ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1996, p. 909–913 Vol. 40, No. 4
0066-4804/96/$04.00+0

Quantitative Correlation between Susceptibility and OprJ Production in NfxB Mutants of *Pseudomonas aeruginosa*

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Received 6 September 1995/Returned for modification 24 November 1995/Accepted 26 January 1996

Various *Pseudomonas aeruginosa* PAO1 NfxB mutants were isolated on agar plates containing cefpirome and ofloxacin. They were classified into type A and type B, based on the degrees of changes in their susceptibilities. Type A mutants were four to eight times more resistant to ofloxacin, erythromycin, and new zwitterionic cephems, i.e., cefpirome, cefclidin, cefozopran, and cefoselis, than was the parent strain, PAO1. In contrast, type B mutants were more resistant to tetracycline and chloramphenicol, as well as ofloxacin, erythromycin, and the new zwitterionic cephems, than was PAO1, and they were four to eight times more susceptible to carbenicillin, sulbenicillin, imipenem, panipenem, biapenem, moxalactam, aztreonam, gentamicin, and kanamycin than was PAO1. The changes in susceptibilities of type B mutants were greater than those of type A mutants. The susceptibilities of both type A and type B mutants were restored to the level of PAO1 by transformation with plasmid pNF111, which contained the wild-type *nfxB* gene, demonstrating that they are NfxB mutants. Immunoblot analysis with a monoclonal antibody to OprJ revealed that type B mutants produced larger amounts of outer membrane protein OprJ than did type A mutants and that PAO1 produced an undetectable amount of it. Moreover, transconjugants obtained with the different types of NfxB mutants as the donor strains showed almost the same phenotypes as the corresponding donor strains. These results suggest that there are at least two *nfxB* mutations that show different phenotypes and that production of OprJ is associated with changes in susceptibilities of NfxB mutants.

Pseudomonas aeruginosa is a clinically significant pathogen because of its intrinsic resistance to many antimicrobial agents. Although this organism has an outer membrane with low permeability (1, 28), this fact alone does not adequately explain the intrinsic resistance (21). Poole et al. (25) cloned an operon, *mexA-mexB-oprM* (previously *oprK* [6]), which is believed to belong to a family of active efflux systems in bacteria (12, 22). When *mexA*, *mexB*, or *oprM* was inactivated by a mutation, the *P. aeruginosa* strain became hypersusceptible to many antimicrobial agents (4, 6, 25). Therefore, it is logical to think that the intrinsic resistance is due to a combination of low outer membrane permeability and a multiple-drug efflux transporter(s) (22).

OprM has been identified as an outer membrane protein overproduced in NalB mutants which show cross-resistance to meropenem, cephems, quinolones, and some other antimicrobial agents (15). *nalB* (26), as well as *nfxB* (7) and *nfxC* (3), has been mapped on a chromosome in *P. aeruginosa* as a quinolone resistance locus. These mutants show cross-resistance to structurally diverse antimicrobial agents, although their resistance patterns are different (3, 7, 15, 26). NfxB mutants produce an outer membrane protein with an apparent molecular weight of 54,000 (designated OprJ) (7, 8, 16).

We previously isolated mutants that were phenotypically similar to NfxB mutants and showed that they were resistant to quinolones and new zwitterionic cephems, i.e., cefpirome and cefozopran (16). However, there is some inconsistency among strains of NfxB mutants in susceptibility to β-lactams, tetracycline, chloramphenicol, and aminoglycosides (7, 24). We have attempted to elucidate the reason for this inconsistency. In this study, we isolated various NfxB mutants and showed a quantitative correlation between changes in susceptibility and the production of OprJ in these mutants.

