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Antimicrobial Susceptibility of *Salmonella typhimurium* Carrying the Outer Membrane Permeability Mutation SS-B

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The antibiotic susceptibility profile of *Salmonella typhimurium* SS-B, a mutant susceptible to some antimicrobial agents, was studied in detail. Twenty-eight agents were tested, and eleven of these had MICs significantly lower (32- to >250-fold) for the SS-B strain than for its parent. The drugs were generally hydrophobic or amphiphilic. Polymyxin B nonapeptide, which has a known outer membrane permeabilizing action, further reduced the MIC of several of these agents for the SS-B strain by a factor of approximately 10 to 30. In most cases, the resulting MICs were lower than the corresponding MICs for the parent strain grown in the presence of polymyxin B nonapeptide. In addition, the hydrophobic fluorescent probe *N*-phenyl naphthylamine was rapidly embedded in the membranes of the SS-B strain but was poorly embedded in those of the parent strain.

Low outer membrane (OM) permeability constitutes a major problem for the penetration of antibiotics into gram-negative enteric bacteria. The OM of these bacteria acts as a very effective permeability barrier against hydrophobic antibiotics (such as erythromycin, clindamycin, rifampin, and many others) (15–18). It also acts as a barrier against hydrophilic antibiotics (such as most of the cephalosporins), most of which can, however, traverse the OM more or less rapidly through the porin pores (3, 15–18).

Enterobacterial mutants which have increased permeabilities to antibiotics have proven useful in assessing the actual role of the OM as a permeability barrier. Some of those mutants can also be expected to give valuable information about the mechanisms by which the OM acts as a barrier. Consequently, they might be useful in the design of "permeabilizer" compounds which permeabilize the OM and thus work synergistically with antibiotics (1, 9, 35).

We have earlier described an antibiotic-supersusceptible SS-B mutant strain of *Salmonella typhimurium* (SS for supersusceptibility) (26, 27). In spite of intensive research, no molecular basis for the supersusceptibility has yet been found (17, 26, 27). Unlike many other hyperpermeable OM mutants, the SS-B mutant has no detectable alterations in the lipopolysaccharide (LPS) (26). Neither has it any obvious changes in phospholipids or OM proteins (27). Furthermore, its growth rate and cell morphology are unaltered. Its preliminary phenotypic characterization (with a limited number of antibiotics and the agar diffusion method) indicated a susceptibility pattern which is unique among *Salmonella* mutants (27, 39). Thus, the SS-B strain appeared to be much more susceptible to benzylpenicillin and the hydrophobic penicillin derivative tested (methicillin) than are, for instance, the LPS-defective deep rough mutants (27). This paper is a systematic and detailed characterization of the antibiotic susceptibility phenotype of the SS-B mutant.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* LT2 strain SH5014 (LPS chemotype Rb_2) is a typical and well-characterized (25, 26, 36–38) representative of rough salmonellae. SH7616 is its supersusceptible SS-B mutant (27). The antibiotic susceptibility phenotype of SH7616 was repeatedly rechecked by the agar diffusion method, using antibiotic disks (Neo-Sensitabs) from A/S Rosco, Copenhagen, Denmark, as described elsewhere (27). The bacterial strains were preserved at −70°C in 20% skim milk (27).

Antibiotics. The following antibiotics and other drugs were used: benzylpenicillin (Na salt), Novo Industri, Copenhagen, Denmark; cloxacillin (Na salt) and ampicillin (Na salt), Astra, Sodertalje, Sweden; piperacillin (Na salt), Lederle, Carolina, P.R.; cephalothin (Na salt) and vancomycin hydrochloride, Eli Lilly & Co., Indianapolis, Ind.; cefuroxime (Na salt), Glaxo, Greenford, Middlesex, England; nafcillin (Na salt), Wyeth, Philadelphia, Pa.; cefotaxime (Na salt) and cefpirome sulfate (HR810), Hoechst, Frankfurt (Main), Federal Republic of Germany; cefepime sulfate (BMY-28142), Bristol-Myers Co., Syracuse, N.Y.; imipenem, MSD, Rahway, N.J.; meropenem, ICI, Macclesfield, Cheshire, England; fusidic acid (Na salt), novobiocin (Na salt), nalidixic acid (Na salt), gentamicin sulfate, amikacin, rifampin, chloramphenicol, and bacitracin, Sigma Chemical Co., St. Louis, Mo.; erythromycin ethylsuccinate and tetracycline hydrochloride, Orion, Helsinki, Finland; clindamycin hydrochloride, Upjohn, Kalamazoo, Mich.; ciprofloxacin hydrochloride, Bayer, Leverkusen, Federal Republic of Germany; and sodium dodecyl sulfate and Triton X-100, BDH, Poole, England.

