ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1986, p. 684–688
0066-4804/86/110684-05$02.00/0

Vol. 30, No. 5

Multiply Resistant Mutants of *Enterobacter cloacae* Selected by β-Lactam Antibiotics

RUDOLF L. THEN* AND PETER ANGEHRN

Pharmaceutical Research Department, F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland

Received 5 March 1986/Accepted 4 August 1986

Mutants of *Enterobacter cloacae*, selected in vitro with ceftriaxone, ceftazidime, carumonam, or aztreonam, fell into several distinct classes. Three mutants highly resistant to nearly all β-lactam antibiotics were stably derepressed for β-lactamase production. Although no other changes could be detected, virulence in a mouse septicemia model was decreased in two of these mutants. One mutant, 908-S_{si}, showed selectively decreased susceptibility to ampicillin and cefotetan. A change in β-lactamase expression was thought to be responsible for this. Alterations in the production of two outer membrane proteins with molecular sizes of 36.5 and 39 kilodaltons were responsible for multiple antibiotic resistance in two mutants, both of which acquired a low level of resistance to β-lactam antibiotics. Whereas one of the mutants, AMA-R, simultaneously acquired resistance to chloramphenicol and trimethoprim, the other, AZT-R, became hypersusceptible to these and other hydrophobic agents. Both strains had drastically reduced virulence in mice.

With the more frequent use of broad-spectrum cephalosporins such as cefamandole, cefuroxime, cefotaxime, and others, resistant strains of several gram-negative species, especially *Enterobacter cloacae*, have been more frequently isolated (5, 15). These strains exhibit a high level of resistance to almost all β-lactam antibiotics, except imipenem, penems, or amdinocillin (13). Resistance is restricted to β-lactam antibiotics and based on the production of large amounts of the derepressed, chromosomally coded β-lactamase (12, 17, 21). In *E. cloacae* the frequency of mutation to the derepressed state is high and may be strain-specific (14, 17). It seems that cephalosporinase-mediated resistance accounts for the majority of resistant strains isolated in vitro or in the hospital under β-lactam exposure.

A different type of resistant mutant, however, has recently been characterized in several enterobacteria and in *Pseudomonas aeruginosa* (6, 7, 14, 24). These mutants exhibit a moderate degree of resistance to β-lactam antibiotics, coupled with resistance to unrelated drugs such as trimethoprim, chloramphenicol, or nalidixic acid. Alterations in the outer membrane proteins, presumably the porins, are thought to be responsible for this novel type of multiply resistant mutants. The design and use of potent β-lactamase-stable β-lactam antibiotics, as well as highly active new fluoroquinolone antibacterial agents that inhibit DNA replication (25), may be expected to select for such mutants more frequently in the future.

During our studies on β-lactam resistance in *E. cloacae* we isolated several mutants after in vitro exposure to different β-lactam antibiotics. Some of these strains exhibited unusual features and were characterized in more detail, especially with respect to changes in virulence. Their properties are described in this study.

MATERIALS AND METHODS

Strains. *E. cloacae* 908-S (908-S_{wi}) and its β-lactamase overproducing mutant 908-R have been described previously (21). A variant of 908-S (designated S_{si}) was detected during induction experiments and differs from 908-S_{wi} by a twofold increased β-lactamase level in the uninduced state. All other strains were selected in vitro by serial passage of 908-S_{wi} on β-lactam antibiotics incorporated into Diagnostic Sensitivity Test (DST)-agar (Oxoid Ltd., London, England) (see below). All strains were identified with the Enterotube II (Roche Diagnostics, Div. Hoffmann-La Roche Inc., Nutley, N.J.) or the API 20E System (API System S.A., Montaleu Vercieu, France).

Antibiotics. Most antibiotics and fine chemicals were supplied by commercial sources. Ceftriaxone, carumonam (20, 23), aztreonam, norfloxacin, trimethoprim, and brodimoprim (22) were synthesized at Roche (Basel, Switzerland). Imipenem was kindly provided from Merck & Co., Inc. (Rahway, N.J.), and cefotetan was obtained from Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan).