MATERIALS AND METHODS

Antimicrobial agents. The antimicrobial agents used in this study were obtained as follows: piperacillin and cefoperazone, Toyama Chemical Co., Tokyo, Japan; carbenicillin and cefoselis (FK 037) (2), Fujisawa Pharmaceutical Co., Osaka, Japan; sulbenicillin, cefsulodin, and cefozopran (SCE-2787) (10), Takeda Chemical Industries, Osaka, Japan; moxalactam, gentamicin, and erythromycin, Shionogi Pharmaceutical Co., Osaka, Japan; ceftazidime, Glaxo Japan, Tokyo, Japan; cefpirome, Chugai Pharmaceutical Co., Tokyo, Japan; cefepime (BMY-28142), Bristol-Myers Squibb, Tokyo, Japan; ME-1228 (20) and streptomycin, Meiji Seika Kaisha, Tokyo, Japan; imipenem, BO-2727 (19), and kanamycin, Banyu Pharmaceutical Co., Tokyo, Japan; panipenem and chloramphenicol, Sankyo Co. Ltd., Tokyo, Japan; meropenem, Sumitomo Pharmaceutical Co., Osaka, Japan; biapenem (L-627) and tetracycline, Lederle Japan, Tokyo, Japan; ofloxacin, Daiichi Pharmaceutical Co., Tokyo, Japan; polymyxin B, Pfizer Pharmaceuticals Inc., Tokyo, Japan; novobiocin, coumermycin, and rifampin, commercial sources.

Bacterial strains, bacteriophage, and plasmids. The *P. aeruginosa* strains used in this study are listed in Table 1. Phage G101 was used for transduction (17). Plasmids pME294 and pNF111 were provided by Y. Itoh (9) and T. Okazaki (24), respectively. pNF111, constructed from pME294, contains the wild-type *nfxB* gene. Both plasmids carry a carbenicillin resistance gene and a kanamycin resistance gene. Plasmid FP5, with chromosome mobilization ability (18), was used for conjugation.

Isolation of cefpirome-ofloxacin-resistant mutants. Cefpirome-ofloxacin-resistant mutants were isolated by plating PAO1 on Mueller-Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) plates containing 2 μg of cefpirome per ml and 1 μg of ofloxacin per ml. They were obtained at a frequency of 1×10^{-8} to 2×10^{-7}.

Susceptibility testing. MICs were determined by the usual twofold agar dilution technique with Mueller-Hinton II agar and an inoculum size of 10^4 cells.

Assays of outer membrane proteins. Outer membrane proteins of exponentially growing cells in Mueller-Hinton broth (Becton Dickinson Microbiology Systems) were prepared as described previously (16). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with 10% (wt/vol) acrylamide and 0.27% (wt/vol) *N*,*N*′-methylenebisacrylamide slab gels as

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TABLE 1. *P. aeruginosa* strains used in this study

Strain(s)	Genotype or derivation	Source
PAO1	Prototroph	
COR1, COR2, COR3	Type A NfxB mutants of PAO1	This study
COR4, COR5, COR6	Type B NfxB mutants of PAO1	This study
PAO969	*proC130*	D. Haas
PAO1816 (FP5$^+$)	*his-9004*	H. Matsumoto
PAO4031	*arg-9040 catA1 his-9015 ilv-9023 met-9020 mtu-9001 nad-9003 nar-9011 pro-9031*	H. Matsumoto
KG2500	Spontaneous rifampin-resistant mutant of PAO4031	This laboratory
TC01, TC02, TC03, TC04, TC05, TC06	*ilv*$^+$ *pro*$^+$ *nfxB* transconjugants of KG2500	This study

described by Laemmli (11). Samples for SDS-PAGE were treated with 2% SDS–5% 2-mercaptoethanol at 100°C for 5 min and then subjected to electrophoresis. After electrophoresis, the gels were stained with Coomassie brilliant blue or subjected to immunoblot analysis with a monoclonal antibody to OprJ as described previously (8). The amount of OprJ was determined with a scanning densitometer (CS-910; Shimadzu Corporation, Kyoto, Japan).