Polymyxin B nonapeptide (PMBN) was prepared essentially as described by Viljanen and Vaara (41) and was obtained from Farmos Group Ltd. (Turku, Finland). Its residual content of polymyxin B was approximately 0.1% as determined by reversed-phase high-pressure liquid chromatography (33).

Susceptibility determinations. The MICs of the antibiotics and other drugs were measured in L broth (14). The inoculum was L agar (14)-grown cells, and the inoculum size was 10^4 cells per ml. The incubation was for 18 h at 37°C. The lowest concentration that completely inhibited visual growth was recorded and interpreted as the MIC. The MIC of methicillin was measured by using a Sensititre Apo1 microdilution plate (Sensititre, Salem, N.H.) and the same growth medium and inoculum as described above.

The MICs of the antibiotics in the presence of PMBN were measured by using Luria broth, an inoculum of 10^4 cells per

ml, and microdilution plates exactly as described earlier (41). After the incubation for 18 h at 37°C, the A_{405} in each microdilution well was measured by a Titertek Multiscan spectrophotometer (Labsystems, Helsinki, Finland). Before the reading, the spectrophotometer was blanked with corresponding uninoculated assay media.

***N*-Phenyl naphthylamine fluorescence experiments.** These experiments were done essentially as described earlier (34) by using the method of Uratani (32). Bacteria were grown in L broth to the early logarithmic phase of growth (40 Klett units, Klett-Summerson colorimeter, red filter), washed in 0.02 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4) containing 0.5 mM $MgCl_2$ and 5 mM glucose, and suspended in the same buffer to a final absorbance of 80 Klett units. To a 2-ml aliquot of this suspension, *N*-phenyl naphthylamine (NPN; 20 μl of a 10^{-3} M solution in methanol) was added, and the fluorescence was then immediately monitored by a spectrofluorometer (MPF-44B; Perkin Elmer Corp., Norwalk, Conn.) equipped with a Perkin Elmer 150 Xenon Power supply, a Perkin Elmer X-Y recorder, and a cuvette with rotating magnetic bar (excitation wavelength, 360 nm; emission was at 420 nm; slits, 6 nm). Both the bacterial background fluorescence (the fluorescence of the suspension without NPN) and the NPN background fluorescence (the fluorescence of the sample without any bacteria) were reduced (both constituted less than 5% of the total fluorescence of SH7616 in the presence of NPN).

RESULTS

We determined the MICs of 28 antibiotics and other inhibitors for the SS-B strain and its parent (Table 1). Eleven of those drugs were remarkably more active (32 to >250 times more active) against the SS-B strain than against the parent strain. Those drugs were benzylpenicillin, the hydrophobic penicillin derivatives (methicillin, cloxacillin, and nafcillin), cefuroxime, erythromycin, clindamycin, fusidic acid, novobiocin, the anionic detergent sodium dodecyl sulfate, and the nonionic detergent Triton X-100. Another cluster of drugs comprised those which were only slightly more active (4 to 8 times more active) against the SS-B strain; those drugs included ampicillin, piperacillin, cephalothin, cefpirome, cefepime, nalidixic acid, ciprofloxacin, chloramphenicol, and tetracycline. The third group comprised the drugs against which the SS-B strain displayed no clear-cut susceptibility increase; they included cefotaxime, imipenem, meropenem, rifampin, gentamicin, amikacin, bacitracin, and vancomycin. Thus, it appears that the SS-B strain was much more susceptible to amphipathic, hydrophobic, or relatively hydrophobic drugs (rifampin being the only exception) than the parent strain, whereas notably hydrophilic drugs were equally effective against both strains.

