to third-generation cephalosporins [18], data very similar to those reported in North America.

The increase in resistance amongst Enterobacteriaceae has been correlated with an increase in the use of broad-spectrum antimicrobial agents. For example, resistance amongst *E. cloacae* to ceftazidime has been shown to be directly related to the use of ceftazidime (Figure 2) [2]. As the use of ceftazidime increased steadily, the susceptibility to ceftazidime declined ($p < 0.02$). To examine temporal trends in ceftazidime resistance, susceptibility data reported to the NNIS survey (CDC) during 1987–1991 were analyzed among nosocomial *Enterobacter* spp., *K. pneumoniae* and *P. aeruginosa.* Progressive increases in resistance were observed for *Enterobacter* spp. and *K. pneumoniae* over time, with the percentage of resistant strains of *Enterobacter* spp. increasing significantly during 1989–1991 [35]. The increase in ceftazidime resistance in *K. pneumoniae* was related to plasmid-mediated extended spectrum β-lactamases [4–6,12,13].

Resistance to third-generation agents caused by derepressed species appears to be greatest amongst the most seriously ill patients, such as those in the ICU setting [40]. Furthermore, *E. cloacae* consistently has the highest rates of resistance (ceftazidime) in general practice (GP) patients, hospitalized patients and those within the ICU (Figure 3; personal communication from the Paul Ehrlich Society, B. Wiedermann).

Resistance development may be particularly devastating in patients with serious infections, e.g. neutropenic and immunocompromized patients, especially if prior antimicrobial therapy has been given. Numerous cases of breakthrough bacteremia with multiply-resistant *Enterobacter* spp. in febrile neutropenic cancer patients and other patients receiving broad-spectrum cephalosporins have been reported [34]. The results of studies that have assessed the rates of resistance emerging among Enterobacteriaceae during or shortly after therapy with a number of cephalosporins are listed in Table 4 [8]. Resistance emerged in 16–44% of treated

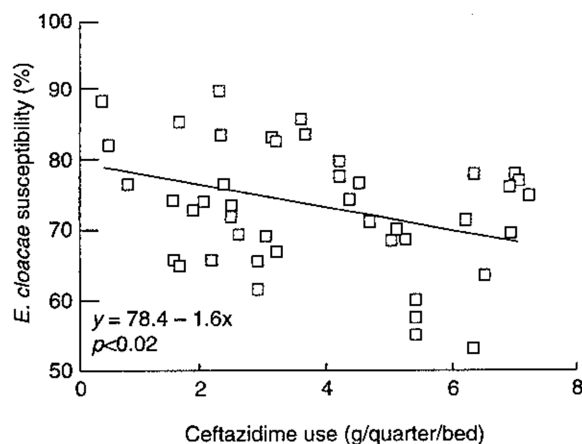


Figure 2 Relationship between ceftazidime use and susceptibility of *Enterobacter cloacae* to ceftazidime [2]. With permission of Diagn Microbiol Infect Dis.

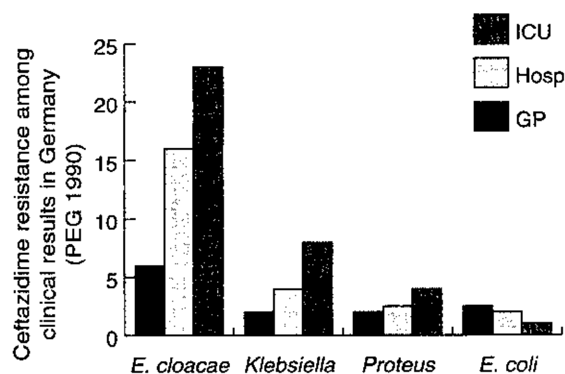


Figure 3 Ceftazidime resistance among clinical isolates in Germany (PEG 1990).

Table 4 Rates of emergence of resistance in patients infected with Enterobacteriaceae organisms possessing inducible β-lactamases and treated with newer cephalosporins. Adapted from Sanders et al. with permission [8].

Drug	Organism[a]	Total no. of patients	No.(%) of patients with emerging resistance	Frequency of clinical failure or relapse[b]
Ceftriaxone	Several	29	8 (28)	6 (21/75)
Moxalactam	*Serratia marcescens*	10	3 (30)	1 (10/33)
Moxalactam	Several	10	4 (40)	1 (10/25)
Several	*Enterobacter* species	9	4 (44)	–
Several	Several	44[c]	7 (16)[c]	3 (7/43)[c]

[a]Data summarized for enteric bacilli from four earlier publications (102 patients, not all of whom received a cephalosporin).
[b]Results are expressed as the number of patients with therapy failure or relapse (percentage of total number of patients/percentage of those with emerging resistance). Minus signs indicate that no data were provided.
[c]Includes *P. aeruginosa* (24 of 49 strains in 44 patients). Only one of the resistant enteric bacilli cases received an extended spectrum β-lactam.

patients (highest among *Enterobacter* spp.), with a mean rate of 25%. The rates were generally consistent among the various drugs examined. A more comprehensive review by Fish et al. documented a lower rate of emerging resistance (7.7–10.1%) for *Citrobacter* spp. and *Enterobacter* spp. [41]. Among patients in whom the emergence of resistance was detected, failure/relapse rates ranged from 25% to 75%, but emerging resistance did not predict clinical failure. The greatest risk of resistance and frequency of pathogen occurrence appear to occur with isolates of *E. cloacae* and *E. aerogenes*, especially those cultured from respiratory tract sites (Tables 1 and 4). High morbidity and mortality cases were also associated with bone and joint infections and in patients with neutropenia and cystic fibrosis [9]. In one investigation, 15 of 16 isolates of *Enterobacter* spp. from neutropenic patients were resistant to extended-spectrum cephalosporins. In contrast, only 12 of 35 isolates from non-neutropenic patients were resistant ($p < 0.05$) [34]. The neutropenic patients had received more β-lactam therapy than the non-neutropenic patients. The authors concluded that prior β-lactam exposure may predispose neutropenic patients to develop resistant *Enterobacter* bacteremia. Other studies have described patients where cephalosporin-resistant Gram-negative bacteria have emerged during treatment, resulting in life-threatening secondary infections [8,31,33]. A total of 18 patients who were infected initially with susceptible organisms exhibited emergence of resistant strains during administration of ceftriaxone, cefotaxime or ceftazidime, some despite combination therapy with aminoglycosides [33]. Resistant strains of *E. cloacae, S. marcescens, K. oxytoca, P. aeruginosa* and *C. freundii* emerged, probably by the selection of stably derepressed mutants, after 9 days of treatment. Thus, the selection of resistant bacteria may have serious clinical consequences in patients with risk factors, such as impaired host-defence mechanisms, as the selection of resistance is associated with a significant rate of therapy failure and relapse.

Risk of AmpC induction

The extent of AmpC induction is dependent upon both the β-lactam-inducing agent and the inducer concentration [9,42–45]. At sub-MIC concentrations, cefoxitin, long regarded as a potent inducing agent, has been shown to induce AmpC by 100- to 600-fold in strains of *E. cloacae, C. freundii, P. stuartii, S. marcescens, M. morganii* and *P. aeruginosa* [44]. However, the carbapenems, imipenem and meropenem, may prove to be at least as potent as cefoxitin as inducing agents for AmpC in *C. freundii* [9].

A consensus of published reports ranks the AmpC inducing potential for β-lactam classes [42–45]. On this basis, carbapenems and cephamycins are the most potent inducing agents (Table 5), followed by penicillins and the older cephalosporins. The fourth-generation cephalosporins, cefpirome and cefepime, have a lower risk of inducing AmpC than the β-lactamase inhibitor, clavulanic acid. Induction itself, however, does not imply a clinical risk, since the greatest inducers produce increased amounts of enzyme without a significant effect on the initial MIC (i.e. rapid bactericidal action becomes manifest before induction of the enzyme has been efficiently produced).

Risk of AmpC selection

Some β-lactam antimicrobials are more likely than others to select mutant subpopulations of resistant organisms and their widespread use in the hospital environment has resulted in the emergence of clinically important endemic bacterial resistances [46]. These selection potential differences in individual inducible strains that cause infection (susceptible by reference test) remains unclear.

The frequency of stably derepressed AmpC mutants in a bacterial population can be as high as 10^{-5} [4]. Such mutants have serious clinical implications and are isolated in approximately 20% of infections involving AmpC-producing strains during selective therapy with broad-spectrum β-lactams [4]. Factors favoring the

Table 5 Induction potential at concentrations below MIC (consensus from the reported literature [42–45])

Induction Potential	Rank
Highest	carbapenems and cephamycins
	aminopenicillins
	carboxy-penicillins
	ureidopenicillins
	older cephalosporins (1st, 2nd and 3rd)
	clavulanic acid
	newer cephalosporins (4th)
	sulphones
Lowest	monobactams

occurrence and selection of such mutants include high bacterial inoculum at the infection site, bacterial species and strain involved.

In an in vitro investigation of resistance development to third- and fourth-generation cephalosporins in 10 strains of *E. cloacae*, full resistance to ceftriaxone and ceftazidime occurred in at least half of the strains within 1–3 days of passage (Figure 4) [46]. This resistance development was associated with greatly enhanced AmpC production, but had only a modest effect upon outer-membrane protein profile as a resistance mechanism. In contrast, at least five passages were required before the majority of strains acquired resistance to fourth-generation cephalosporins. Resistance to the fourth-generation cephalosporins was associated with changes in the outer membrane proteins, but involved little alteration of AmpC expression. The latter results suggest that at least two genetic mutations, altered permeability and high Km, may be necessary to achieve resistance to newer zwitterionic cephalosporins.

The dramatic impact of inducible AmpC β-lactamase-producing strains upon β-lactam susceptibility and clinical outcome makes it essential that clinical microbiology laboratories can identify such strains reliably. The primary difficulties caused by Gram-negative pathogens with inducible β-lactamases stem from their apparent susceptibility, when tested against third-generation cephalosporins, in routine in vitro tests. However, accurate bacterial identification should be sufficient to raise the possibility of selecting derepressed AmpC mutants. Identification of the 'at risk' species is well within the specifications of most commonly used commercial kits (Vitek, MicroScan, Sensident, Micronaut, API, etc.). Information provided by computerized 'Expert Systems' for the interpretation of antimicrobial susceptibility testing, frequently coupled with the above cited commercial diagnostic systems, may also be useful. As confirmation, standardized susceptibility tests can accurately determine β-lactam susceptibility for the selected derepressed mutants without the need for elaborate or time-consuming induction or other non-standardized tests [42]. In a survey of over 8,500 strains conducted by 43 laboratories in the USA, the observed rates (i.e. local center results) for ceftazidime resistance in *E. cloacae* (28.4%) and *C. freundii* (31.0%) [3] were very similar to rates obtained (29.8% and 33.2%, respectively) by reference methods in the monitoring laboratory [47].

