a very moderate susceptibility increase only. Even less truncated types (Rb_2, Rb_1, and Ra) are as resistant as the wild-type strains. A recent analysis of a series of LPS-defective mutants of *E. coli* strongly suggests that the phosphate residue of the proximal heptose is essential for normal structure and/or biogenesis of the OM (38).

The sequence of events ultimately leading to the antibiotic-supersusceptible phenotype in the deep rough mutants has been revealed to a considerable extent. It was recently shown that the LPS isolated from these mutants does not facilitate trimerization of the monomeric OmpF porin secreted by spheroplasts of *E. coli* (44). This lack of trimerization might inhibit protein translocation to the OM (45) and could therefore explain the observed (33–35) relative lack of porins and the compensatory appearance of phospholipid bilayers in the OM of the Re-type mutants. Since hydrophobic probe compounds do not partition significantly better to the LPS of the deep rough mutants than to that of the Rb_2-type mutants (62), these phospholipid bilayers are apparently the principal reason for the increase in susceptibility to hydrophobic antibiotics (33).

OTHER SUPERSUSCEPTIBLE MUTANTS

The supersusceptibility of the SS-A mutant of *S. typhimurium* to antibiotics was recently shown to be caused by a point mutation in the plasmid gene *traT*, which encodes the 24-kDa OM lipoprotein (48). In addition, studies with other *traT* mutants indicated that the production of an altered OM protein can seriously damage the OM permeability barrier (48). With regard to the degree of supersusceptibility, it should be noted, however, that the *traT* mutants are less supersusceptible than the deep rough mutants (Table 2) (50).

In contrast to the situation for the SS-A mutant, the biochemical defects in the SS-B mutant of *S. typhimurium* as well as in the *abs* mutant (DC2) and *acrA* mutant of *E. coli* have remained unelucidated (49). The latter two mutant types have been used, to some extent, as supersusceptible indicator strains in past antibiotic screening studies, but neither appears to be as supersusceptible to hydrophobic

TABLE 2. Susceptibility of *E. coli* CL94 (*acrA*) and DC2 (*abs*) as well as *S. typhimurium* SH7620 (*traT*) and SH7616 (supersusceptibility class SS-B) to hydrophobic antibiotics[a]

Antibiotic	Susceptibility index[b] for:			
	CL94 (*acrA*)	DC2 (*abs*)	SH7620 (*traT*)	SH7616 (SS-B)
Rifampin	3	4	30	2
Fusidic acid	<2		≥24	≥128
Erythromycin	3	4	9	32
Novobiocin	>80	40		128

[a] Data are from references 6, 8, 49, and 53.
[b] Approximate ratio between the MIC for the wild-type parent and that for the corresponding mutant.

antibiotics as the most supersusceptible mutants (deep rough mutants and *lpxA*, *firA*, and *envA* mutants) discussed above (Table 2) (8, 28). Furthermore, the SS-B mutant and *acrA* mutant are as resistant as are their parents to vancomycin and bacitracin (8, 53) and as the *abs* mutant (DC2) is to vancomycin (46) (the susceptibility of DC2 to bacitracin has not been published).

The antibiotic-supersusceptible phenotypes of the mutants defective in the cell division genes *lkyD*, *envB*, and *envC* have been only partially characterized. The *lkyD* mutant of *S. typhimurium*, unable to produce the last step in cell division, i.e., OM invagination (9), leaks its periplasmic constituents and is supersusceptible to rifampin and anionic detergents, as is the *envC* mutant of *E. coli* (49). The *envB* mutants of *S. typhimurium* display a less supersusceptible phenotype; they are supersusceptible to deoxycholate but not to rifampin or novobiocin (37). The function of these genes has not yet been elucidated. While *envB* (*mreB* [67]) and *envC* map at 73 min on the *E. coli* chromosome (23), *lkyD* maps at 6.5 min on the *S. typhimurium* chromosome (5). Other still poorly characterized supersusceptible mutants include the *imp* mutant of *E. coli* (43) as well as mutant strain 16 of *E. coli* (46). The latter resembles the *lpxA*, *firA*, and *envA* mutants (see above) in being susceptible not only to hydrophobic antibiotics but also to vancomycin (46). The supersusceptible phenotypes of several OM protein mutants as well as of the *pss* mutant (defective in phosphatidylserine synthetase) have been reviewed elsewhere (49). In most instances, those phenotypes have not yet been characterized in detail. The *dye* mutant of *E. coli*, mutated in the aerobic respiration control and plasmid transfer gene *arc* (*sfrA*, *dye* [47]), displays a slight increase in susceptibility to some inhibitors, including the redox dyes toluidine blue and methylene blue, but not to a representative set of hydrophobic antibiotics (4).

CONCLUDING REMARKS

It can be summarized that from the large selection of OM-defective, antibiotic-supersusceptible enterobacterial mutants now known, the most supersusceptible include those mutated in *lpxA*, *firA*, or *envA*. Whereas the *lpxA* mutant is defective in the synthesis of lipid A, as are probably also the *firA* mutants, the biochemical defect in the *envA* mutant is not yet known. Both *lpxA* and *firA* mutants are thermosensitive for growth, and *envA*, too, has been shown to be an essential gene. It is clear that various mutant alleles can certainly be expected to differ in the extents of their functional lesions and, hence, in their degrees of supersusceptibility. However, it can be concluded that the *lpxA2* mutant of *E. coli* appears to be the most supersusceptible mutant thus far described.

Even though strains with a defective OM permeability barrier are undoubtedly valuable tools in several fields of research (see Introduction), perhaps one of the most interesting applications is their use in antibiotic screening panels. Such panels need gram-negative indicator bacteria, since several attractive targets for antibacterial action (such as the biosynthesis of LPS and its lipid A component) are lacking in gram-positive bacteria. Furthermore, many drugs can be much less effective against gram-positive bacteria than against susceptible gram-negative bacteria (known examples include monobactams, amidinopenicillins, quinolones, and fluoroquinolones). The use of very susceptible gram-negative indicator strains is indeed meaningful in such screenings to detect antibiotic substances present in low concentrations in culture supernatants and extracts. Wild-type gram-negative bacteria that possess an effective OM permeability barrier are understandably not as suitable for this purpose. The analysis (59) of reports of new antibacterial agents published in the *Journal of Antibiotics* in 1990 and 1991 indicated that more than 90% of the new antibacterial agents reported lacked activity against wild-type *E. coli* but inhibited gram-positive bacteria; at least in most of these cases, the resistance of *E. coli* could be expected to have been due to its OM. On the other hand, numerous gram-negative bacteria living in aquatic or terrestrial habitats appear to differ from enteric bacteria in having a much less effective OM permeability barrier (39, 40, 59); many of the natural antibacterial substances could be directed against those bacteria. Accordingly, new important antibiotics against gram-negative bacteria could perhaps be discovered, provided that maximally susceptible target strains, such as the *lpxA2* mutant of *E. coli*, are used in the screenings. Subsequent chemical modification would then potentially widen the spectra of those agents to include clinically relevant bacteria as well.

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