Susceptibility tests. MICs were determined on DST-agar (Oxoid) by applying 10^4 CFU per spot with a multipoint inoculating device (Dynatech Laboratories, Inc., Alexandria, Va.) The plates were read after 18 to 24 h of incubation at 35°C. Other media used were tryptic soy broth (TSB), nutrient agar, nutrient broth, Mueller-Hinton broth (MH), blood agar base, and brain heart infusion agar (all from Difco Laboratories, Detroit, Mich.).

β-Lactamase assays. β-Lactamase was assayed at 37°C in crude sonicated bacterial extracts, using nitrocefin as the substrate (21). Hydrolysis of various β-lactams was measured by a spectroscopic method (20). Isoelectric points were determined in crude extracts on PAG plates (LKB Instruments, Inc., Rockville, Md.) with a pH range from 3.5 to 9.5. In some instances a pI marker kit (BDH Chemicals Ltd., Poole, United Kingdom) was used during focusing. Inducibility of the β-lactamase was measured as described recently (10, 21) in TSB or MH (both from Difco). Inducer was added to a 30-ml culture in the late-logarithmic phase (at approximately 10^9 CFU per ml). The cells were harvested 2 h later and analyzed for β-lactamase.

Penicillin-binding proteins. The conditions for the isolation of membranes, the binding assay, and polyacrylamide gel electrophoresis were described recently (23). Penicillin-

* Corresponding author.

TABLE 1. Selection of some *E. cloacae* mutants on antibiotic-containing agar plates

Selecting agent	MIC (μg/ml) for selecting agent after passage no.:										Mutant designation
	1	2	3	4	5	6	7	8	9	10	
Ceftriaxone	0.25	0.5	256	>256	>256	>256	>256	>256	>256	>256	CRO-R
Ceftazidime	0.5	128	256	256	256	>256	>256	>256	>256	>256	CAZ-R
Carumonam	0.12	0.5	1	2	2	4	4	8	32	16	AMA-R
Aztreonam	0.12	0.5	1	1	1	2	4	8	16	8	AZT-R

binding proteins (PBPs) were characterized by competition of aztreonam or carumonam with [1-^{14}C]benzylpenicillin (54 mCi/mmol; Amersham International) or by direct labeling with [1-^{14}C]benzylpenicillin, [2-^{14}C]thiazolylceftriaxone, or [2-^{14}C]thiazolylcarumonam (48.2, and 39.9 mCi/mmol for ceftriaxone and carumonam, respectively). The last two compounds were synthesized at Roche (Basel).

Analysis of outer membrane proteins. Cell envelopes were prepared from cells grown in TSB to the stationary phase. Membranes were obtained by differential centrifugation after ultrasonic disruption of the cells (20). The Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.) insoluble fraction, obtained essentially as described previously (6), was analyzed on 12% acrylamide gels, containing 0.1% sodium dodecyl sulfate (SDS). Alternatively, outer membranes were obtained after passing the cells through a French pressure cell (American Instrument Co., Silver Spring, Md.), followed by differential and sucrose density gradient centrifugation, as described previously (2, 16). Membrane samples were solubilized in 58 mM Tris hydrochloride (pH 6.8)–5% glycerol–50 mM dithiothreitol–2% SDS–0.02% bromophenol blue at 95°C for 90 s before electrophoresis.

Determination of virulence. Virulence was tested in albino mice (Füllinsdorf strain: Animal and Laboratory Service, Roche) that weighed between 17 and 20 g. Twofold serial dilutions of overnight cultures were injected intraperitoneally as suspensions in 2% hog gastric mucin (American Laboratories Inc., Omaha, Nebr.), starting with a 25-fold dilution. Eight mice were used at each dilution level. The number of viable bacteria injected was determined by plating. The 50% lethal infectious dose (LD_{50}) was defined as the viable count resulting in death in 50% of the animals as a consequence of bacteremia and was calculated by the Probit method from the survival rates on day 3 after infection. Probit analysis techniques were also used to look for significant differences in LD_{50}s between mutant strains and the parent.