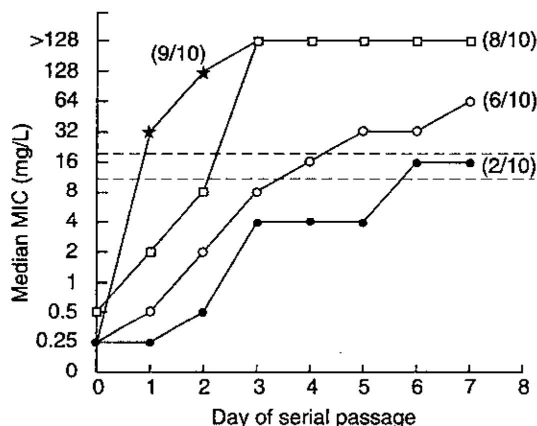


Figure 4 Median MICs for 10 *E. cloacae* strains during 7-day serial passage with a cephalosporin. The median MIC represents the sixth MIC observation when the MICs for the 10 strains on each day of testing are listed from the lowest to the highest value. The values in parentheses are the number of strains among the 10 strains tested for which the MIC was in the resistant range (≥ 32 mg/L) for the 7-day serial passage. ★ ceftriaxone; □ ceftazidime; ○ cefpirome; ● cefepime. The upper and lower broken lines in the figure are cut-offs for resistance and susceptibility (NCCLS criteria), respectively. With permission of Am Soc Microbiol J Div [46].

SIGNIFICANCE OF INDUCIBLE AND STABLY DEREPRESSED RESISTANCE

Induction potential does not necessarily translate to reduced efficacy in either the laboratory or clinical situation [48]. Confounding variables, such as the presence of multiple resistance mechanisms, outer membrane penetration, PBP affinity, enzyme inhibition by the inducer and, most importantly, the β-lactamase stability of the inducer, can affect the periplasmic concentration of the β-lactam and hence bactericidal activity. Some compounds both strongly induce and are hydrolyzed by chromosomally-mediated enzymes of Gram-negative bacteria (e.g. the aminopenicillins and the cephamycins for *E. cloacae*). Other compounds (e.g. piperacillin and other cephalosporins), although poor inducers, are labile so that greatly increased MICs are observed, despite relatively modest levels of AmpC induction. In contrast, the high AmpC-inducing potential of the carbapenems does not compromize their efficacy due to high bacterial membrane penetration and relative β-lactamase stability. The fourth-generation cephalosporins also combine high penetration rates and β-lactamase stability with low induction potential [49].

ROLE OF NEW CEPHALOSPORINS IN THERAPY

In common with third-generation cephalosporins, the fourth-generation cephalosporins have an aminothiazolyl (or amino thiadiazolyl)-methoximino group at the C-7 position of the cephem nucleus (Figure 5) [50].

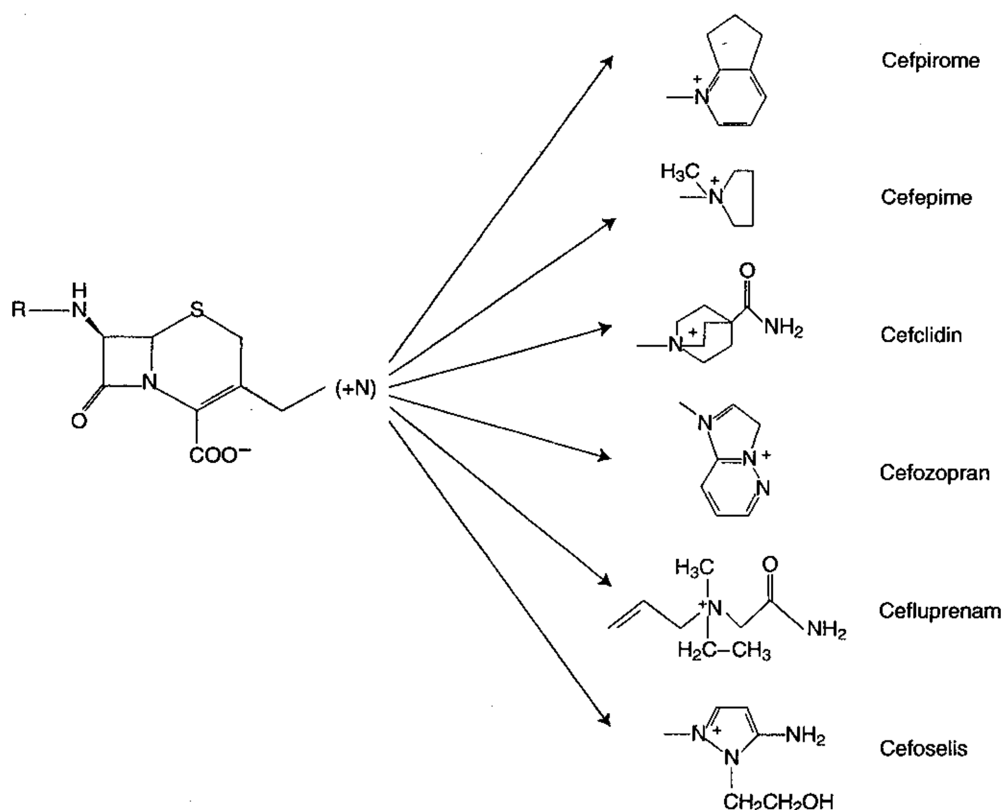


Figure 5 C-3' quaternary ammonium cephems [50].

However, these newer cephalosporins possess a quaternary ammonium group at the C-3' position which confers a considerable increase in potency and has led to these compounds being termed 'fourth-generation' cephalosporins. These C-3' substitutions confer a more balanced antimicrobial spectrum compared to ceftazidime and maintain stability to, and low affinity for, clinically important β-lactamases. They also give these compounds the properties of a zwitterion which enhances outer membrane permeability. The principal candidates for inclusion in the group are listed in Table 6 and include cefpirome and cefepime.

Both cefpirome and cefepime have been shown to penetrate the outer membrane of *E. cloacae* approximately 5- to 6-fold faster than cefotaxime. This, coupled with much lower affinity (high Km) for and higher stability towards the AmpC β-lactamase, results in higher periplasmic concentrations than those achieved by cefotaxime [51]. Consequently, MIC_{90} values of 0.5–1 mg/L are routinely achieved against *E. cloacae*, as opposed to > 32 mg/L for cefotaxime and ceftazidime [52]. Pooling of data from nine studies produced a median MIC_{90} value of 1 mg/L for cefpirome against *E. cloacae*, compared to 50 mg/L for ceftazidime [53].

Fourth-generation cephalosporins have also demonstrated excellent activity against *Enterobacter* spp. isolated from ICU [18] and other units [54]. In one study in ICU infections, cefpirome and imipenem were the most potent against ceftazidime-resistant isolates, with 94% and 97%, respectively, of strains susceptible [18]. With the exception of cefpirome, there was significant cross-resistance among the cephalosporins tested.

Fourth-generation cephalosporins generally

Table 6 List of candidate 'fourth-generation' cephalosporins for human use [50]

C-7, 2-amino-5-thiazolyl	C-7, 5-amino-2-thiadiazolyl
Cefpirome (HR-810)	Cefclidin (E-1040)
Cefepime (BMY-28142)	Cefozopran (SCE-2787)
Cefoselis (FK-037)	Cefluprenam (E-1077)

maintain good activity against ceftazidime-resistant (MIC > 16 mg/L) Enterobacteriaceae with inducible AmpC β-lactamases. In an international study of 160 ceftazidime-resistant strains [55], 74% were inhibited by cefpirome at ≤ 8 mg/L (Table 7). An identical rate of cefpirome susceptibility was noted in a five-nation survey (Table 7; Australia, France, Germany, Italy and UK) and in the USA [55]. In another 11-nation study of ceftazidime-resistant Enterobacteriaceae, > 80% of strains were inhibited by cefepime (≤ 8 mg/L), with the exception of some strains from Brazil (48%) and Italy (55%). Overall, cefpirome and cefepime display similar activities against Enterobacteriaceae which produce inducible AmpC β-lactamases, while cefocelis (FK 037) appeared slightly less active [57,58].

As yet, there are limited published clinical studies to assess the efficacy of fourth-generation cephalosporins against serious *Enterobacter* infections and especially against strains resistant to third-generation cephalosporins [59–61]. However, early indications are promising [61]. From pooled comparative clinical trials using cefepime (2,487 patient cases), 17 infections with *Enterobacter* spp. caused by organisms tested as susceptible to cefepime, but resistant to ceftazidime were observed [61]. Cefepime therapy resulted in clinical cure in all patients and an 88.2% bacteriological eradication rate. Also, no emergence of resistance was noted. In a study of 276 hospitalized patients with severe infections, three were attributable to *E. cloacae*, and were eradicated following treatment with cefpirome at 1 or 2 g bid (Table 8) [59]. In another study involving less serious infections [60], ceftazidime produced bacterial eradication in 70% of patients, whereas cefpirome at 1 g bid achieved 100% eradication (Table 8). In a Scandinavian study, cefpirome dosed at 1 g bid was found to be at least as effective as ceftazidime 1 g tid in eradicating *Citrobacter* and *Enterobacter* spp. from the urinary and respiratory tracts [62].

Another recent multicenter study compared the efficacy and safety of cefpirome and ceftazidime in the empiric treatment of nosocomial and community-acquired pneumonia in the ICU [63]. A satisfactory bacteriological response was achieved in 73% and 64% of patients receiving cefpirome (2 g bid) and ceftazidime (2 g tid), respectively, for infections caused by *Enterobacter* spp. Similarly, cefepime has demonstrated favourable results compared to ceftazidime in the treatment of infections caused by Enterobacteriaceae [64].

Table 7 Distribution of Gram-negative ceftazidime-resistant (MIC > 16 mg/L) Enterobacteriaceae strains by cefpirome MICs[a]

		Strains with following cefpirome MIC (mg/L)						
Organism	No. of strains	≤ 0.5	1	2	4	8	16	> 16
Citrobacter spp.[b]	23	6	0	6	3	5	1	2
E. cloacae	99	15	14	12	17	14	5	22
Enterobacter spp.[c]	19	12	1	1	3	1	1	0
H. alvei	7	2	0	1	1	0	0	3
M. morganii	6	2	1	0	0	0	1	2
P. stuartii	1	0	0	1	0	0	0	0
S. marcescens	5	0	0	0	0	1	0	4
Total[d]	160	37	16	21	24	21	8	33

[a]Modified from [55,56] for strains from the USA, Australia, France, Germany, Italy and the UK.
[b]Includes *Citrobacter freundii* (20 strains) and *Citrobacter* spp. (three strains, not speciated).
[c]Includes *Enterobacter aerogenes* (15 strains) and *Enterobacter* spp. (four strains, not speciated).
[d]74.4% of tested strains were susceptible (≤ 8 mg/L).

Table 8 Eradication rates for cefpirome used against infections caused by *Enterobacter* spp. [59,60]

	No. eradicated/No. treated		
Study (year)	1 g bid	2 g bid	All cases
Carbon et al. (1992)	2/2	1/1	3/3
Study group (1992)	15/15[a]	–	15/15[a]

[a]Comparator (ceftazidime) eradication rate = 70%.