We next proceeded to test whether PMBN, the known very potent sensitizer (17, 35–37, 40), could further sensitize even this very susceptible SS-B strain, as it appeared to do in our preliminary test with antibiotic-containing disks (39). A relatively small concentration of PMBN (3 μg/ml) was sufficient to decrease the MIC of fusidic acid for the SS-B strain by a factor of approximately 30 (Fig. 1). In Table 2, the MICs of other hydrophobic drugs have been included as well; the MICs of fusidic acid and erythromycin decreased by a factor of 30, and those of novobiocin and nafcillin decreased by a factor of 10. The resulting MICs were very low. As shown earlier (Table 1), the SS-B strain was as susceptible to rifampin as its parent; Table 2 shows that PMBN sensitized both strains equally, by a factor of approximately 300. As is also evident from the values in Table 2, the SS-B mutant in the presence of PMBN was 1,000 to 10,000 times more susceptible to five of the tested hydrophobic drugs (fusidic acid, cloxacillin, nafcillin, erythromycin, and novobiocin) than was the intact parent strain (e.g., the parent strain in the absence of PMBN).

We have probed the SS-B strain and its parent with the hydrophobic fluorescent probe NPN (Fig. 2). NPN is an uncharged, very hydrophobic compound which, when excited in a hydrophobic milieu, has a characteristic bright fluorescence emission peak at 420 nm. That peak is lacking when NPN is in an aqueous milieu. NPN has earlier proven to be a useful probe with which to detect bacteriocin- or polycation-damaged OM (10, 31, 32). The damaged OM allows the instantaneous penetration of NPN into the hydrophobic membrane environments of the OM and the cytoplasmic membrane (10, 31, 32). NPN embedded rapidly in the membranes of the SS-B strain but poorly in those of the parent strain (Fig. 2). This most probably indicates that the outer leaflet of the SS-B OM binds NPN and allows its diffusion to the hydrophobic membrane environments.

TABLE 1. MICs of various antibacterial agents for the SS-B mutant SH7616 and its parent SH5014

Antibacterial agent	MIC (μg/ml) for:		Ratio[a]
	Parent strain SH5014	SS-B mutant SH7616	
Beta-lactams			
Benzylpenicillin	8	0.25	32
Methicillin	≥32	1	≥32
Cloxacillin	>256	1	>256
Nafcillin	≥500	4	≥125
Ampicillin	1	0.25	4
Piperacillin	1	0.125	8
Cephalothin	4	1	4
Cefuroxime	8	0.125	64
Cefotaxime	0.125	0.060	2
Cefpirome	0.25	0.06	4
Cefepime	0.125	0.03	4
Imipenem	0.25	0.25	1
Meropenem	0.03	0.03	1
Other antibiotics			
Erythromycin	64	2	32
Clindamycin	128	4	32
Fusidic acid	>512	4	>128
Novobiocin	128	1	128
Nalidixic acid	8	1	8
Ciprofloxacin	0.015	0.004	4
Chloramphenicol	4	1	4
Tetracycline	1	0.25	4
Rifampin	8	4	2
Gentamicin	2	2	1
Amikacin	4	4	1
Bacitracin	>128	>128	NA[b]
Vancomycin	>128	>128	NA
Detergents			
Sodium dodecyl sulfate	>16,000	64	>250
Triton X-100	>16,000	256	>60

[a] The approximate ratio between the MIC for the strain SH5014 and that for the strain SH7616.
[b] NA, Not applicable.