Genetic techniques. Plasmid DNA was prepared by the boiling method described by Maniatis et al. (13). Transformation of *P. aeruginosa* was performed as described previously (8). pME294 and pNF111 transformants were selected on Mueller-Hinton II agar containing 400 μg of carbenicillin per ml. Phage G101-mediated transduction and plasmid FP5-mediated conjugation were performed as described by Matsumoto et al. (17).

RESULTS

Isolation and characterization of cefpirome-ofloxacin-resistant mutants of PAO1. Fifty-eight cefpirome-ofloxacin-resistant *P. aeruginosa* PAO1 mutants were isolated, and their susceptibilities to 12 representative agents, listed in Table 2, were determined. These mutants were grouped into two types, based on their susceptibilities to meropenem. One type of mutant (27 strains) was four to eight times more resistant to meropenem than the parent strain, while the other type (31 strains) showed the same susceptibility to meropenem as the parent strain. The mutants that were more resistant to meropenem than PAO1 showed cross-resistance to ofloxacin, tetracycline, chloramphenicol, and the β-lactams (except imipenem), like NalB mutants (15). Cell-free lysate of G101 phage was prepared from three representative mutants of this type and used for transduction to a recipient, PAO969 (*proC130*). The drug resistance and *proC* in these mutants were cotransducible at a frequency of 24%, suggesting that these mutants have mutations at the *nalB* locus, as described previously (15, 26). The other mutants, whose susceptibilities to meropenem were comparable to that of PAO1, showed a susceptibility pattern similar to that of NfxB mutants isolated previously (7, 16, 24). They were grouped into type A and type B, based on the degrees of changes in their susceptibilities to agents other than meropenem. The susceptibilities of representative type A strains COR1, COR2, and COR3 and type B strains COR4, COR5, and COR6 are shown in the upper part of Table 2. Type A mutants were eightfold more resistant to cefpirome and ofloxacin than was the parent strain. In contrast, type B mutants were more resistant to tetracycline and chloramphenicol, as well as ofloxacin and cefpirome, than was PAO1, and they were fourfold more susceptible to carbenicillin, imipenem, moxalactam, aztreonam, and kanamycin than was PAO1. Moreover, type B mutants were fourfold more resistant to ofloxacin than type A mutants were. In general, the changes in susceptibility of type B mutants were greater than those of type A mutants. Among the isolated mutants, there were also a few exceptional strains that showed susceptibilities to these agents that were intermediate between the susceptibilities of type A and B mutants (data not shown). To determine the gene responsible for resistance in type A and B mutants, the six representative strains were transformed with either plasmid pNF111 containing the wild-type *nfxB* gene or pME294 (vector). The susceptibilities of the transformants are given in the middle part of Table 2. When pNF111 was intro-

TABLE 2. Susceptibilities of *P. aeruginosa* PAO1, its cefpirome-ofloxacin-resistant mutants and transformants, KG2500, and its *nfxB* transconjugants to various antimicrobial agents

Strain(s)	Phenotype	MIC (μg/ml)[a]											
		CPR	MEPM	CFS	CBPC	IPM	MOX	AZT	OFLX	TC	CP	GM	KM
PAO1		1.56	0.39	1.56	25	1.56	6.25	3.13	0.39	12.5	50	1.56	50
COR1–COR3	A	12.5	0.39	0.78–1.56	12.5–25	0.78	3.13	1.56	3.13	12.5–25	50	1.56	25
COR4–COR6	B	12.5	0.39	0.78	6.25	0.39	1.56	0.78	12.5	50	200	0.39–0.78	12.5
COR1–COR3 (pME294)	A	ND	ND	ND	ND	0.78	ND	ND	3.13–6.25	25	50–100	1.56–3.13	>200
COR1–COR3 (pNF111)		ND	ND	ND	ND	1.56	ND	ND	0.78	12.5–25	25	3.13	>200
COR4–COR6 (pME294)	B	ND	ND	ND	ND	0.39	ND	ND	12.5	50	200	0.78–1.56	>200
COR4–COR6 (pNF111)		ND	ND	ND	ND	1.56	ND	ND	0.78	12.5	25	3.13	>200
KG2500		6.25	0.78	6.25	100	1.56	12.5	12.5	0.39	25	100	1.56	100
TC01–TC03	A	12.5	0.39–0.78	1.56	12.5–25	0.78	3.13–6.25	1.56–3.13	3.13	25–50	100	1.56	50–100
TC04–TC06	B	25	0.39	1.56	6.25	0.20–0.39	1.56	0.78	6.25–12.5	50–100	200	0.78	25