ROLE OF ALTERNATIVE AGENTS

A number of alternative agents are available for the treatment of serious Gram-negative infections, although these too have resistance problems. Indeed, strains resistant to third-generation cephalosporins show a higher rate of resistance to other antibiotics of unrelated classes, such as amikacin, gentamicin and ciprofloxacin [Privitera, personal communication] (Table 9). Amongst the β-lactam antimicrobials, the carbapenems (imipenem, meropenem) have the broadest antimicrobial spectrum. Imipenem readily enters the periplasmic space of *Enterobacter* spp. via a different porin channel to that used by cephalosporins and inhibits the PBPs; it is also highly β-lactamase stable. However, clinical isolates of *Enterobacter* spp. and *P. aeruginosa* that are resistant to imipenem have been isolated recently [65]. In the USA, resistance to imipenem among Enterobacteriaceae (*Proteus* spp.) varied from 1–46%, depending on the species [66]. However, these figures also include false-positive results from some commercial systems (Vitek), emphasizing the need for in vitro monitoring methods using reference standards [3,66].

Aminoglycoside resistance continues to be a problem in the treatment of nosocomial infections. Modest increases in aminoglycoside resistance over time have occurred, even with acceptable infection control practices and therapeutic drug level monitoring. Current resistance problems with aminoglycosides include resistance mediated by reduced drug uptake in Enterobacteriaceae and *Pseudomonas* spp. and plasmid-mediated modifying enzymes (often multiple) in Enterobacteriaceae, *Pseudomonas* spp. and Gram-positive species.

Most parenteral fluoroquinolones are characterized by their broad-spectrum activity, although recent years have seen the emergence of resistant strains. Current resistance problems associated with the fluoroquinolones include resistance among methicillin-resistant *Staphylococcus aureus* (MRSA). Future problems which may become more common include resistance among *Pseudomonas* spp. and Enterobacteriaceae attributed to altered DNA topoisomerases or modified drug permeability. Ciprofloxacin resistance has been reported in *C. freundii* (9.9%), *S. marcescens* (6.8%) and *P. aeruginosa* (14.9%) in the USA in 1993–1994 [3,66] and in other countries [64].

CONCLUSIONS

Emerging resistance among Enterobacteriaceae will continue to compromize therapy with existing third-generation cephalosporins. The fourth-generation cephalosporins penetrate the bacterial outer-membrane more rapidly, have greater β-lactamase stability and, therefore, have a broader antimicrobial spectrum and higher intrinsic activity than third-generation agents. These features will sustain the class therapeutic efficacy against strains involved in serious infections in hospitalized patients.

Fourth-generation cephalosporins are active against the majority of *P. aeruginosa* and could be used as an alternative to ceftazidime as the cephalosporin of choice in combination regimens for such infections. Cefpirome and some other fourth-generation compounds have potent activity against oxacillin-susceptible staphylococci [65] and the majority of penicillin and multidrug-resistant streptococci [67,68]. Despite the improved activity and spectrum of cefpirome, it is likely that co-drugs will continue to be necessary for maximal empiric therapy of serious nosocomial infections including bacteremia, pneumonia and mixed anaerobic infections such as those in surgery patients.

Other factors, for example less frequent dosing, safety, cost and favorable interactions with other drugs (i.e. synergistic killing) will also be important factors in selecting alternative agents to complement or replace third-generation cephalosporins or other β-lactams in the treatment of infections caused by strains producing Bush Group 1 enzymes (inducible or derepressed expression).

Table 9 Association of resistance to other antimicrobial classes among 252 strains of Enterobacteriaceae having resistance to third-generation cephalosporins (Italy, 1995)

Organism	(No. tested)	% Resistance[a]		
		Amikacin	Gentamicin	Ciprofloxacin
C. freundii	(44)	13.6	31.8	28.6
E. aerogenes	(71)	24.3	15.5	48.5
E. cloacae	(100)	3.1	29.0	25.6
S. marcescens	(37)	13.9	63.9	55.2

[a]Susceptibility interpretation criteria published by the NCCLS (1995).

DISCUSSION

Prof. B. Weidemann: There may be differences in the induction potential within the cephamycin group of cephalosporins and possibly among the carbapenems, for instance, imipenem has a greater induction potential than meropenem.

Prof. F. Baquero: It remains unclear whether differences in the induction potential between strains of a particular species are important. For cefpirome, the low induction can be partially explained by the rapid bactericidal activity, as both cefoxitin and cefpirome are equally effective against the cell wall. The inducer is produced at the same rate for both cephalosporins, therefore, the observed differences are related to the relative speeds of killing; rather than differences in induction potential.

Prof. B. Wiedemann: Differences in the induction potential of the drugs are related to binding to PBP 5. The stronger the binding to PBP 5, then the more intense the induction.

Prof. K. Klugman: The increasing worldwide importance of the extended-spectrum β-lactamases (ESBLs), should not be overlooked, particularly regarding the impact on MIC values.

Prof. R. Jones: Yes I agree, the overall pattern of emerging resistance in *E. coli*, or *Klebsiella* spp. is going to mimic the pattern among stably-derepressed β-lactamase-producing *Enterobacter* or *Citrobacter* to the clinical microbiologist. Would Dr Bauernfeind address this issue?

Dr. A. Bauernfeind: To be more specific, the incidence of AmpC genes on plasmids is increasing world-wide. However, one advantage of the fourth-generation cephalosporins is that they retain good in vitro activity against *ampC* plasmid containing strains.

Prof. F. Baquero: The activity against plasmid mediated *ampC* producing Enterobacteriaceae is a potential advantage for the fourth-generation cephalosporins.

Prof. R. Jones: The number of strains with ESBL phenotypes is becoming alarmingly high in the USA. Dr Pfaller, do you have any comment on this?

Dr. M. Pfaller: Recent data demonstrate that ≥ 40% of *Klebsiella* spp. in individual institutions are ESBL-producing strains. Not all these strains are the result of an outbreak of a single clone, and the percentage varies from one institution to another and between strains in the same medical center. There is considerable variation in the incidence of ESBLs and the incidence should be closely monitored.

Prof. R. Jones: In hospitals with a high incidence of ESBL phenotypes, approximately 50% of strains are cefoxitin-resistant, often carrying multiple resistance phenotypes. This appears to be due to mobilization of the *ampC* gene into *K. pneumoniae*. Approximately 17% of current bacteremias in a large hospital sample (60 medical centers) in the USA, due to *K. pneumoniae*, are ESBL or *ampC* phenotypes.

Prof. F. Baquero: In the study by Dr. E. Sanders, the emergence of *ampC* mutants were not detected following a 1 g bid dose of cefepime. In an analysis of the ceftazidime-resistant strains a trimodal MIC distribution was observed for cefepime; one peak was at 0.5 mg/L, one at about 4 mg/L and one at ≥ 8 mg/L. These strains may also exhibit increased MIC values the carbapenems.

Prof. R. Jones: Examination of the susceptibility testing data demonstrates that the usual cefepime MIC was in the 'first mode' (previously mentioned). All the fourth-generation cephalosporins tested against these ceftazidime-resistant strains exhibit a trimodal effect, although there is variation of the MIC values of particular agents.

Prof. J. Turnidge: The main problem with the emergence of resistance is with *Enterobacter cloacae*, which is the most prevalent of pathogens and also seems to have the highest propensity for the development of resistance.

References

1. Brown EH, Spencer RC, Brown JMC. The emergence of bacterial resistance in hospitals: A need for continuous surveillance. J Hosp Infect 1990; 125 (suppl A): 35–9.
2. Ballow CH, Schentag JJ. Trends in antimicrobial utilization and bacterial resistance: Report of the National Resistance Surveillance Group. Diagn Microbiol Infect Dis 1992; 15 (suppl): 37S–42S.
3. Jones RN, Kehrberg EN, Erwin ME, Anderson SC, and the Fluoroquinolone Resistance Surveillance Group. Prevalence of important pathogens and antimicrobial activity of parenteral drugs at numerous medical centers in the United States. Study on the threat of emerging resistances: Real or perceived? Diagn Microbiol Infect Dis 1994; 19: 203–15.
4. Livermore DM. β-lactamases in laboratory and clinical resistance. Clin Microbiol Rev 1995; 8: 557–84.
5. Chanal C, Sirot D, Romaszko JP, Bret L, Sirot J. Survey of extended β-lactamases among Enterobacteriaceae. J Antimicrob Chemother 1996; 38: 127–32.
6. Philippon A, Arlet G, Lagrange PH. Origin and impact of plasmid-mediated extended-spectrum β-lactamases. Eur J Clin Microbiol Infect Dis 1994; 13: 17–29.
7. Sanders CC. Inducible β-lactamase and non-hydrolytic resistance mechanisms. J Antimicrob Chemother 1984; 13: 1–3.
8. Sanders CC, Sanders WE Jr. Clinical and epidemiological implications for use of newer cephalosporins. Rev Infect Dis 1988; 10: 830–6.
9. Stapleton P, Shannon K, Phillips I. The ability of β-lactam

antimicrobials to select mutants with derepressed β-lactamase synthesis from *Citrobacter freundii*. J Antimicrob Chemother 1995; 36: 483–96.