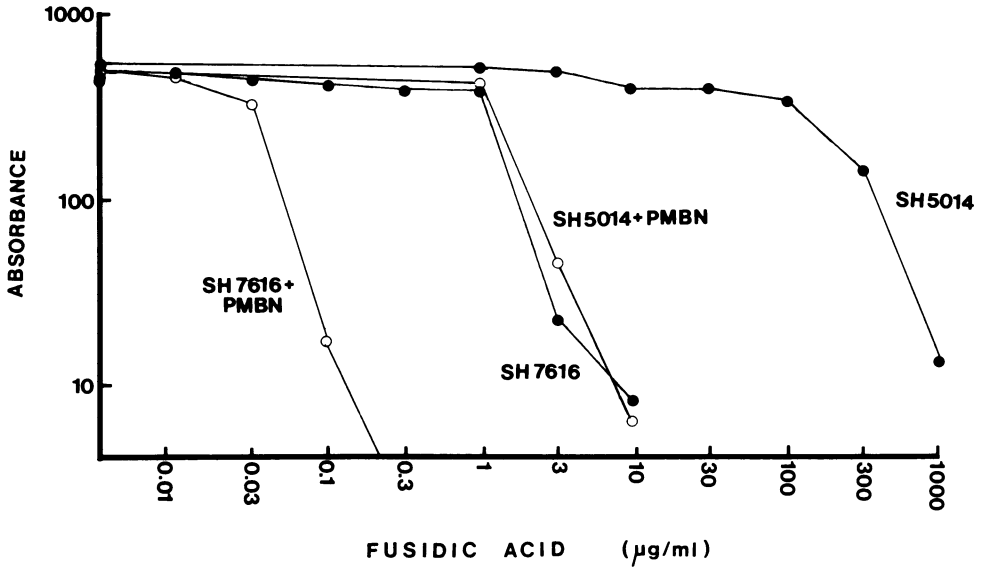

FIG. 1. The susceptibility of the SS-B mutant (SH7616) and its parent (SH5014) to fusidic acid in the absence and presence of PMBN. Bacteria were grown in the wells of a microdilution plate in L broth which contained increasing concentrations of fusidic acid as well as a constant amount (3 μg/ml) of PMBN or no PMBN. The bacterial growth was quantified after an 18-h incubation by measuring A_{405}. A typical experiment (of a total of three) is shown.

DISCUSSION

This paper shows that the SS-B mutant strain of *S. typhimurium* is extremely susceptible to a number of antibiotics and other inhibitors, all typically very or moderately hydrophobic. Moreover, the strain can further be sensitized to several such drugs by PMBN. The resulting MICs are very low and of the same order of magnitude as are the MICs of the same drugs for susceptible gram-positive bacteria, such as the susceptible strains of *S. aureus* (see reference 2 for the corresponding MICs for staphylococci).

The increased susceptibility of the SS-B mutant to various, unrelated hydrophobic agents can be explained only by assuming that this mutant has a defective OM which is not able to act as an effective permeability barrier against hydrophobic solutes. Consistent with this assumption, the experiment shown in Fig. 2 reveals that there is an instantaneous and rapid diffusion of the hydrophobic probe NPN through the outer membrane of the SS-B mutant. Both the kinetics and the quantity of the NPN uptake are similar to those observed earlier for the deep rough LPS mutants of *S. typhimurium* (M. Vaara, unpublished data).

Yet, the antibiotic supersusceptibility phenotype of the SS-B mutant can clearly be differentiated from that of the deep rough *S. typhimurium* (15, 17) and *Escherichia coli* (17, 29, 30) strains. Even though both types of strains are equally susceptible to many of the drugs included in the present study, the deep rough strains are not or are only slightly sensitized to benzylpenicillin (30, 36). Furthermore, and again in contrast to the SS-B mutant, they remain relatively resistant to cloxacillin and nafcillin (15, 36). On the other

TABLE 2. MICs of various hydrophobic antibacterial agents for the SS-B mutant and its parent in the absence and presence of PMBN