[a] Abbreviations: CPR, cefpirome; MEPM, meropenem; CFS, cefsulodin; CBPC, carbenicillin; IPM, imipenem; MOX, moxalactam; AZT, aztreonam; OFLX, ofloxacin; TC, tetracycline; CP, chloramphenicol; GM, gentamicin; KM, kanamycin; ND, not determined.

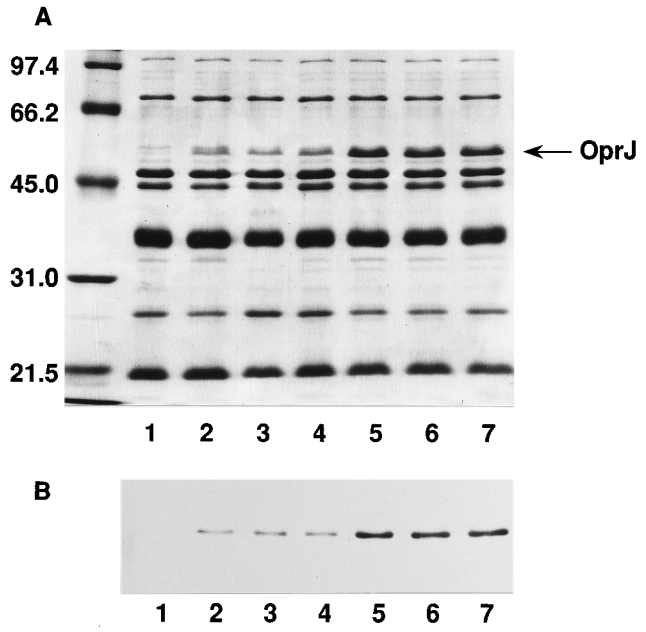

FIG. 1. Outer membrane proteins of *P. aeruginosa* PAO1 (lane 1) and its NfxB mutants, COR1 through COR6 (lanes 2 through 7), in an SDS-polyacrylamide gel stained with Coomassie brilliant blue (A) and immunoblots with a monoclonal antibody to OprJ (B). The numbers on the left are molecular weights in thousands.

duced into mutants COR1 through COR6, the susceptibilities to imipenem, ofloxacin, tetracycline, chloramphenicol, and gentamicin reverted to those of parent strain PAO1. In contrast, introduction of the vector pME294 did not alter the susceptibilities to these compounds significantly. The MICs of kanamycin, which is another marker for both plasmids, against these transformants were increased to >200 μg/ml, showing that these strains possess the plasmid. These results demonstrate that both type A and B mutants are NfxB mutants.

Outer membrane protein profiles of NfxB mutants. Figure 1A shows outer membrane protein profiles of each of three representative strains of, respectively, type A and B NfxB mutants and that of their parent strain, PAO1. The amounts of all proteins except OprJ, an outer membrane protein with an apparent molecular weight of 54,000, were almost the same among the tested strains. Immunoblot analysis with a monoclonal antibody to OprJ (8) showed that both types of mutants produced OprJ, although no band was observed for PAO1 (Fig. 1B). Analysis by densitometry showed that type B NfxB mutants produced fourfold to sevenfold greater amounts of OprJ than type A NfxB mutants did.