10. Richmond MH, Sykes RB. The β-lactamases of Gram-negative bacteria and their possible physiological role. Adv Microbiol Physiol 1973; 9: 31–88.
11. Sykes RB, Matthew M. The β-lactamases of Gram-negative bacteria and their role in resistance to β-lactam antibiotics. J Antimicrob Chemother 1976; 2: 115–57.
12. Bush K. Characterization of β-lactamases: Groups 1, 2a, 2b and 2c. Antimicrob Agents Chemother 1989; 33: 264–70.
13. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 1995; 39: 1211–33.
14. Ambler RP. The structure of β-lactamases. Philos Trans R Soc London Ser B 1980; 289: 321–31.
15. Gallagher PG. *Enterobacter* bacteremia in pediatric patients. Rev Infect Dis 1990; 12: 808–12.
16. Jones RN, Pfaller MA, Marshall S, Erwin M, Edmund M, Wenzel R. Nosocomial enterococcal blood stream infections in the SCOPE Survey: Drug resistance, species occurrence and identification accuracy (abstract 168). In: Abstracts of the IDSA 34th Annual Meeting, New Orleans, 1996.
17. Jarvis WR, Martone WJ. Predominant pathogens in hospital infections. J Antimicrob Chemother 1992; 29 (suppl A): 19–24.
18. Verbist L. Epidemiology and sensitivity of 8625 ICU and haematology/oncology bacterial isolates in Europe. Scand J Infect Dis 1993; 91(suppl): 14–24.
19. Lindquist S, Lindberg F, Normark S. Binding of the *Citrobacter freundii* AmpR regulator to a signal DNA site provides both autoregulation and activation of the inducible ampC β-lactamase gene. J Bacteriol 1989; 171: 3746–53.
20. Livermore DM. Clinical significance of beta-lactamase induction and stable derepression on Gram-negative rods. Eur J Clinical Microbiol 1987; 6: 439–45.
21. Peter K, Korfmann G, Wiedeman B. Impact of the ampD Gene and its Product on β-lactamase Production in *Enterobacter cloacae*. Reviews of Infectious Diseases 1988; 10: 800–5.
22. Normark S, Bartowsky E, Erickson J, et al. Bacterial Cell Wall. In Ghuysen J-M, Hakenbeck R, eds. Amsterdam: Elsevier Science BV, 1994: 485–503.
23. Jacobs C, Joris B, Jamin M, et al. AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase. Mol Microbiol 1995; 15: 553–9.
24. Höltje J, Kopp V, Ursinus A, Wiedeman B. The negative regulator of β-lactamase induction AmpD is a N-acetyl-anhydromuramyl-L-alanine amidase. FEMS Microbiol Lett 1994; 122: 159–64.
25. Jacobs C, Huang L, Bartowsky E, Normark S, Park JT. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J 1994; 13: 4684–94.
26. Korfmann G, Sanders CC. *AmpG* is essential for high-level expression of AmpC β-lactamase in *Enterobacter cloacae*. Antimicrob Agents Chemother 1989; 33: 1946–51.
27. Lindqvist S, Weston-Hafer K, Schmidt H, et al. AmpG, a signal transducer in chromosomal β-lactamase induction. Mol Microbiol 1993; 9: 703–15.
28. Bennett PM, Chopra I. Molecular basis of β-lactamase induction in bacteria. Antimicrob Agents Chemother 1993; 37: 153–8.
29. Dietz H, Pfeifle D, Wiedeman B. Location of N-acetyl-anhydromuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid, the presumed signal molecule for β-lactamase induction, in the bacterial cell. Antimicrob Agents Chemother 1996; 40: 2173–7.
30. Dietz H, Wiedeman B. The role of N-acetylglucosaminyl-1,6 anhydro N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid-D-alanine for the induction of β-lactamase in *Enterobacter cloacae*. Zbl Bakt 1996; 284: 207–17.
31. Sanders CC, Sanders WE. Type 1 β-lactamases of Gram-negative bacteria: Interactions with β-lactam antibiotics. J Infect Dis 1986; 154: 792–800.
32. Jones RN, Thornsberry C. Cefotaxime: A review of *in vitro* antimicrobial properties and spectrum of activity. Rev Infect Dis 1982; 4 (suppl): S300–15.
33. Follath F, Costa E, Thommen A, Frie R, Burdeska A, Meyer J. Clinical consequences of development of resistance to third-generation cephalosporins. Eur J Clin Microbiol 1987; 6: 446–50.
34. Johnson MP, Ramphol R. β-lactam-resistant *Enterobacter* bacteremia in febrile neutropenic patients receiving monotherapy. J Infect Dis 1990; 162: 981–3.
35. Burwen DR, Banerjee SN, Gaynes RP and the National Nosocomial Infections Surveillance System. Ceftazidime resistance among selected nosocomial Gram-negative bacilli in the United States. J Infect Dis 1994; 170: 1622–5.
36. Jones RN. The antimicrobial activity of cefotaxime: Comparative multinational hospital isolate surveys covering 15 years. Infection 1994; 22 (suppl 3): S152–60.
37. Murray PR, Cantrell HF, Lankford RB. Multicenter evaluation of the *in vitro* activity of piperacillin/tazobactam compared with 11 selected β-lactam antimicrobials and ciprofloxacin against more than 42,000 Gram-positive and Gram-negative bacteria. Diagn Microbiol Infect Dis 1994; 19: 111–20.
38. Baron EJ, Jones RN. National Survey of the *in vitro* spectrum of piperacillin-tazobactam tested against more than 40,000 aerobic clinical isolates from 236 medical centers. Diagn Microbiol Infect Dis 1995; 21: 141–51.
39. Marshall SA, Aldridge KE, Allen SD, Fuchs PC, Gerlach EH, Jones RN. Comparative antimicrobial activity of piperacillin-tazobactam tested against more than 5000 recent clinical isolates from five medical centers. A reevaluation after five years. Diagn Microbiol Infect Dis 1995; 21: 153–68.
40. Stratton CW, Ratner H, Johnston PE, Schaffner W. Focused microbiologic surveillance by specific hospital unit as a sensitive means of defining antimicrobial resistance problems. Diagn Microbiol Infect Dis 1992; 15: S11–8.
41. Fish DN, Piscitelli SC, Danziger LH. Pharmacy Practice Insights: Development of resistance during antimicrobial

therapy: A review of antibiotic classes and patient characteristics in 173 studies. Pharmacother 1995; 13: 279–91.
42. Minami S, Yotsugi A, Inoue H, Mitsuhashi S. Induction of β-lactamase by various β-lactam antimicrobials in *Enterobacter cloacae*. Antimicrob Agents Chemother 1980; 18: 382–5.
43. Farmer TH, Reading C. Induction of the β-lactamases of a strain of *Pseudomonas aeruginosa, Morganella morganii* and *Enterobacter cloacae*. J Antimicrob Chemother 1987; 19: 401–8.
44. Curtis NAC, Eisenstadt RL, Rudd C, White AJ. Inducible Type 1 β-lactamases of Gram-negative bacteria and resistance to β-lactam antibiotics. J Antimicrob Chemother 1986; 17: 51–61.
45. Sanders CC, Sanders WE Jr. Microbial resistance to newer generation β-lactam antimicrobials: Clinical and laboratory implications. J Infect Dis 1985; 151: 399–406.
46. Fung-Tomc JC, Gradelski E, Huczko E, Dougherty TJ, Kessler RE, Bonner DP. Differences in the resistant variants of *Enterobacter cloacae* selected by extended-spectrum cephalosporins. Antimicrob Agents Chemother 1996; 40: 1289–93.
47. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7–A3. National Committee for Clinical Laboratory Standards 1993; Wayne PA.
48. Phillips I, Shannon K. Importance of Beta-lactamase Induction. Eur J Clin Microbiol Infect Dis 1993; (suppl 1): 19–26.
49. Hancock REW, Bellido F. Factors involved in the enhanced efficacy against Gram-negative bacteria of fourth-generation cephalosporins. J Antimicrob Chemother 1992; 29 (suppl A): 1–6.
50. Bryskier A, Aszodi J, Chantot JF. Parenteral cephalosporin classification. Expt Opin Invest Drugs 1994; 3: 145–71.
51. Hancock REW, Bellido F. Antibacterial *in vitro* activity of fourth-generation cephalosporins. J Chemother 1996; 8: 31–6.
52. Pechere J-C, Wilson W, Neu H. Laboratory assessment of antibacterial activity of zwitterionic 7-methoxyimino cephaloporins. J Antimicrob Chemother 1995; 36: 757–71.
53. Wiseman LR, Bryson HM. Cefpirome: A review of its antibacterial activity, pharmacokinetic properties and clinical efficacy in the treatment of severe nosocomial infections and febrile neutropenia. Drugs 1996; 52: (in press).
54. Jones RN, Fuchs PC. Activity of cefepime (BMY-28142) and cefpirome (HR 810) against Gram-negative bacilli resistant to cefotaxime or ceftazidime. J Antimicrob Chemother 1989; 23: 163–5.
55. Sader HS, Jones RN. The fourth generation cephalosporins: Antimicrobial activity and spectrum definitions using cefpirome as an example. Antimicrob Nwsltr 1993; 9: 9–16.
56. Sader HS, Jones RN. *In vitro* antimicrobial activity of cefpirome against ceftazidime-resistant isolates from two multicenter studies. Eur J Clin Microbiol Infect Dis 1994; 13: 675–9.
57. Sanchez ML, Jones RN. Antimicrobial activity of FK-037 against class 1 β-lactamase producing species resistant to ceftazidime: A multi-laboratory clinical isolate sample. J Antimicrob Chemother 1993; 32: 654–6.
58. Jones RN, Marshall SA. Antimicrobial activity of cefepime tested against Bush group 1 β-lactamase-producing strains resistant to ceftazidime. A multilaboratory national and international clinical isolate study. Diagn Microbiol Infect Dis 1994; 19: 33–8.
59. Carbon C and the Cefpirome Study Group. Prospective randomised phase II study of intravenous cefpirome 1g or 2g in the treatment of hospitalised patients with different infections. J Antimicrob Chemother 1992; 29 (suppl A): 87–94.
60. Study Group. Cefpirome versus ceftazidime in the treatment of urinary tract infections. J Antimicrob Chemother 1992; 29 (suppl A): 95–104.
61. Sanders WE, Tenney JH, Kessler RE. Efficacy of cefepime in the treatment of infections due to multiply resistant *Enterobacter* species. Clin Infect Dis 1996; 23: 454–61.
62. Norrby SR. Cefpirome: Efficacy in the treatment of urinary and respiratory tract infections and safety profile. Scand J Infect Dis 1993; 91 (suppl): 41–50.
63. Giamarellou H. Clinical experience with fourth-generation cephalosporins. J Chemother 1996; 8 (suppl 2): 91–104.
64. Wolff M, Carbon C, Falissard B, Geddes A, Verhoef J, Wilson W. Comparative study of cefpirome and ceftazidime in ICU patients with pneumonia. (Abstract), 36th ICAAC, 1996.
65. Reeves DS, Bywater MJ, Holt HA. The activity of cefpirome and ten other antibacterial agents against 2858 clinical isolates collected from 20 centres. J Antimicrob Chemother 1993; 31: 345–62.
66. Jones RN. Impact of Changing Pathogens and Antimicrobial Susceptibility Patterns in the Treatment of Serious Infections in hospitalized Patients. Am J Med 1996; 100: 6A3S–6A12S.
67. Frèmaux A, Sissia G, Geslin P. *In vitro* antibacterial activity of cefotaxime, cefpirome and four other beta-lactam antibiotics against penicillin-resistant *Streptococcus pneumoniae*. Poster, 6th Int Cong Infect Dis, Prague 1994; PCS 26.
68. Klugman K, Goldstein F, Kohno S, Baquero F. The role of fourth-generation cephalsporins in the treatment of penicillin-resistant streptococcal infections [This supplement].

β-ラクタマーゼの酵素阻害剤によるクラス鑑別法の検討

澤井　哲夫・小原　康治・大沼　雅江・中村　昭夫・内藤　泰代・仲澤今日子
千葉大学薬学部微生物薬品化学研究室*

【原著・基礎】

β-ラクタマーゼの酵素阻害剤によるクラス鑑別法の検討

澤井　哲夫・小原　康治・大沼　雅江・中村　昭夫・内藤　泰代・仲澤今日子

千葉大学薬学部微生物薬品化学研究室*

(平成11年9月13日受付・平成12年1月12日受理)

臨床分離菌の生産するβ-ラクタマーゼのクラスA, B, CおよびDへのクラス鑑別を，3種のβ-ラクタマーゼ阻害剤を利用して簡便，迅速に行う方法を考案した。阻害剤としてクラブラン酸，カルバペネム誘導体（J-110, 441）およびモノバクタム誘導体（Syn-2161）を用いた。J-110,441およびSyn-2161はそれぞれ，クラスBβ-ラクタマーゼおよびクラスCβ-ラクタマーゼに対する新阻害物質である。アンピシリン抗菌活性（MIC）への阻害剤併用効果から，被験菌の生産するβ-ラクタマーゼのクラス鑑別を行った。生産するβ-ラクタマーゼ活性とそのクラスの明らかな7菌種，計14菌株を被験菌として本鑑別法を適用し，クラスA/D, クラスB, クラスCの各生産菌をMICパターンから鑑別できた。MIC測定は一般的な微量液体希釈法を用いたが，ATP-bioluminescence法（Hattori N., et al. Antimicrob. Agents Chemother., 42: 1406～1411, 1998）を適用すると，鑑別時間は5分の1以下に短縮でき，同一の判定結果が得られた。