RESULTS

Selection of strains. Besides those strains obtained earlier (see above), more mutants were obtained by the passage of the parent strain 908-S_{wi} on agar plates containing graded concentrations of ceftazidime, ceftriaxone, aztreonam, or carumonam. The plates were inoculated with 10^4 CFU per spot, and after each passage those colonies growing on the highest concentrations were taken for the next passage on the same antibiotic without previous subculturing. The MICs obtained after 10 passages remained constant after 10 additional transfers on antibiotic-free medium. The majority of mutants obtained with all agents in this way seemed to produce large quantities of stably derepressed β-lactamase, as deduced from the rapid color change after single colonies were checked with a drop of nitrocefin. CRO-R (selecting agent ceftriaxone) and CAZ-R (selecting agent ceftazidime) belong in this class. Only those few mutants which seemed to differ from this type were analyzed further. Among those were AMA-R (selecting agent carumonam) and AZT-R (selecting agent aztreonam), which acquired a moderate resistance level in a gradual way (Table 1).

Growth properties. All strains formed smooth colonies. On several media tested (e.g., DST-agar, blood agar, or brain heart infusion agar) the colonies of AZT-R and AMA-R were consistently smaller than those of all other strains. These two strains also grew slower in TSB than the parent strain. The mass doubling times in this medium were 56 min for AZT-R and 57 min for AMA-R, as compared with 19 min for the parent 908-S_{wi}. Both mutants grew very poorly on nutrient agar or in nutrient broth, in contrast with the parent S_{wi}. AMA-R failed to grow even after 48 h, whereas AZT-R showed faint growth after 24 h. Since, in contrast with nutrient broth, TSB contains 5 g of NaCl per liter, which might influence the outer membrane protein composition, the effect of a change in the osmotic pressure was studied. The addition of 1% NaCl or 10% sucrose to nutrient broth or nutrient agar was seen to restore the ability to grow. Under these conditions AZT-R gave large, mucoid colonies.

Susceptibility to antibiotics. All strains were tested against a large number of β-lactam antibiotics. The results obtained with a representative number of them are shown in Table 2. CRO-R, CAZ-R, and 908-R exhibited a high level of resistance to nearly all compounds tested, except imipenem. S_{si}, interestingly, showed a distinctly reduced susceptibility only to ampicillin and cefotetan. AMA-R and AZT-R were both moderately resistant to cephalosporins and monobactams, but there were clear differences in their response to the penicillins. In addition, imipenem showed a fourfold reduced activity against these strains.

AMA-R and AZT-R displayed altered susceptibility to a number of antibiotics with unrelated modes of action. AMA-R was more resistant than the parent S_{wi} to chloramphenicol, nalidixic acid, norfloxacin, trimethoprim, and brodimoprim; whereas the activity of amikacin or gentamicin (data not shown) remained unaffected. AZT-R, in contrast, became more susceptible to nearly all of these agents and, in addition, to erythromycin and clindamycin

Susceptibility to detergents and dyes. The multiple drug resistance displayed by AMA-R, as well as the unusual increase in susceptibility to several unrelated drugs in AZT-R, suggests that envelope alterations are the basis for this behavior. To test this possibility further, the response to some detergents and dyes was also evaluated. In Table 2 it is shown that AZT-R was hypersusceptible to SDS, benzalkonium chloride, methylene blue, crystal violet, and acridine orange.

β-Lactamase. All strains produced a typical *E. cloacae* cephalosporinase with a pI of 8.2 ± 0.2, as measured with a surface electrode (Table 3). A pI of 7.9 ± 0.2 was obtained when a pI marker kit was used for calibration. This agreed with the pI of the *E. cloacae* P99 β-lactamae (17). No change in pI occurred on induction. The enzyme was inducible in

TABLE 2. Susceptibilities to antibiotics, detergents, and dyes of *E. cloacae* 908-S_{wi} and mutants derived from it

Agent	MIC (μg/ml) for the following strains:						
	S_{wi}	S_{si}	CRO-R	CAZ-R	AMA-R	AZT-R	908-R
Ampicillin	16	>256	>256	>256	256	8	>256
Aztreonam	0.25	0.25	256	128	32	16	64
Carumonam	0.12	0.12	64	128	16	16	64
Cefotetan	0.5	64	>128	>128	128	128	>128
Ceftazidime	0.5	0.5	>256	>256	8	8	>256
Ceftriaxone	0.12	0.5	>256	>256	4	4	>256
Imipenem	0.5	0.5	1	0.25	2	2	0.5
Moxalactam	0.05	0.12	64	32	32	64	8
Piperacillin	2	4	>128	>128	32	2	>128
Amikacin	2	2	2	2	2	1	2
Brodimoprim	1	1	2	1	32	0.12	1
Chloramphenicol	8	8	8	8	32	2	8
Clindamycin	128	128	64	64	128	4	64
Erythromycin	128	128	128	64	128	16	128
Nalidixic acid	4	4	ND[a]	ND	16	2	4
Norfloxacin	0.05	0.05	0.1	0.1	1.6	0.05	0.1
Trimethoprim	0.5	0.5	0.5	0.5	16	0.12	0.5
Acridine orange[b]	500	500	500	500	500	7.8	500
Benzalkonium hydrochloride[b]	39	39	39	19.5	39	9.8	39
Crystalviolet[b]	250	125	125	125	125	2	125
Methylene blue[b]	>1,000	>1,000	2,000	2,000	1,000	3.9	>2,000
SDS[b]	>10,000	>10,000	>10,000	>10,000	>10,000	78	>10,000