Transfer of gene *nfxB* by conjugation. To elucidate the variety of mutations in gene *nfxB*, we attempted to transfer the resistance gene from each type of NfxB mutant to another strain by conjugation. First, we carried out conjugations with PAO1816 ($FP5^+$) as the donor strain to introduce plasmid FP5 into each of the three representative strains of, respectively, type A and B NfxB mutants. Selection was made for mercury resistance (18), and the NfxB mutants carrying FP5 were obtained. Subsequently, we crossed KG2500 and each of the six representative NfxB mutants ($FP5^+$) and selection was made for *pro-9031*$^+$*–ilv-9023*$^+$ to obtain the transconjugants that inherited the mutant *nfxB* gene. Transconjugants were isolated on minimal medium plates containing 20 mM sodium gluconate; 1 mM each arginine, histidine, methionine, and nicotinamide; and 300 μg of rifampin per ml. The recombinants obtained in crosses between KG2500 and each of the mutants COR1 ($FP5^+$) through COR6 ($FP5^+$) were designated transconjugants TC01 through TC06, respectively. The susceptibilities of each transconjugant and recipient strain KG2500 are shown in the lower part of Table 2. Each transconjugant showed almost the same susceptibility pattern as its donor strain. The transconjugants made with KG2500 and type B mutants produced 3 to 12 times greater amounts of OprJ than did those made with KG2500 and type A mutants. No band was observed for KG2500 in immunoblot analysis. Thus, each type of transconjugant showed almost the same phenotypes as the corresponding donor strain.

TABLE 3. Susceptibilities of PAO1 and its type B NfxB mutants to various antimicrobial agents

Antimicrobial agent	MIC (μg/ml)	
	PAO1	COR4–COR6
Piperacillin	6.25	3.13
Sulbenicillin	25	3.13
Cefoperazone	6.25	3.13
Ceftazidime	1.56	0.78
ME-1228	1.56	1.56
Cefepime	3.13	12.5
Cefclidin	0.39	1.56
Cefozopran	0.78	6.25–12.5
Cefoselis	1.56	6.25
Cefluprenam	1.56	3.13
Panipenem	6.25	1.56
Biapenem	0.39	0.05
BO-2727	0.39	0.20
Streptomycin	50	12.5–25
Erythromycin	400	3,200
Polymyxin B	0.78	0.78
Novobiocin	1,600	800
Coumermycin	12.5	12.5
Rifampin	12.5	25

Susceptibilities of cefpirome-ofloxacin-resistant mutants to various antimicrobial agents. To determine further the susceptibility pattern of NfxB mutants, we examined the susceptibilities of PAO1 and both types of NfxB mutants to all of the antimicrobial agents listed in Materials and Methods. Table 3 shows the susceptibilities of the representative type B NfxB mutants. Type B NfxB mutants were 4 to 16 times more resistant to cefepime, cefclidin, cefozopran, and cefoselis, as well as cefpirome, than PAO1 was. In contrast, type B NfxB mutants were four to eight times more susceptible to sulbenicillin, panipenem, and biapenem, as well as carbenicillin and imipenem, than PAO1 was. Furthermore, type B NfxB mutants showed decreases in susceptibility to erythromycin, compared with that of PAO1. Type A NfxB mutants showed susceptibilities to all of the tested agents that were intermediate between the susceptibilities of PAO1 and type B NfxB mutants (data not shown).

DISCUSSION

Okazaki and Hirai cloned both wild and mutant *nfxB* genes and determined their nucleotide sequences (23). They reported that *nfxB* had a region homologous to that of helix-turn-helix DNA-binding domains of bacterial regulator proteins such as LacI, DeoR, CytR, and AsnC, and they proposed that the NfxB protein negatively regulates the production of a 54-kDa outer membrane protein, OprJ. In this study, we isolated various NfxB mutants that showed different levels of

changes in antibiotic susceptibility and production of OprJ. Transconjugants obtained with the NfxB mutants as the donor strains showed almost the same phenotypes as the corresponding donor strains. These results suggest that various isolated NfxB mutants have different mutations in the *nfxB* gene. These mutated gene products may show various affinities for the promoter region, for which the wild-type NfxB suppressor shows high affinity, and cause various levels of expression of OprJ (and other genes involved in susceptibility).