Key words: β-lactamase class, β-lactam-resistance, β-lactamase inhibitors, rapid MIC assay

β-ラクタマーゼは多くの病原細菌のβ-ラクタム薬耐性の原因となる薬剤不活化酵素群である[1]。酵素タンパク質アミノ酸配列の相同性から，クラスA～Dの4クラスに分類される[2,3]。このクラス分けはβ-ラクタマーゼ分類法として現在広く用いられ，またもっとも客観的な分類法である。クラスA, C, Dは活性中心にセリンを持つセリンβ-ラクタマーゼに属し，クラスBは活性中心に亜鉛を持つメタロβ-ラクタマーゼであり，セリンβ-ラクタマーゼとは異なる加水分解酵素である。基質特異性では，クラスAはペニシリナーゼ，クラスCはセファロスポリナーゼ，クラスDはオキサシリン分解型ペニシリナーゼ，にそれぞれ相当する。クラスBのメタロβ-ラクタマーゼはカルバペネム薬を含む広範な基質特異性を持つβ-ラクタマーゼである。これらに加え近年，クラスAおよびCではオキシイミノ系β-ラクタム薬などの難分解性β-ラクタム薬へ適応した変異酵素（基質特異性拡張型β-ラクタマーゼ）が出現している[4,5]。特にクラスA変異酵素はESBL（Extended Spectrum Beta-Lactamase）の名称で知られる。

細菌感染症に対する適切なβ-ラクタム薬選択には，起因菌の生産するβ-ラクタマーゼの酵素化学的特性の情報が必要であり，臨床分離株の生産するβ-ラクタマーゼの簡便なクラス鑑別法が望まれる。本研究では，クラスAβ-ラクタマーゼの阻害剤として知られるクラブラン酸（CVA），新たに開発されたクラスBβ-ラクタマーゼ阻害剤（J-110,441）とクラスCβ-ラクタマーゼ阻害剤（Syn-2161）の3阻害剤を用い，アンピシリン（ABPC）のMICにおよぼす阻害剤併用効果からβ-ラクタマーゼのクラス鑑別を試みた。さらに，細菌細胞内ATP量を指標としてMICを測定するATP-bioluminescence法[6]を適用することにより，鑑別時間を大幅に短縮することを行った。

I. 材料と方法

1. 菌株とRプラスミド

Table 1に示した7菌種，計16菌株およびRプラスミド宿主として*Escherichia coli* ML 1410を使用した。*E. coli* ML 1410は*E. coli* K 12由来のナリジクス酸耐性変異株である[7]。5種のプラスミド（RGN 823, RGN 14, RDK 4, RGN 238, pMTY 010）はそれぞれTEM-IIβ-ラクタマーゼ，TEM-Iβ-ラクタマーゼ，メタロβ-ラクタマーゼ，OXA-1β-ラクタマーゼ，基質特異性拡張型クラスAβ-ラクタマーゼ（Toho-1 ESBL），の酵素遺伝子を持つ。*Citrobacter freundii* GN 346/RGN 823は染色体性クラスCβ-ラクタマーゼ生産菌にRGN 823を導入して作成した2種β-ラクタマーゼ生産株である。その他の菌株は染色体性β-ラクタマーゼ生産株である。

2. β-ラクタム薬とβ-ラクタマーゼ阻害剤

ABPCは明治製菓，CVAはスミスクライン・ビーチャム製薬，カルバペネム誘導体（J-110,441）は萬有製薬，モノバクタム誘導体（Syn-2161）は大鵬薬品工業よりそれぞれ分与された原末を使用した。

3. 最小発育阻止濃度（MIC）の測定

MICの測定は日本化学療法学会の規定に準じ，微量液体希釈法[15]により行いμg/mLで表した。菌体内ATPの定量にもとづくATP-bioluminescence法によるMIC

*千葉県千葉市稲毛区弥生町1-33

Table 1. List of the β-lactamase-producing bacteria employed

β-lactamase	Strain	Ref.
Class A	*E. coli* ML 1410/RGN 823	Sawai et al.[8,9]
	E. coli ML 1410/RGN 14	Sawai et al.[9]
	K. pneumoniae GN 69	Sawai et al.[9]
	P. mirabilis N-29	Sawai et al.[8,9]
	P. vulgaris GN 76/C 1	Sawai et al.[8,9]
Class B	*E. coli* ML 1410/RDK 4	Yamaguchi et al.[10]
Class C	*E. coli* 255	Sawai et al.[8,9]
	C. freundii GN 346	Sawai et al.[8,9]
	E. cloacae P 99	Galleni et al.[11]
	E. cloacae 363	Sawai et al.[8,9]
	M. morganii 1510	Sawai et al.[8,9]
Class D	*E. coli* ML 1410/RGN 238	Sawai et al.[9]
Extended-spectrum Class A (Toho-1, ESBL)	*E. coli* ML 1410/pMTY 010	Ishii et al.[12]
Extended-spectrum Class C	*E. cloacae* GC 1	Nukaga et al.[13]
	C. freundii GC 3	Haruta et al.[14]
Production of two kinds of β-lactamases (class A and C)	*C. freundii* GN 346/RGN 823	This paper

測定はHattoriらの改良法[6]を用いた。

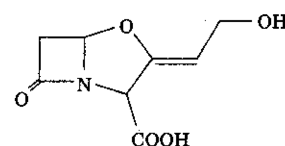
II. 結　果

1. β-ラクタマーゼ阻害剤の性質

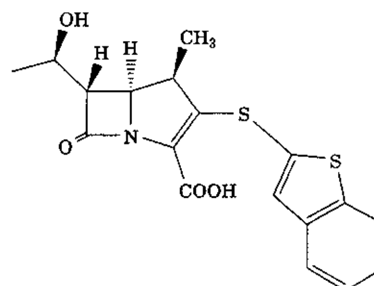
CVAはクラスAβ-ラクタマーゼの阻害剤として利用されている。その酵素阻害様式は安定な酵素—阻害剤の共有結合体形成による progressive inactivation（漸進的不活化）である[16]。J-110, 441[17]はクラスBβ-ラクタマーゼ，Syn-2161[18]はクラスCβ-ラクタマーゼに対するそれぞれ特異的な新阻害剤である。これら3種のβ-ラクタマーゼ阻害剤の化学構造をFig. 1に示す。カルバペネム誘導体であるJ-110, 441は，基質アナローグとしてクラスBβ-ラクタマーゼに対し拮抗阻害剤として作用する[17]。アズトレオナムなどのモノバクタム薬は強い抗菌力と共に，クラスCβ-ラクタマーゼの強力な阻害剤として作用し，その阻害様式はprogressive inactivationである[19]。モノバクタム薬の抗菌力を著しく低め，阻害剤としての性質を残した誘導体がSyn-2161である。本研究の使用菌株に対する3阻害剤のMIC値は50 μg/mLまたはそれ以上であり，阻害剤としての使用濃度はMIC値の5分の1以下を用いた。

2. ABPCとの併用による阻害剤最適濃度の設定

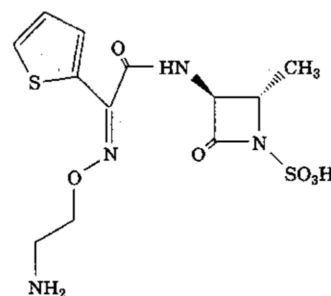
阻害剤と併用するβ-ラクタム薬としては，クラスA～Dβ-ラクタマーゼのいずれの生産によってもそのMIC値が大きく影響を受けるものが望ましい。また，臨床分離菌の薬剤感受性測定で一般的に使用される薬剤であることが実用的である。以上の要件を考慮し，ABPCを併用薬とした。微量液体希釈法による阻害剤効果測定では，阻害剤濃度を1濃度に固定することが実用的である。使用菌株より代表的な8菌株を選択し，各阻害剤濃度でのABPCのMICを測定し，効果的な阻害剤濃



Clavulanic acid (CVA)



J-110,441



Syn-2161

Fig. 1. Structures of the inhibitors used in this study.

度を定めた。Table 2 に Syn-2161 の測定例を示した。この場合，Syn-2161 濃度は 20 μg/mL を採用した。同様に CVA と J-110,441 の使用濃度を実験的に検討し，それぞれ 10 μg/mL とした。

3. β-ラクタマーゼ生産菌に対する阻害剤併用効果

クラスA～D の生産菌およびβ-ラクタム薬感受性 *E. coli* に対して，各阻害剤一定濃度下での ABPC の MIC を測定した (Table 3)。これら菌株または R プラスミドの β-ラクタマーゼの酵素化学的性質はすでに解析されている。クラス A 生産菌に対して，CVA はすべてに阻害剤効果を示し，Syn-2161 ではまったく観察されなかった。J-110,441 では *Klebsiella pneumoniae* および *Proteus vulgaris* の染色体性クラス Aβ-ラクタマーゼ生産菌で阻害剤効果が見られるが，その他のプラスミド性β-ラクタマーゼ生産菌では観察されない。クラス B 生産菌では J-110,441 により明瞭な阻害剤効果が観察された。クラス C 生産菌はすべて Syn-2161 による顕著な阻害剤効果が観察された。クラス Cβ-ラクタマーゼはすべて染色体性酵素であるが，*Morganella morganii* のみが J-110,441 の影響を受けた。クラス Dβ-ラクタマーゼは典型的なペニシリナーゼ型基質特異性を持つプラスミド性酵素であり，阻害剤効果はプラスミド性クラス A 酵素生産菌と差異がみられなかった。

Toho-1 はわが国でグラム陰性菌に広く分布するプラスミド性クラス A の ESBL である。主に染色体性であるクラス C では著者らの分離した *Enterobacter cloacae* GC 1 と *C. freundii* GC 3 の β-ラクタマーゼが基質特異性拡張型の変異酵素である。一方，臨床分離の高度耐性菌では複数の β-ラクタマーゼ遺伝子を持つものが少なくない。*C. freundii* GN 346/RGN 823 は染色体性クラス C とプラスミド性クラス A をそれぞれ構成的に生産する 2 種 β-ラクタマーゼ生産の高度耐性菌のモデル菌株である。Table 4 に示すように，クラス A の ESBL では CVA の阻害剤効果がみられるのに対し，クラス C の基質特異性拡張型変異酵素を生産する *E. cloacae* GC 1，*C. freundii* GC 3 とクラス A/C 生産株の *C. freundii* GN 346/RGN 823 では CVA に弱い感受性を示す以外は阻害剤に不感受性を示した。

それぞれ特徴のある β-ラクタマーゼ生産型を持つ菌株に対する 3 阻害剤の効果の総括を Table 5 に示した。

Table 2. Combined effect of Syn-2161 on MIC of ampicillin in bacteria producing β-lactamases

β-Lactamase class	Strain	MIC of ampicillin (μg/mL)				
	Syn-2161 (μg/mL)	0	5	10	20	50
non	*E. coli* ML 1410	6.3	3.1	3.1	3.1	<1.6
A	*E. coli* ML 1410/RGN 823	>1,600	>1,600	>1,600	>1,600	>1,600
B	*E. coli* ML 1410/RDK 4	25	25	25	25	25
C	*C. freundii* GN 346	800	25	12.5	1.6	<1.6
C	*E. cloacae* P 99	>1,600	100	25	12.5	3.1
D	*E. coli* ML 1410/RGN 238	800	800	800	800	800
ES-C*	*E. cloacae* GC 1	>1,600	>1,600	>1,600	>1,600	800
ES-C*	*C. freundii* GC 3	>1,600	>1,600	>1,600	>1,600	800

*ES-C: Extended-spectrum class C β-lactamase.