[a] ND, Not done.
[b] Tested in a concentration range from 0.0001 to 1%, depending on the solubility.

the parent S_{wi} and in the mutants S_{si}, AMA-R, and AZT-R. A large amount of β-lactamase was produced constitutively in CRO-R, CAZ-R and 908-R (Table 3).

S_{si} differed from the wild-type S_{wi} by a twofold increased basal enzyme level. This difference was highly significant by the Student *t* test ($P < 1\%$). The enzyme activity reached higher values after induction with imipenem, cefoxitin, or cefotetan (Tables 3 and 4). β-Lactamase expression in *E. cloacae* was seen to be dependent on the medium used, but as shown in Table 4, in all cases 908-S_{si} produced higher levels than 908-S_{wi}. The basal enzyme level of AMA-R and the induced enzyme level in AMA-R and AZT-R seem also to be different from that of the parent, but this was not studied further.

PBPs. The unusual susceptibility pattern of AMA-R, and especially of AZT-R, prompted an investigation of their PBPs. The PBP patterns obtained after fluorography were identical for both the parent and daughter strains. PBPs of the β-lactamase overproducing variants could not be reliably visualized with [^{14}C]benzylpenicillin because of residual β-lactamase in the membrane fraction. Competition experiments with aztreonam and carumonam, as well as direct labeling of the PBPs with [^{14}C]ceftriaxone and [^{14}C]carumonam, did not reveal any significant differences in the PBPs of AMA-R an AZT-R and the parent S_{wi}.

Changes in membrane proteins. Coomassie-blue stained polyacrylamide gels of membrane fractions obtained by sonication revealed differences among the parent, AMA-R, and AZT-R. The Sarkosyl-insoluble fraction gave three major protein bands with molecular sizes of approximately 36, 37.5, and 40 kilodaltons (kDa) in the parent. The 36- and 40-kDa proteins were missing in both mutants. Analysis of the outer membrane proteins obtained after separation of the inner and the outer membrane by sucrose density gradient centrifugation also showed clear differences between these mutants and the parent (Fig. 1). The two major outer membrane proteins of 36.5 and 39 kDa were diminished or completely absent. It was seen that most of the 37.5-kDa protein band observed after Sarkosyl extraction migrated at 28.8 kDa if dithiothreitol instead of mercaptoethanol was

TABLE 3. β-Lactamase levels, inducibility, and pIs in mutants of *E. cloacae* 908-S_{wi}

Strain	Basal level of β-lactamase[a]	β-Lactamase activity[a] after induction with imipenem at:				pI
		0.1 μM	1 μM	10 μM[b]	100 μM[b]	
S_{wi}	14.48 ± 1.21	22.2	84.3	403	729	8.3/8.3[c]
S_{si}	27.1 ± 1.20	111.2	1,261	1,628	2,057	8.3/8.3[c]
CRO-R	36,578	37,472	39,294	36,362	29,394	8.2
CAZ-R	22,566	24,946	28,711	17,448	11,093	8.3
AMA-R	6.2	14.2	61.8	1,137	719	8.2
AZT-R	14.6	11.4	31.6	137	191	8.3
908-R	25,380	36,120	36,591	31,843		8.3

[a] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein in crude extract.
[b] Considerable inhibition of growth and lysis occurred at these inducer concentrations.
[c] Not induced/induced; mean of three to six determinations.