Type B NfxB mutants produced greater amounts of OprJ and showed larger changes in susceptibility than did type A NfxB mutants. Revertants obtained from type B NfxB mutants on agar plates containing carbenicillin showed susceptibilities comparable to that of PAO1, and they produced undetectable amounts of OprJ (14). These results suggest that the production of OprJ is associated with changes in the susceptibilities of NfxB mutants. *mexA-mexB-oprM* is believed to belong to a family of active efflux systems in bacteria (12, 22). Gotoh and Poole have recently observed (5) that OprJ is highly homologous to OprM. Thus, OprJ, as well as OprM, may act as a component of a multiple-drug efflux transporter. However, the mechanism(s) by which OprJ causes increases in susceptibility to some antimicrobial agents is unclear. There are several possibilities. One is that OprJ enhances outer membrane permeability to these agents, serving as a porin or interacting with lipopolysaccharides or phospholipids. Another possibility is that OprJ causes suppression of some efflux system(s) that pumps out these agents. Further, another possibility, that mutation of *nfxB* causes not only induction of OprJ but also suppression of β-lactamase, cannot be excluded immediately. However, the finding that NfxB mutants isolated from β-lactamase-deficient strains were also more susceptible to carbenicillin, imipenem, moxalactam, and aztreonam than their parent strains were (14) rules out the last possibility.

Both type A and B NfxB mutants showed cross-resistance to cefpirome, cefepime, cefclidin, cefozopran, cefoselis, and cefluprenam, although they showed slight increases in susceptibility to cefsulodin, cefoperazone, and ceftazidime and no changes in susceptibility to ME-1228. These results are consistent with the previous hypothesis that NfxB mutants only show cross-resistance to cephems that possess a positively charged substitution at position C-3, a negatively charged carboxyl group at position C-2, and no additional negative charge in the substitution at position C-7 (16). The structures of these new zwitterionic cephems are shown in Fig. 2. Cefsulodin, ceftazidime, and ME-1228 possess an additional negative charge in the substitution at position C-7, and cefoperazone possesses only one negatively charged carboxyl group at position C-2. In addition, carbenicillin, sulbenicillin, moxalactam, and aztreonam possess two negative charges, and increases in the susceptibilities of NfxB mutants to these drugs were greater than those to the other β-lactams tested, except carbapenems. However, we cannot explain the changes in the susceptibilities of NfxB mutants to β-lactams only on the basis of molecular charge, because carbapenems possess one positive charge and one negative charge, but NfxB mutants show increases in susceptibility to some carbapenems, such as imipenem, panipenem, and biapenem.

The multiple-drug-resistant NalB, NfxB, and NfxC mutants show a unique pattern of resistance, especially to β-lactams. NalB mutants show cross-resistance to most β-lactams, except some carbapenems. NfxB mutants show cross-resistance to the new zwitterionic cephems. No NfxC mutants show cross-resistance to β-lactams, except that diminished OprD causes resistance to carbapenems (3). As stated above, each mutant showed decreased or increased susceptibility to specific β-lactams, although these mutants showed cross-resistance to structurally diverse antimicrobial agents. This is very interesting, but the reason is unclear. In previous studies, when *oprM* was inactivated by a mutation, *P. aeruginosa* became hypersusceptible to many antimicrobial agents (4, 25). These results suggest that OprM is involved in the intrinsic resistance of *P. aeruginosa*. Wild-type strains PAO1 and KG2500 produced undetectable amounts of OprJ in immunoblot analysis, in agreement with a previous report (8), although wild-type strains produce small amounts of OprM (4). Therefore, OprJ may not play an important role in the intrinsic resistance of *P. aeruginosa*. However, the different specificities of agents to which susceptibilities were affected by *nalB*, *nfxB*, and *nfxC* mutations may be useful in clarifying the function of OprM and OprJ in multiple-drug resistance.