Table 3. Effect of specific inhibitors on MIC of ampicillin in bacteria producing four class β-lactamases

β-Lactamase class	Strain	MIC of ampicillin (μg/mL)			
		0	CVA	Syn-2161	J-110,441
non	*E. coli* ML 1410	6.3	3.1	3.1	3.1
A	*E. coli* ML 1410/RGN 823	>800	12.5	>800	>800
	E. coli ML 1410/RGN 14	>800	12.5	>800	>800
	K. pneumoniae GN 69	>800	1.6	>800	100
	P. mirabilis N-29	>800	12.5	>800	>800
	P. vulgaris GN 76/C 1	800	1.6	800	6.3
B	*E. coli* ML 1410/RDK 4	25	12.5	25	3.1
C	*E. coli* 255	400	400	6.3	400
	C. freundii GN 346	800	200	1.6	400
	E. cloacae P 99	>800	800	12.5	800
	E. cloacae 363	800	400	3.1	400
	M. morganii 1510	800	400	1.6	6.3
D	*E. coli* ML 1410/RGN 238	800	25	800	800

The inhibitor concentrations were fixed as follows: CVA; 10 μg/mL, Syn-2161; 20 μg/mL, J-110,441; 10 μg/mL.
CVA: clavulanic acid

Table 4. Effect of the specific inhibitors on MIC of ampicillin in bacteria producing extended-spectrum β-lactamases or two kinds of β-lactamases

β-Lactamase	Strain	MIC of ampicillin (μg/mL)			
		0	CVA	Syn-2161	J-110,441
Toho-1 ESBL	*E. coli* ML 1410/pMTY 010	>800	6.3	>800	>800
Extended-spectrum class C	*E. cloacae* GC 1	>800	800	>800	>800
	C. freundii GC 3	>800	800	>800	>800
Class A/C simultaneous production	*C. freundii* GN 346/RGN 823	>800	400	>800	>800

The inhibitor concentrations were fixed as follows: CVA; 10 μg mL, Syn-2161; 20 μg mL, J-110,441; 10 μg mL.
CVA: clavulanic acid

Table 5. Summary of the inhibitory effect on MIC of ampicillin in β-lactamase-producing bacteria

β-Lactamase production	Inhibitor effect*		
	CVA	Syn-2161	J-110,441
Class A	++	−	−(v)**
Class B	+	−	++
Class C	+	++	−(v)**
Class D	++	−	−
Toho-1 ESBL	++	−	−
Extended-spectrum Class C	+	−	−
Class A/C production	+	−	−

*The degree of effect was expressed as follows; significant effect (++), weak effect (+), no effect (−).
**varied according to the species.
CVA: clavulanic acid

ABPCのMIC値が8分の1以下に低下する顕著な阻害剤効果を++で表示し，MIC値低下が2分の1〜4分の1程度の弱い効果を+と表示した。なお，同一クラスのβ-ラクタマーゼの中のごく一部のものが阻害剤感受性を示す場合は −(v) と表している。J-110,441ではクラスAおよびクラスC生産菌の一部菌種で阻害効果が見られるが，クラスAあるいはD，クラスB，クラスC，基質特異性拡張型クラスCあるいはクラスA・C複合生産の分類が可能である。

4. ATP-bioluminescence法によるMIC判定の迅速化

微量液体希釈法はMIC測定法として汎用されるが，結果の判定には約18時間を要する。クラス鑑別の迅速化の方策として，ATP-bioluminescence法によるMIC測定を行い，微量液体希釈法と比較した(Table 6)。ATP-bioluminescence法を用いても微量液体希釈法とほぼ同一の結果が得られ，さらに鑑別所要時間は3.5時間であり，微量液体希釈法の約5分の1に短縮が可能であった。

III. 考　　察

医療現場における臨床分離菌の薬剤感受性試験法として，ディスク法に代わり微量液体希釈法による定量的測定が普及しつつある。自動測定機器の改良によりさらに一般化することと思われる。この測定法を利用して，阻害剤一定濃度下におけるABPCのMIC値から被験菌株の生産するβ-ラクタマーゼの酵素化学的特徴を判定する本鑑別法は，MIC測定作業へ組み入れることにより医療現場での実施が容易と思われる。臨床利用されているβ-ラクタム薬の各クラスβ-ラクタマーゼに対する安定性などについては十分な情報があり，本鑑別法の結果を加えることにより，β-ラクタマーゼ生産菌に対する適切なβ-ラクタム薬選択が可能と思われる。また，特に迅速な鑑別が要求される場合は，ATP-bioluminescence法の利用により鑑別時間を大幅に短縮可能である。

本鑑別法ではクラスAβ-ラクタマーゼ生産菌とクラスDβ-ラクタマーゼ生産菌の区別ができない。Rプラスミドに支配されるクラスDβ-ラクタマーゼはオキサシリン分解型ペニシリナーゼとも呼ばれる。基質特異性からはもっとも典型的なペニシリナーゼ型に属し，クラス分類初期にはクラスAに分類されている。このβ-ラ

Table 6. Comparison of the MIC values measured by ATP-bioluminescence method with those by microdilution method

β-Lactamase class	Strain	MIC of ampicillin (μg/mL)			
		ATP-bioluminescence		microdilution	
		ABPC	ABPC+CVA*	ABPC	ABPC+CVA*
non	*E. coli* ML 1410	3.1	3.1	6.3	3.1
A	*E. coli* ML 1410/RGN 823	>800	12.5	>800	12.5
B	*E. coli* ML 1410/RDK 4	25	12.5	25	12.5
C	*E. cloacae* P 99	>800	800	>800	>800
ES-C	*E. cloacae* GC 1	>800	>800	>800	>800

*The CVA concentration was 10 μg/mL.
ABPC: ampicillin, CVA: clavulanic acid

クタマーゼの酵素化学的特徴から，クラスD酵素生産菌に対するβ-ラクタム薬の適用ではクラスA酵素生産菌と同様に扱ってもよいと考えられる。クラスAβ-ラクタマーゼ生産菌とクラスAのESBL生産菌の区別は本鑑別法ではできなかったが，オキシイミノ系薬剤のMIC値を参照すれば区別は可能である。J-110,441はクラスBβ-ラクタマーゼ阻害剤として用いたが，それぞれクラスAおよびCに属する*P. vulgaris*と*M. morganii*の生産する染色体性β-ラクタマーゼに対して強い阻害効果を示した。*P. vulgaris*のβ-ラクタマーゼはセファロスポリナーゼ型の基質特異性を持ちながら，クラスAに属する特異なβ-ラクタマーゼとして知られる[20]。また*M. morganii*は以前の菌種分類では*Proteus*グループに分類された菌種である。これら菌種の生産するβ-ラクタマーゼがJ-110,441に対して他のクラスAあるいはクラスCβ-ラクタマーゼと異なる挙動を示すことは，酵素活性中心における性質の差異を反映しているものと考えられ興味深い。これらのバリエーションはCVAまたはSyn-2161の明確な阻害パターンから，クラス鑑別の障害にはならないと考えられる。

基質特異性拡張型クラスCβ-ラクタマーゼとクラスA/Cβ-ラクタマーゼ複合生産の菌株では用いた阻害剤への感受性が低く，これら高度耐性菌のより細分化した鑑別はできなかった。これらの耐性菌に対してはより慎重なβ-ラクタム薬の選択あるいは他薬剤の使用が必要となる。新たな阻害剤の採用により，高度耐性菌の分類を試みる予定である。なお，3阻害剤を用いる鑑別法をディスク法に適用して，定性的であるがより簡便なクラス鑑別法の作成は原理的に十分に可能である。この点については現在検討中である。

謝　辞

本研究は平成9年度および10年度厚生省科学研究補助金（新興・再興感染症研究事業）による研究の一環として実施されたものである。β-ラクタマーゼ阻害剤を提供していただいた大鵬薬品工業株式会社（Syn-2161），萬有製薬株式会社（J-110,441）およびスミスクライン・ビーチャム製薬株式会社（クラブラン酸）に感謝致します。またアンピシリンを提供していただいた明治製菓株式会社に御礼申し上げます。さらに，ATP-bioluminescence法の利用について，キッコーマン株式会社のご協力に感謝致します。

文　献

1) 澤井哲夫: β-ラクタマーゼによるβ-ラクタム剤耐性メカニズム。日本臨林 55: 1225～1230, 1997
2) Ambler R P: The structure of β-lactamases. Philos. Trans. R. Soc. Lond. Biol. 289: 321～331, 1980
3) 澤井哲夫: β-ラクタマーゼの新しい分類。検査と技術 21: 1118, 1993
4) Livermore D M: β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. 8: 557～585, 1995
5) 澤井哲夫，額賀路嘉，春田　伸: β-ラクタマーゼとその分子進化。蛋白質 核酸 酵素 40: 1887～1899, 1995
6) Hattori N, Nakajima M, O' Hara K, et al.: Novel antibiotic susceptibility tests by the ATP-bioluminescence method using filamentous cell treatment. Antimicrob. Agents Chemother. 42: 1406～1411, 1998
7) Egawa R, Sawai T, Mitsuhashi S: Drug resistance of enteric bacteria. XII. Unique substrate specificity of penicillinase produced by R factor. Japn. J. Microbiol. 11: 173～178, 1967
8) Sawai T, Kanno M, Tsukamoto K: Characterization of eight β-lactamases of Gram-negative bacteria. J. Bacteriol. 152: 567～571, 1982
9) Sawai T, Yoshida T, Tsukamoto K, et al.: A set of bacterial strains for evaluation of β-lactamase-stability of β-lactam antibiotics. J. Antibiot. 34: 1318～1326, 1981
10) Yamaguchi H, Nukaga M, Sawai T: Appearance of an R plasmid mediated metallo-β-lactamase in gram-negative enteric bacteria. DNA Data Base of Japan (DDBJ) entry name KPNRD 4 and accession no.D 29636. 1994
11) Galleni M, Lindberg F, Normark S, et al.: Sequence and comparative analysis of three *Enterobacter cloacae ampC* β-lactamase genes and their products. Biochem. J. 250: 753～760, 1988
12) Ishii Y, Ohno A, Taguchi H, et al.: Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A β-lactamase isolated from *Escherichia coli*. Antimicrob. Agents Chemother. 39: 2267～2275, 1995
13) Nukaga M, Haruta S, Tanimoto K, et al.: Molecular evolution of a class C β-lactamase extending its substrate specificity. J. Biol. Chem. 270: 5729～5735, 1995
14) 春田　伸，額賀路嘉，澤井哲夫: オキシイミノ系β-ラクタム剤高度耐性菌，*Citrobacter freundii* GC 3の耐性機構の解析。日本薬学会第115年会講演要旨集 3: 210 p. 1995
15) 日本化学療法学会: 微量液体希釈によるMIC測定法（微量液体希釈法）。Chemotherapy 38: 103～104, 1990
16) Sawai T, Yamaguchi A: Mechanism of beta-lactamase inhibition: Differences between sulbactam and other inhibitors. Diagn. Microbiol. Infect. Dis. 12: 121 S～129 S, 1989
17) Nagano R, Adachi Y, Imamura H, et al.: Carbapenem derivatives as potential inhibitors of various β-lactamases including class B metallo-β-lactamases. Antimicrob. Agents Chemother. 43: 2497～2503, 1999
18) Maiti S N, Reddy A V, Micetich R G, et al.: Azetidinone derivatives as β-lactamase inhibitors. PCT International Publication No.WO 99/10324, 1999
19) Sakurai Y, Yoshida Y, Saitoh K, et al.: Characteristics of aztreonam as a substrate, inhibitor and inducer for β-lactamases. J. Antibiot. 43: 403～410, 1990