Antibiotic	MIC (μg/ml) for:			
	Parent strain SH5014		SS-B mutant SH7616	
	Without PMBN	With PMBN (3 μg/ml)	Without PMBN	With PMBN (3 μg/ml)
Fusidic acid	1,000	3	3	0.1
Cloxacillin	1,000	10	1	0.3
Nafcillin	1,000	30	3	0.3
Erythromycin	100	3	3	0.1
Novobiocin	100	1	1	0.1
Rifampin	10	0.03	10	0.03
Cefuroxime	8	4	0.1	0.05
Clindamycin	≥100	3	3	1
Nalidixic acid	10	1	1	1

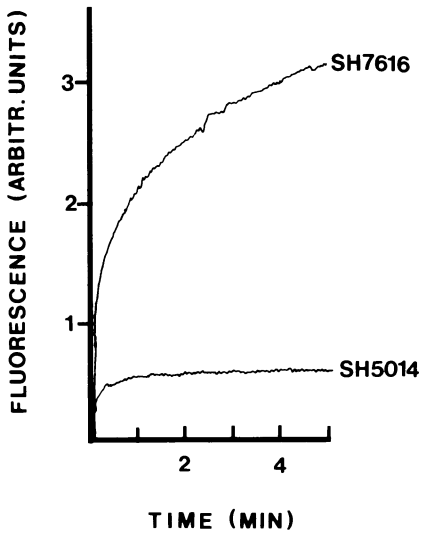

FIG. 2. Time course of the uptake of the fluorescent hydrophobic probe NPN by the cells of the SS-B strain SH7616 and the parent strain SH5014. For experimental details, see Materials and Methods. Fluorescence was measured in arbitrary units.

hand, the SS-B mutant is not sensitized to rifampin (Table 1), while the deep rough mutants are (15).

Other, earlier-described antibiotic-supersusceptible enterobacterial mutants include the *abs*, *acrA*, *envA*, and *envC* mutants. The susceptibility pattern of the *E. coli* K-12 mutant DC2, which bears the *abs* mutation, has been determined in detail (4, 6, 11, 21, 23), and it strikingly resembles that now determined for the *S. typhimurium* SS-B mutant. Both are very susceptible to benzylpenicillin, methicillin, and cloxacillin, and both are practically resistant to rifampin. The drug susceptibility phenotypes of the *acrA* (5), the *envA* (7, 8, 19), and especially the *envC* (22, 24) mutants have been only partially evaluated, so comparisons are less easily made. However, it appears that the SS-B mutant is much more susceptible than the *acrA* mutant of *E. coli*.

The molecular mechanism underlying the drastic OM permeability increase in the SS-B mutant is not yet known, as in the *acrA* mutant and in the *abs* mutant. However, the SS-B mutation is probably distinct from the *abs* mutation, because while the phenotype of the DC2 strain is suppressed by fucose and other deoxysugars, that of the SS-B mutant is not (SS-B strain SH7616 was tested; M. Vaara, unpublished data). As reviewed earlier in this report and in contrast to the deep rough mutations, the SS-B mutation does not involve any detectable alteration in LPS (26). In contrast to the cell division defects (i.e., defects in the formation of a normal septum) found in the *envA* and *envC* strains (19, 20, 22), no electron microscopically detectable morphological alterations are to be seen in the SS-B mutant (27).

The SS-B mutant has no detectable changes in phospholipid or OM protein composition (27). Quite recently, we analyzed its OM lipoprotein pattern by using palmitic acid labeling (12) and its cell wall penicillin-binding protein pattern by using radiolabeled penicillin (11). Both patterns were identical with those found in the parent strain (J. Kantele and M. Vaara, unpublished data). The SS-B mutant SH7616 also possesses the cationic 16-kilodalton OM protein OmpH which we have recently isolated, cloned, and sequenced from *S. typhimurium* SH5014 (13; P. Koski and M. Vaara, unpublished data).

In conclusion, the antibiotic supersusceptibility phenotype of the SS-B mutant strain SH7616 has now been evaluated in a detailed manner. As far as we know, this *Salmonella* mutant, the *abs* mutant of *E. coli*, and the deep rough LPS mutants are at present the phenotypically best-characterized extremely supersusceptible enterobacterial strains.

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