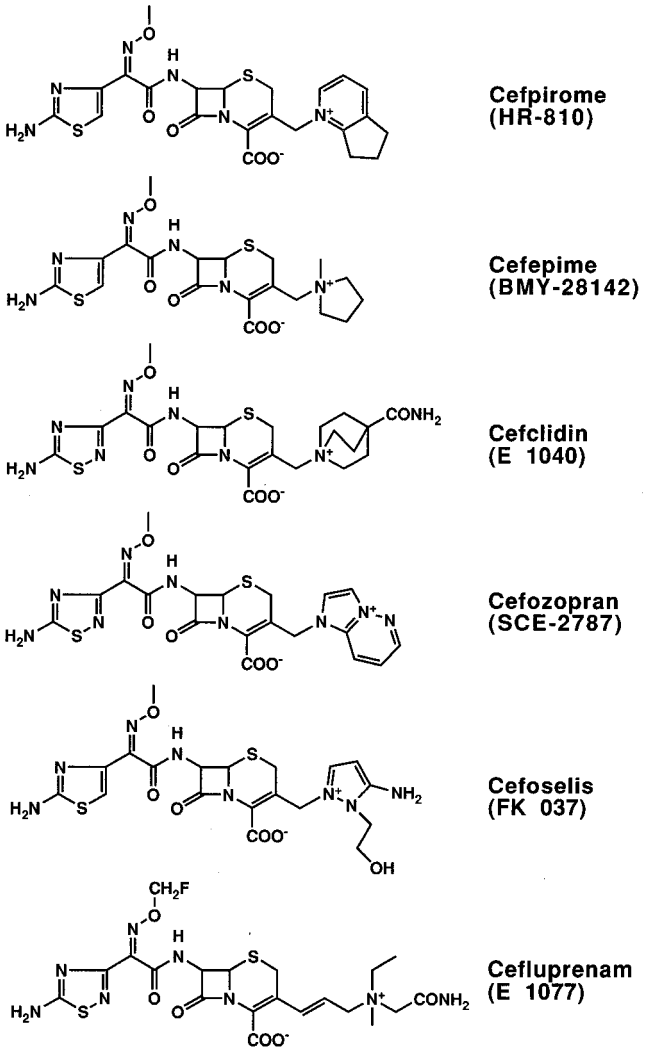

FIG. 2. Structures of new zwitterionic cephems to which NfxB mutants showed cross-resistance.

ACKNOWLEDGMENTS

This research was supported by a grant-in-aid from The Ministry of Education, Science and Culture of Japan.

We are grateful to E. Ibuki and H. Miyoshi for excellent technical assistance.