20) Tamaki M, Nukaga M, Sawai T: Replacement of serine 237 in class A β-lactamase of *Proteus vulgaris* modifies its unique substrate specificity. Biochemistry 33: 10200~10206, 1994

Classification of β-lactamases by utilizing the enzyme inhibitors

Tetsuo Sawai, Koji O'hara, Masae Ohnuma, Akio Nakamura, Yasuyo Naitou and Kyoko Nakazawa

Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Inage-ku, Chiba 263-8522, Japan

A simple and rapid method for classification of β-lactamases in bacterial cells into classes A, B, C and D was devised by utilizing three β-lactamase-inhibitors clavulanic acid, a carbapenem derivative (J-110,441) and a monobactam derivative (Syn-2161). On the basis of the combined effects of ampicillin with the inhibitor on the MIC of ampicillin, the enzyme class in the test organisms was estimated. This method was evaluated according to its application in 14 bacterial strains, which consisted of seven gram-negative species. For routine assay, a liquid microdilution method for MIC determination was employed. However, the time required for assay was reduced to one-fifth by employing an ATP-bioluminescens method developed by Hattori N, et al. (Antimicrob. Agents Chemother., 42: 1406~1411, 1998).

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1999, p. 2063–2065 Vol. 43, No. 8
0066-4804/99/$04.00+0
Copyright © 1999, American Society for Microbiology. All Rights Reserved.

Identification of Functional Amino Acids in the Macrolide 2′-Phosphotransferase II

KAZUO TANIGUCHI, AKIO NAKAMURA, KAZUE TSURUBUCHI, AKI ISHII, KOJI O'HARA,* AND TETSUO SAWAI

Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Inage-ku, Chiba 263-8522, Japan

Received 9 November 1998/Returned for modification 21 March 1999/Accepted 27 May 1999

Macrolide 2′-phosphotransferase [MPH(2′)] transfers the γ phosphate of ATP to the 2′-OH group of macrolide antibiotics. The role of aspartic acids in the putative ATP-binding site of MPH(2′)II was investigated through the substitution of alanine for aspartate by site-directed mutagenesis. D200A, D209A, D219A, and D231A mutant strains were unable to inactivate the substrate oleandomycin, while a D227A mutant retained 7% of the activity of the original enzyme.

Reported macrolide resistance mechanisms are as follows: (i) methylation (2) of adenine in 23S rRNA (2058 in *Escherichia coli*), (ii) efflux protein (13), and (iii) inactivation of macrolide by erythromycin esterase (1, 3, 17) or macrolide 2′-phosphotransferase [MPH(2′)]. MPH(2′) is divided into MPH(2′)I (14, 18) and MPH(2′)II (10, 15) on the basis of its substrate specificity and primary amino acid sequence. The phosphotransferases are encoded by *mphA* and *mphB*, respectively. The former inactivates 14-membered ring macrolides more effectively than 16-membered ring macrolides, whereas the latter does not show this substrate preference. The primary amino acid sequence similarity between MPH(2′) and aminoglycoside phosphotransferase (APH) is poor, but the C-terminal regions have highly conserved motifs 1 and 2 in common (Fig. 1). They are the putative ATP-binding sites of APH, in which several functional amino acids have already been identified (4–8, 23, 24).

From these reports, it was expected that there are functional amino acids in the same region as MPH(2′). Five aspartic acids (D200, D209, D219, D227, and D231) were noted, and among these, three were highly conserved not only in bacterial phosphotransferase but also in the eukaryotic protein kinase family (4). To identify the functional aspartic acids, they were each replaced with alanine by site-directed mutagenesis.

(This work was presented in part at the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, Calif., 25 September 1998.)

E. coli TG1 (22), a derivative of K-12, was employed for DNA technology, for measurement of the macrolide susceptibility of cells bearing cloned *mphB*, and as a host for crude-enzyme preparation.

Bacteria were grown on LB broth and agar (12). Sources for antibiotics were the following: oleandomycin, erythromycin, roxithromycin, troleandomycin, spiramycin, and tylosin, Sigma, St. Louis, Mo.; kitasamycin, rokitamycin, and josamycin, Wako Pure Chemical Industries Ltd., Osaka, Japan; clarithromycin, Taisho Pharmaceutical Co., Ltd., Tokyo, Japan; azithromycin, Pfizer Pharmaceutical Co., Ltd., Tokyo, Japan. ATP disodium salt was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan, and restriction enzymes and DNA modification enzymes were purchased from Toyobo Co., Osaka, Japan.

The MPH(2′)II-encoding gene, *mphB*, used in this study was cloned from *E. coli* CU1, which was isolated in 1997 from clinical material in Japan (20, 21). An approximately 1.0-kb DNA fragment carrying *mphB* was inserted into the multiple cloning site of pHSG398 (Takara Shuzo Co., Ltd., Tokyo, Japan), and the resultant plasmid was designated pKTA321. Using the specially designed primers ECMPHBF-1 (5′-GCG ATAGAATTCAAGGAGAAATAATATGACCGTAGTCA CGACCGCCGAT-3′) and ECMPHBR-1 (5′-GTTTTCCCA GTCACGACGTTGT-3′), *mphB* was amplified by the PCR method. The DNAs so produced were digested with *Eco*RI and *Pst*I. The resulting DNA fragments were inserted into the *Eco*RI-*Pst*I site of pKF18k (Takara Shuzo Co., Ltd.) to construct a template, designated pKFB280, for site-directed mutagenesis.

Chemically synthesized mutant primers for site-directed mutagenesis (11) in the *mphB* gene were designed from published sequence data and purchased from Life Technologies, Inc. The sequences of the primers were 5′-GATTCATGGCGCCGTA CATGCCGG-3′, 5′-ACTATGATCGCGAAGGATGCCAAT G-3′, 5′-AATGTGACAGGCCTAATCGCTTGGAC-3′ 5′-A AGGTTACAGCTGTTTCGCATGAC-3′, and 5′-GTTTCGC ACGCGTTTATTTTCAAC-3′, and they were used to create D200A, D209A, D219A, D227A, and D231A mutants, respectively. The underlined letters indicate base mismatches compared to the wild-type sequence. A Mutan-Super Express kit (Takara Shuzo Co., Ltd.) with *E. coli* MV1184 for the Oligonucleotide-directed Dual Amber-Long and Accurate (ODA-LA) method was used for construction of mutant *mphB* by using pKFB280 as a template. The cycling program consisted of an initial incubation at 94°C for 5 min; 30 cycles of 93°C for 1 min, 52°C for 2 min, and 72°C for 2 min; and a final step of 72°C for 6 min. The mutant genes were completely sequenced by the chain termination method using a DSQ-1000 DNA sequencer (Shimadzu Co., Kyoto, Japan) and specific fluorescein-labelled primers designated RV22-FITC (5′-CACACAG GAAACAGCTATGACC-3′) and M422-FITC (5′-CCAGGG TTTTCCCAGTCACGCC-3′) to confirm the desired change in the nucleotide sequence. These mutant plasmids were digested with *Eco*47III and *Pst*I. The resulting approximately 520-bp DNA fragment was used to displace the corresponding region of *mphB* on pKTA321.

Macrolide-inactivating activity was measured (10) in sam-

* Corresponding author. Mailing address: Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan. Phone: 81-43-290-2930. Fax: 81-43-290-2929. E-mail: oharak@p.chiba-u.ac.jp.

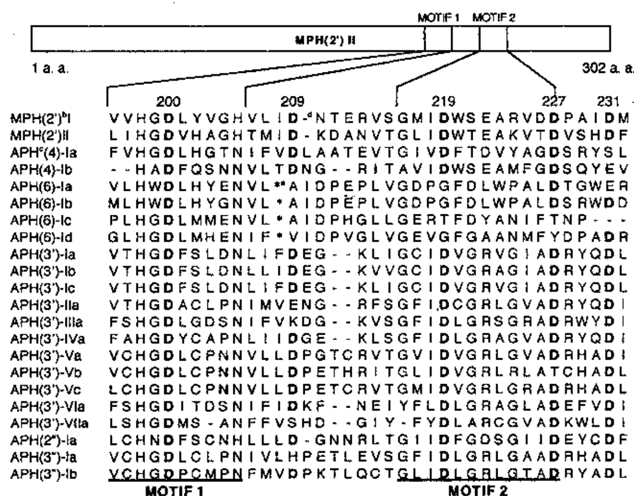


FIG. 1. Amino acid alignment of conserved motifs I and II (15, 19) in the MPH(2') and APH family. a. a., amino acid. Superscripts: b, MPH(2'); c, APH; d, deletion of an amino acid; e, insertion of an amino acid. Aspartic acid residues (D) are in boldface.

ples consisting of 50 μl of 40 mM ATP, 50 μl of macrolide antibiotics, and 400 μl of crude extract diluted with TMK buffer (0.06 M KCl, 0.01 M $MgCl_2$, 0.006 M 2-mercaptoethanol in 0.1 M Tris-HCl buffer, pH 7.8) that were mixed and allowed to react at 37°C for 0, 0.5, 1, 2, and 4 h. A 30-μl sample of the reaction mixture was spotted on a paper disk (8-mm diameter; thin; TOYO Filter Co., Ltd., Tokyo, Japan), and the disk was heated in a microwave oven to stop the reaction. The residual potency of the antibiotics was determined by microbioassay using *Bacillus subtilis* ATCC 6633 (9) as an indicator organism on a nutrient soft agar upper layer and a nutrient agar lower layer.

Change in D200. In a previous study of aminoglycoside 3'-phosphotransferase IIa [APH(3')IIa], D190, which corresponds to D200 of MPH(2'), was replaced with glutamine (Q). The affinity of D190Q for ATP was the same as that of the wild type, and there was a slight retention of enzymatic activity (6).

It has also been reported that a D190A mutant of APH(3')IIIa had no enzymatic activity (4), and it was proposed that D190 of APH(3')IIIa was a general base activating the 3'-OH group to attack the γ phosphate of ATP.

In the case of MPH(2')II, the specific enzyme activity of D200A was less than 0.1% of the original activity, thereby demonstrating that D200 is essential for the catalytic activity of MPH(2')II. These results suggested that D200 might similarly be a general base activating the 2'-OH group of macrolide antibiotics.