TABLE 4. Differences in inducibility of β-lactamase in *E. cloacae* 908-S_{wi} and 908-S_{si}

Inducer	Concn (μM)	Medium (lot no.)	Sp act of β-lactamase[a]	
			908-S_{wi}	908-S_{si}
Cefoxitin	0	MH (619507)	16.5	32.8
	1		42	889
	10		1,477	2,568
	0	TSB (728779)	9	24.3
	1		10.5	30.1
	10		48.9	77
Cefotetan	0	MH (619507)	19	28.8
	1		51.7	970
	10		208	1,496
	0	TSB (728779)	11.0	21.0
	1		10.7	21.7
	10		7.0	45.6

[a] Nanomoles of nitrocefin hydrolyzed per minute per milligram of protein in crude extract.

used as the reducing agent. The membranes of these strains have been analyzed in more detail, and results will be described elsewhere (R. Aggeler, R. L. Then, and R. Ghosh, manuscript in preparation).

Virulence. The individual isolates displayed considerable difference in virulence. The LD_{50}s of strains S_{si} and CRO-R were comparable with that of the parental strain S_{wi} (Table 5). A decreased, though still pronounced, virulence was found for the mutant CAZ-R. The mutant AMA-R was more than 100-fold less virulent than S_{wi}. AZT-R and 908-R reproducibly did not induce lethal septicemia, even when injected in amounts 100-fold greater than the LD_{50} for S_{wi}.

DISCUSSION

E. cloacae normally produces an inducible cephalosporinase. Derepression of this enzyme is a frequent mutational event and results in a high level of resistance against nearly all β-lactam antibiotics, except imipenem, penems, amdinocillin, temocillin (BRL 17421), or quaternary ammonium-substituted cephalosporins, such as BMY 28142 (9, 13, 18). Three of the six mutants studied, viz., CRO-R, CAZ-R, and 908-R belong to this class of mutants.

The β-lactam-resistant mutants AMA-R and AZT-R, on the other hand, also exhibited distinct susceptibility changes to other unrelated drugs. AZT-R became hypersusceptible to chloramphenicol and brodimoprim, while AMA-R demonstrated increased resistance to these agents. The selection of multiply resistant mutants by β-lactam antibiotics has been observed both in vitro and during therapy. In several gram-negative species, altered production of outer membrane proteins, presumably the porins, are thought to be responsible for this resistant phenotype (6, 7, 14, 24).

The occurrence of mutants of both types during therapy shows that both can be virulent, at least under the selection pressure imposed by the antibiotic. One interesting result of this study is, however, that the mutants AMA-R and AZT-R exhibited drastically reduced virulence in mice. The presumed membrane alterations, which not only impair the penetration of antibiotics but also that of nutrients into the bacterial cell, resulting in diminished growth rates, offer a reasonable explanation for their reduced virulence.

It was surprising that two of the three mutants for which no changes besides β-lactamase overproduction were observed also exhibited reduced virulence. Other, more subtle changes which have not been detected may be responsible for this phenomenon. This correlates with recent findings that in mixed cultures a growth disadvantage of hyperproducers can be demonstrated (K. Buchanan, S. Swanzy, F. D. Schoenknecht, and J. C. Sherris, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, A-113, p. 19). In contrast, results of a recent study (1) with *P. aeruginosa* showed no decrease in virulence for several β-lactam-persistent mutants.

The mutant S_{si} exhibited selectively reduced susceptibility for ampicillin and cefotetan and a twofold elevated basal level of β-lactamase which reached much higher levels than in the parent S_{wi} on induction with imipenem, cefoxitin, or cefotetan. This strain seems to have undergone a mutation affecting β-lactamase expression. The mechanisms responsible for the impressive differences in β-lactamase activity observed during induction in either MH or TSB remains to be investigated. The dependence of β-lactamase expression on the medium used has also been observed by others (3).

AZT-R exhibited unusual features in that it became resistant to most β-lactams (excluding ampicillin and piperacillin), whereas it became more susceptible to erythromycin, clindamycin, and trimethoprim. It was also hypersusceptible to several detergents and dyes. It is speculated that these features are probably the result of more than one mutation, as deduced from the slow and gradual acquisition of resistance (Table 1). A porin mutation and an additional, so far unknown, but compensatory mutation in the lipopolysaccharide may have occurred in this strain.