REFERENCES

1. **Angus, B. L., A. M. Carey, D. A. Caron, A. M. B. Kropinski, and R. E. W. Hancock.** 1982. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. **21:**299–309.
2. **Fu, K. P., B. D. Foleno, S. C. Lafredo, J. M. LoCoco, and D. M. Isaacson.** 1993. In vitro and in vivo antibacterial activities of FK037, a novel parenteral broad-spectrum cephalosporin. Antimicrob. Agents Chemother. **37:**301–307.
3. **Fukuda, H., M. Hosaka, K. Hirai, and S. Iyobe.** 1990. New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **34:**1757–1761.
4. **Gotoh, N., N. Itoh, H. Tsujimoto, J. Yamagishi, Y. Oyamada, and T. Nishino.** 1994. Isolation of OprM-deficient mutants of *Pseudomonas aeruginosa* by transposon insertion mutagenesis: evidence of involvement in multiple antibiotic resistance. FEMS Microbiol. Lett. **122:**267–274.
5. **Gotoh, N., and K. Poole.** Unpublished data.
6. **Gotoh, N., H. Tsujimoto, K. Poole, J. Yamagishi, and T. Nishino.** 1995. The outer membrane protein OprM of *Pseudomonas aeruginosa* is encoded by *oprK* of the *mexA-mexB-oprK* multidrug resistance operon. Antimicrob. Agents Chemother. **39:**2567–2569.
7. **Hirai, K., S. Suzue, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1987. Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31:**582–586.
8. **Hosaka, M., N. Gotoh, and T. Nishino.** 1995. Purification of a 54-kilodalton protein (OprJ) produced in NfxB mutants of *Pseudomonas aeruginosa* and production of a monoclonal antibody specific to OprJ. Antimicrob. Agents Chemother. **39:**1731–1735.
9. **Itoh, Y., L. Soldati, T. Leisinger, and D. Haas.** 1988. Low- and intermediate-copy-number cloning vectors based on the *Pseudomonas* plasmid pVS1. Antonie van Leeuwenhoek **54:**567–573.
10. **Iwahi, T., K. Okonogi, T. Yamazaki, S. Shiki, M. Kondo, A. Miyake, and A. Imada.** 1992. In vitro and in vivo activities of SCE-2787, a new parenteral cephalosporin with a broad antibacterial spectrum. Antimicrob. Agents Chemother. **36:**1358–1366.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
12. **Lewis, K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem. Sci. **19:**119–123.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 366–367. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Masuda, N., N. Gotoh, and T. Nishino.** Unpublished data.
15. **Masuda, N., and S. Ohya.** 1992. Cross-resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**1847–1851.
16. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
17. **Matsumoto, H., S. Ohta, R. Kobayashi, and Y. Terawaki.** 1978. Chromosomal location of genes participating in the degradation of purine in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **167:**165–176.
18. **Matsumoto, H., and T. Tazaki.** 1973. FP5 factor, an undescribed sex factor of *Pseudomonas aeruginosa*. Jpn. J. Microbiol. **17:**409–417.
19. **Nakagawa, S., T. Hashizume, K. Matsuda, M. Sanada, O. Okamoto, H. Fukatsu, and N. Tanaka.** 1993. In vitro activity of a new carbapenem antibiotic, BO-2727, with potent antipseudomonal activity. Antimicrob. Agents Chemother. **37:**2756–2759.
20. **Neu, H. C., G. Saha, and N. X. Chin.** 1989. In vitro activity of ME1228, a new parenteral cephalosporin. Antimicrob. Agents Chemother. **32:**535–539.
21. **Nikaido, H.** 1985. Role of permeability barriers in resistance to β-lactam antibiotics. Pharmacol. Ther. **27:**197–231.
22. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
23. **Okazaki, T., and K. Hirai.** 1992. Cloning and nucleotide sequence of the *Pseudomonas aeruginosa nfxB* gene, conferring resistance to new quinolones. FEMS Microbiol. Lett. **97:**197–202.
24. **Okazaki, T., S. Iyobe, H. Hashimoto, and K. Hirai.** 1991. Cloning and characterization of a DNA fragment that complements the *nfxB* mutation in *Pseudomonas aeruginosa* PAO. FEMS Microbiol. Lett. **79:**31–36.
25. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
26. **Rella, M., and D. Haas.** 1982. Resistance of *Pseudomonas aeruginosa* PAO to nalidixic acid and low levels of β-lactam antibiotics: mapping of chromosomal genes. Antimicrob. Agents Chemother. **22:**242–249.
27. **Watanabe, N., R. Hiruma, and K. Katsu.** 1992. In vitro evaluation of E1077, a new cephalosporin with a broad antibacterial spectrum. Antimicrob. Agents Chemother. **36:**589–597.
28. **Yoshimura, F., and H. Nikaido.** 1982. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J. Bacteriol. **152:**636–642.