Changes in D209, D219, and D231. In earlier work, D208 and D220 of APH(2')IIa [corresponding to D219 and D231 of MPH(2')II, respectively] were each replaced with glycine (6). The results suggested that D208 and D220 are involved in the binding of ATP. Our testing determined that the specific activities of D219A and D231A for oleandomycin were less than 0.1 U (nanomoles of oleandomycin inactivated per hour), which suggested that D219 and D231 were an essential for enzymatic activity. These aspartic acids were highly conserved in motif II in the same way as D208 and D220 of APH(3'), so that the role of D219 and D231 might be correspondingly similar.

The specific activity of the D209A mutant was also less than 0.1 U, which suggested that D209 is crucial for catalysis. The amino acid corresponding to D209 in MPH(2')II has not been studied in APH and other phosphotransferases, so further experimentation is needed to clarify the precise role of this residue.

Change in D227. The specific activity of the D227A mutant was greatly reduced, but measurable activity (7%) was retained (Table 1). This suggested that D227 is not essential for, but clearly affects, enzymatic activity. The substrate specificity of D227A for various macrolides was examined (Table 1). The data showed that substitution at D227 resulted in a much less significant alteration of the substrate specificity of MPH(2')II for 14-membered ring macrolides, such as erythromycin, troleandomycin (16), roxithromycin, and clarithromycin and 15-membered ring macrolides such as azithromycin, in contrast to that for 16-membered ring macrolides such as spiramycin and rokitamycin. In the latter two cases, activity was decreased by at least 25-fold compared with that of the wild type. In the other 16-membered ring macrolides, the relative activities of D227A demonstrated a 12-fold reduction for kitasamycin, similar activity for josamycin, and a fourfold reduction for tylosin compared with that of the wild type. These results suggested that D227 participated in the recognition of 16-membered ring macrolides, especially kitasamycin, spiramycin, and rokitamycin.

TABLE 1. Substrate specificity of crude enzyme extract containing wild-type or D227A mutant MPH(2')II

Macrolide	Relative activity (%)[a]	
	Wild type	D227A mutant
14 membered		
Oleandomycin	100	100
Troleandomycin	104	58
Erythromycin	83	83
Clarithromycin	46	49
Roxithromycin	50	9
15 membered azithromycin	88	27
16 membered		
Kitasamycin	202	16
Spiramycin	75	85
Josamycin	75	2
Rokitamycin	50	2
Tylosin	55	13

[a] The specific activity of the wild type enzyme for oleandomycin was 121 nmol/h/mg of protein and that of the D227A mutant was 8.5 nmol/h/mg of protein.

In spite of structural differences among macrolides, specific activities for 14- and 16-membered ring macrolides with the original enzyme were not so very different from each other (about 50~200% of that of oleandomycin). Additionally, there is poor homology between MPH(2') and erythromycin esterase in their primary amino acid sequences, so that it is difficult to identify the 14-membered ring recognition site in these macrolide-modifying enzymes. Kitasamycin, josamycin, and rokitamycin have a bulky side chain at the 4" position of L-mycarose, whereas spiramycin and tylosin have a small OH group at the same position. On the other hand, 14- and 15-membered ring macrolides do not have L-mycarose at the 4' position of D-desosamine.

On the basis of this information, we predict that MPH(2')II might more strongly interact with the sugar moiety than the lactone ring and we speculate that D227 makes a pocket where

the desosamine and mycarose moieties fit. To identify the exact part of the macrolide which interacts with D227, a more detailed examination will follow in our laboratory.

This study was supported by a grant from the Ministry of Health and Welfare, Japan, 1998, for molecular characterization of antibiotic resistance and development of methods for rapid detection of drug-resistant bacteria.

REFERENCES

1. **Andremont, A., H. Sancho-Garnier, and C. Tancrede.** 1986. Epidemiology of intestinal colonization by members of the family *Enterobacteriaceae* highly resistant to erythromycin in a hematology-oncology unit. Antimicrob. Agents Chemother. **29:**1104–1107.
2. **Arthur, M., A. Andremont, and P. Courvalin.** 1987. Distribution of erythromycin esterase and rRNA methylase genes in members of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob. Agents Chemother. **31:**404–409.
3. **Arthur, M., and P. Courvalin.** 1986. Contribution of two different mechanisms to erythromycin resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **30:**694–700.
4. **Hon, W. C., G. A. Mckay, P. R. Thompson, R. M. Sweet, D. S. C. Young, G. D. Wright, and A. M. Berghuis.** 1997. Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. Cell **89:**887–895..
5. **Kocabiyik, S., C. Mullins, C. Breeding, and M. H. Perlin.** 1992. Structure-function analyses for aminoglycoside 3′-phosphotransferase II (APH(3′)II). SAAS Bull. Biochem. Biotech. **5:**58–63.
6. **Kocabiyik, S., and M. H. Perlin.** 1992. Site-specific mutation of conserved C-terminal residues in aminoglycoside 3′-phosphotransferase II: phenotypic and structural analysis of mutant enzymes. Biochem. Biophys. Res. Commun. **185:**925–931.
7. **Kocabiyik, S., and M. H. Perlin.** 1992. Altered substrate specificity by substitution at Tyr 218 in bacterial aminoglycoside 3′-phosphotransferase-II. FEMS Microbiol. Lett. **93:**199–202.
8. **Kocabiyik, S., and M. H. Perlin.** 1994. Amino acid substitutions within the analogous nucleotide binding loop (P-loop) of aminoglycoside 3′-phosphotransferase II. Int. J. Biochem. **26:**61–66.
9. **Kono, M., K. Ohmiya, T. Kanda, N. Noguchi, and K. O'Hara.** 1987. Purification and characterization of chromosomal streptomycin-adenylyltransferase from derivative of *Bacillus subtilis* Marburg 168. FEMS Microbiol. Lett. **40:**233–238.
10. **Kono, M., K. O'Hara, and T. Ebisu.** 1992. Purification and characterization of macrolide 2′-phosphotransferase type II from a strain of *Escherichia coli* highly resistant to macrolide antibiotics. FEMS Microbiol. Lett. **97:**89–94.
11. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Matsuoka, M., K. Endou, S. Saitoh, M. Katoh, and Y. Nakajima.** 1995. A mechanism of resistance to partial macrolide and streptogramin B antibiotics in *Staphylococcus aureus* clinically isolated in Hungary. Biol. Pharm. Bull. **18:**1482–1486.
14. **Noguchi, N., A. Emura, H. Matsuyama, K. O'Hara, M. Sasatsu, and M. Kono.** 1995. Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2′-phosphotransferase I in *Escherichia coli*. Antimicrob. Agents Chemother. **39:**2359–2363.
15. **Noguchi, N., J. Katayama, and K. O'Hara.** 1996. Cloning and nucleotide sequence of the *mphB* gene for macrolide 2′-phosphotransferase II in *Escherichia coli*. FEMS Microbiol. Lett. **144:**197–202.
16. **O'Hara, K.** 1993. Reaction mechanism of macrolide 2′-phosphotransferase from *Escherichia coli* to the 2′-modified macrolide antibiotics. Jpn. J. Antibiot. **46:**818–826.
17. **O'Hara, K., and K. Yamamoto.** 1996. Reaction of roxithromycin and clarithromycin with macrolide-inactivating enzymes from highly erythromycin-resistant *Escherichia coli*. Antimicrob. Agents Chemother. **40:**1036–1038.
18. **O'Hara, K., T. Kanda, K. Ohmiya, T. Ebisu, and M. Kono.** 1989. Purification and characterization of macrolide 2′-phosphotransferase from a strain of *Escherichia coli* that is highly resistant to erythromycin. Antimicrob. Agents Chemother. **33:**1354–1357.
19. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.
20. **Taniguchi, K., A. Nakamura, K. Tsurubuchi, A. Ishii, K. O'Hara, and T. Sawai.** 1999. Appearance in Japan of highly macrolide-resistant *E. coli* producing macrolide 2′-phosphotransferase. Microbios **97:**137–144.
21. **Taniguchi, K., A. Nakamura, K. Tsurubuchi, K. O'Hara, and T. Sawai.** 1998. Identification of *Escherichia coli* clinical isolates producing macrolide 2′-phosphotransferase by a highly sensitive detection method. FEMS Microbiol. Lett. **167:**191–195.
22. **Taylor, J. W., J. Ott, and F. Eckstein.** 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphothioate-modified DNA. Nucleic Acids Res. **13:**8765–8785.
23. **Thompson, P. R., D. W. Hughes, and G. D. Wright.** 1996. Mechanism of aminoglycoside 3′-phosphotransferase type IIIa: His 188 is not a phosphate-accepting residue. Chem. Biol. **3:**747–755.
24. **Thompson, P. R., D. W. Hughes, N. P. Nicholas, and G. D. Wright.** 1998. Spectinomycin kinase from *Legionella pneumophila*. J. Biol. Chem. **273:** 14788–14795.

Appearance of fosfomycin resistant *Rahnella aquatilis* clinically isolated in Japan

Koji O'Hara[1*], Jia Chen[1], Fritz Shigenobu[1], Akio Nakamura[1], Kazuo Taniguchi[1], Masahiro Shimojima[2], Hiroshisa Ida[2], Eiji Yoshikawa[2], Isami Tsuboi[2], Keiji Mizuoka[2] and Tetsuo Sawai[1]

[1]Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan, and [2]BML General Laboratory, Kawagoe-shi, Saitama 350-1101, Japan (*Reprint address)

Key words: fosfomycin, *Rahnella aquatilis*, clinical isolate

Abstract

Among recent clinical isolates in Japan, strain CU264 was discovered which formed unusual colonies. This strain was identified as *Rahnella aquatilis* which is usually found in water. The antibiotic susceptibilities against tetracycline, carbenicillin, chloramphenicol, streptomycin, kanamycin, gentamicin, sulphonamide, neomycin, fosfomycin, rifampicin, norfloxacin and nalidixic acid, were investigated. The result demonstrated that the strain was highly resistant to fosfomycin only. It was further shown that this resistance was transmissible with low frequency to *Serratia marcescens* whereas it was not transmissible to *Escherichia coli*.

Introduction

Rahnella aquatilis is known as a bacterial species which is rarely isolated from clinical samples. The bacterium was classified into the family Enterobacteriaceae in 1979 and is generally found in water (Izard *et al.*, 1985).

In Japan, *Legionella* (Edelstein *et al.*, 1996; Martin *et al.*, 1996; Van, 1996), the cause of Legionnaire's disease, has been found in the cooling water of air conditioners. *Pseudomonas*, which causes infection (Bryan *et al.*, 1984; O'Hara *et al.*, 1997a), has also been discovered in tap water. Such bacteria present in water may invade the human body, which emphasizes the need for extreme caution in the clinical field.

In our work on several *Serratia marcescens* strains clinically isolated in Japan, some uncommon *Serratia* colonies were observed in one strain. Identification showed that it was a very unusual bacterium, *Rahnella aquatilis*. The characteristics of this strain were investigated in this study.

Materials and methods

Bacterial strains

Strains CU264 and CU231 were isolated from patients in the hospital affiliated to the School of Medicine, Gifu University (Gifu, Japan) in the 1990s. *Escherichia coli* K12-W3110rif (O'Hara, 1993; O'Hara and

Published and © 1998 by The Faculty Press
88 Regent Street, Cambridge, Great Britain