β-Lactamase and altered PBPs have been excluded as the basis for β-lactam resistance. The acquisition of resistance for cephalosporins, but not for ampicillin or piperacillin, could indicate that penicillins and cephalosporins use dif-

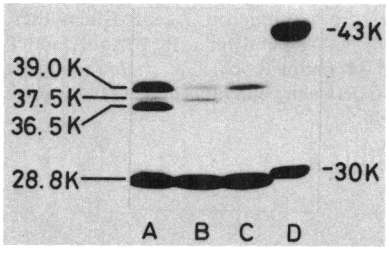

FIG. 1. Coomassie blue-stained 12% SDS-polyacrylamide gel of the porin region of the outer membrane of *E. cloacae* 908-S_{wi} (lane A), mutant AZT-R (lane B), and mutant AMA-R (lane C). Lane D, Molecular size standards:ovalbumin (43 kDa [K]) and carbonic anhydrase (30 kDa [K]).

TABLE 5. Virulence of *E. cloacae* 908-S_{wi} and mutants in mice

Strain	LD_{50} (CFU/mouse) (95% confidence limits)
S_{wi}	6.3×10^4 (2.2×10^4–1.8×10^5)
S_{si}	4.0×10^4 (1.4×10^4–1.2×10^5)
CRO-R	2.3×10^5 (8.7×10^4–5.9×10^5)
CAZ-R	1.2×10^6 (6.3×10^5–2.2×10^6)[a]
908-R	$>6.9 \times 10^6$ (NC[b])
AMA-R	9.1×10^6 (2.8×10^6–2.8×10^7)[a]
AZT-R	$>1.0 \times 10^7$ (NC)

[a] Significant difference to S_{wi} (α = 5%).
[b] NC, Not calculable.

ferent pathways of outer membrane penetration, as suggested by Yamaguchi et al. (26).

Mutants of *Salmonella typhimurium* or *P. aeruginosa* with increased susceptibility to hydrophobic agents have been described (4, 19). Mutations in minor outer membrane proteins or lipopolysaccharide may cause such phenotypes, but in no case are the underlying mechanisms well understood (11). Because colonies of AZT-R have a smooth phenotype, the idea of a mutation in lipopolysaccharides is not supported. As in other similar mutants of *E. cloacae* described by Werner et al. (24), a distinctly diminished expression of major outer membrane proteins of sizes between 35 and 40 kDa was found for AMA-R and AZT-R. The molecular sizes of 36.5 and 39 kDa are very close to those determined by Kaneko et al. (8) for two proteins with porin function in *E. cloacae*. These changes are therefore assumed to be responsible, at least in part, for the resistance phenotype of AMA-R and AZT-R. Meanwhile, more data on the membranes of these mutants have been obtained and will be described elsewhere.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of M. Keller, J. Nell, and H. Straub. We are also indebted to R. Aggeler, who did most of the work on membranes, and to R. Charnas for valuable comments.

LITERATURE CITED

1. **Bryan, L. E., A. J. Godfrey, and T. Schollardt.** 1985. Virulence of *Pseudomonas aeruginosa* strains with mechanisms of microbial persistence for β-lactam and aminoglycoside anitibiotics in a mouse infection model. Can. J. Micobiol. **31**:377–380.
2. **Burnell, E., L. van Alphen, A. Verkleij, B. de Kruiff, and B. Lugtenberg.** 1980. ^{31}P nuclear magnetic resonance and freeze-fracture electron microscopy. Biochim. Biophys. Acta **597**:518–532.
3. **Cullmann, W., A. Dalhoff, and W. Dick.** 1984. Nonspecific induction of β-lactamase in *Enterobacter cloacae*. J. Gen. Microbiol. **130**:1781–1786.
4. **Fyfe, J. A. M., and J. R. W. Govan.** 1984. Chromosomal loci associated with antibiotic hypersensitivity in pulmonary isolates of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **130**:825–834.
5. **Giamarellou, H., G. Koratzanis, K. Kanellakopoulou, and G. K. Daikos.** 1985. Epidemiological study of *Enterobacter cloacae* resistant to 3rd generation cephalosporins: a preliminary report. Chemioterapia **4**:43–46.
6. **Gutmann, L., and Y. A. Chabbert.** 1984. Different mechanisms of resistance to latamoxef (moxalactam) in *Serratia marcescens*. J. Antimicrob. Chemother. **13**:15–22.
7. **Gutmann, L., R. Williamson, N. Moreau, M. D. Kitzis, E. Collatz, J. F. Acar, and F. W. Goldstein.** 1985. Cross resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella*, *Enterobacter*, and *Serratia*. J. Infect. Dis. **151**:501–507.
8. **Kaneko, M., A. Yamaguchi, and T. Sawai.** 1984. Purification and characterization of two kinds of porins from the *Enterobacter cloacae* outer membrane. J. Bacteriol. **158**:1179–1181.
9. **Kessler, R. E., M. Bies, R. E. Buck, D. R. Chisholm, T. A. Pursiano, Y. H. Tsai, M. Misiek, K. E. Price, and F. Leitner.** 1985. Comparison of a new cephalosporin, BMY 28142, with other broad-spectrum β-lactam antibiotics. Antimicrob. Agents Chemother. **27**:207–216.
10. **Minami, S., A. Yotsuji, M. Inoue, and S. Mitsuhashi.** 1980. Induction of β-lactamase by various β-lactam antibiotics in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **18**:382–385.
11. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1–32.
12. **Sanders, C. C.** 1983. Novel resistance selected by the new expanded spectrum cephalosporins: a concern. J. Infect. Dis. **147**:585–589.
13. **Sanders, C. C., and W. E. Sanders.** 1983. Emergence of resistance during therapy with the newer β-lactam antibiotics: role of inducible β-lactamases and implications for the future. Rev. Infect. Dis. **5**:639–648.
14. **Sanders, C. C., W. E. Sanders, R. V. Goering, and V. Werner.** 1984. Selection of multiple antibiotic resistance by quinolones, β-lactams, and aminoglycosides with special reference to cross resistance between unrelated drug classes. Antimicrob. Agents Chemother. **26**:797–801.
15. **Sanders, C. C., and W. E. Sanders.** 1985. Microbial resistance to newer generation β-lactam antibiotics: clinical and laboratory implications. J. Infect. Dis. **151**:399–406.
16. **Schnaitman, C. A.** 1970. Protein composition of the cell wall cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **104**:890–901.
17. **Seeberg, A. H., R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1983. Chromosomal β-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrob. Agents Chemother. **23**:918–925.
18. **Slocombe, B., M. J. Basker, P. H. Bentley, J. P. Clayton, M. Cole, K. R. Comber, R. A. Dixon, R. A. Edmondson, D. Jackson, D. J. Merrikin, and R. Sutherland.** 1981. BRL 17421, a novel β-lactam antibiotic highly resistant to β-lactamases, giving high and prolonged serum levels in humans. Antimicrob. Agents Chemother. **20**:38–46.
19. **Sukupolvi, S., M. Vaara, J. M. Helander, P. Viljanen, and P. H. Mäkelä.** 1984. New *Salmonella typhimurium* mutants with altered outer membrane permeability. J. Bacteriol. **159**:704–712.
20. **Then, R. L.** 1984. Interaction of Ro 17-2301 (AMA-1080) with β-lactamases. Chemotherapy **30**:398–407.
21. **Then, R. L., and P. Angehrn.** 1982. Trapping of nonhydrolyzable cephalosporins by cephalosporinases in *Enterobacter cloacae* and *Pseudomonas aeruginosa* as a possible resistance mechanism. Antimicrob. Agents Chemother. **21**:711–717.
22. **Then, R. L., and F. Hermann.** 1984. Properties of brodimoprim as an inhibitor of dihydrofolate reductases. Chemotherapy **30**:18–25.
23. **Then, R. L., and J. Kohl.** 1985. Affinity of carumonam for penicillin binding proteins. Chemotherapy **31**:246–254.
24. **Werner, V., C. C. Sanders, W. E. Sanders, and R. V. Goering.** 1985. Role of β-lactamases and outer membrane proteins in multiple β-lactam resistance of *Enterobacter cloacae*. Antimicrob. Agents Chemother. **27**:455–459.
25. **Wolfson, J. S., and D. C. Hooper.** 1985. The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity in vitro. Antimicrob. Agents Chemother. **28**:581–586.
26. **Yamaguchi, A., N. Tomiyama, R. Hiruma, and T. Sawai.** 1985. Difference in pathway of *Escherichia coli* outer membrane permeation between penicillins and cephalosporins. FEBS Lett. **181**:143–148.