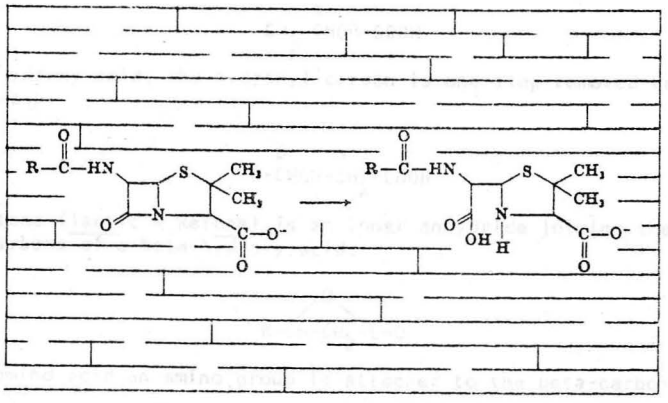
THE NIBBLING OF THE RING: INS AND OUTS OF THE BETA-LACTAMASES

William B. Baine, M.D.

Grand Rounds
Department of Internal Medicine
Southwestern Medical School
The University of Texas Health Science Center at Dallas

January 3, 1985



Bleib' fern! fürchte dies Zeichen!
Zur Schande zwingst du mich nicht,
so lang' der Ring mich schützt.

[Stand back! Bow to this token!
No shame can touch me from thee
While yet this ring is my shield.]

Richard Wagner, Götterdämmerung

I will have my bond.

William Shakespeare, The Merchant of Venice

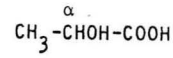
The Lore of the Rings

Der Welt Erbe
gewänne zu eigen,
wer...schüfe den Ring,
der masslose Macht ihm verlieh'.

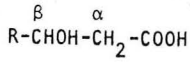
[The world's kingdom
That one can encompass
Who...shapeth the Ring,
Which measureless might can secure.]

Richard Wagner, Rheingold

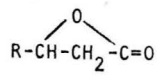
When bacteria of the genus Lactobacillus are permitted to grow in milk (L. lac, lactis), the milk sours with the transformation of glucose to the alpha-hydroxy carboxylic acid, lactic acid:



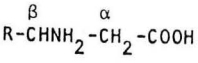
In a beta-hydroxy acid, the hydroxyl carbon is one step removed from the carboxyl group:



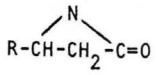
A beta-lactone (lactic + ketone) is an inner anhydride joining the beta and carboxyl carbons of a beta-hydroxy acid:



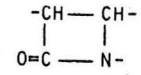
In a beta-amino acid an amino group is attached to the beta-carbon atom:



By analogy with beta-lactones, the inner anhydride of a beta-amino acid is called a beta-lactam (lactone + amino):



All penicillins and cephalosporins contain the 4-membered azetidinone ring:



A moment's inspection makes it apparent that the keto-carbon and the nitrogen molecule in the azetidinone ring are joined by a beta-lactam bond. The

integrity of this bond is essential to the antibacterial action of the beta-lactam antibiotics.

The Mechanism of Action of Beta-lactam Antibiotics

For many years it was believed that the antibacterial action of penicillins and cephalosporins depended on their stereochemical resemblance to the carboxy-terminal D-alanyl-D-alanine region of murein. According to this hypothesis, the beta-lactam antibiotics acted as antimetabolites that interfered with transpeptide cross-linking in the bacterial cell wall (1). In recent years, this concept has been refined to emphasize the interaction of beta-lactam antibiotics with what are termed the penicillin-binding proteins (1-4).

These proteins, located on the cytoplasmic membrane of the bacterial cell, are enzymes responsible for the terminal stages of peptidoglycan assembly during the synthesis of the bacterial cell wall, incorporating precursors into the insoluble murein sacculus (1-4). As an analog of penicillin-binding protein substrate, penicillin can acylate the active site of these enzymes. With opening of the beta-lactam ring, an inactive covalently linked penicilloyl-enzyme complex is formed. Interference with normal cell wall synthesis can thus lead to autolysis or to the production of filamentous or otherwise deformed bacterial cells.

Bacterial Resistance to Beta-lactam Antibiotics

All bacteria have cell walls, but not all bacteria are uniformly susceptible to beta-lactam antibiotics. There are at least four mechanisms by which bacteria may manifest resistance to one or more beta-lactams (4): 1. Permeability barriers may impede access of the drug to the target penicillin-binding proteins. 2. Alterations in penicillin-binding proteins may reduce their affinity for beta-lactam antibiotics or render the penicllin-binding protein targets less susceptible to drug-mediated inactivation. 3. Binding of drug to target may not activate bacterial autolysis because of inhibition of bacterial autolysins. 4. The antibiotics may be inactivated by bacterial enzymes.

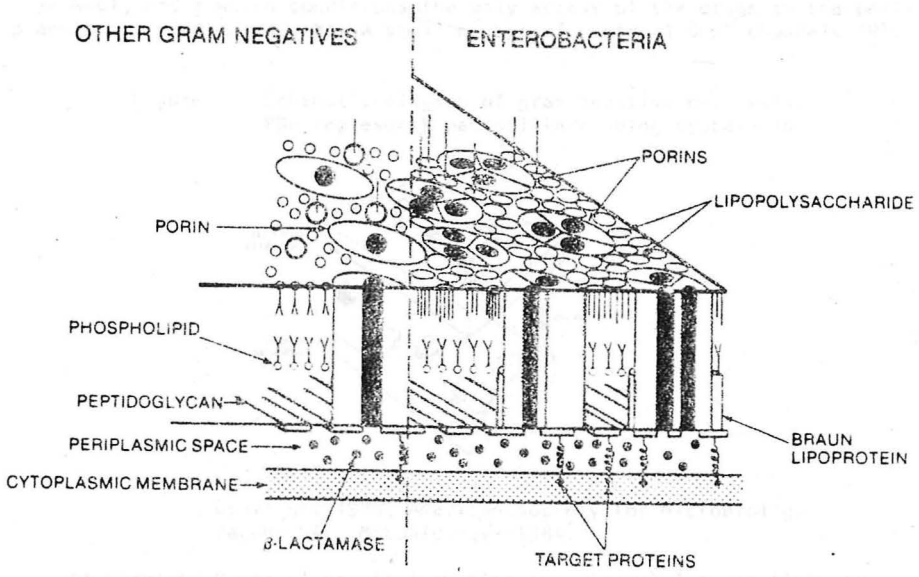
All conventional bacteria possess a cytoplasmic membrane surrounded by a peptidoglycan cell wall--considerably thicker in gram-positive bacteria than in gram-negatives. Gram-negative bacteria also possess an outer membrane external to the cell wall. This outer membrane provides a permeability barrier to hydrophilic antibiotics (Figure 1) (4-6).

Nikaido has reviewed the influence of outer membrane permeability on resistance to beta-lactam antibiotics (7-9). Most hydrophilic solutes, including beta-lactams, cross the outer membrane through fluid-filled channels formed by a class of proteins known as porins. *Escherichia coli* have been shown to produce porins coded for by genes designated *ompF* (outer membrane protein F) and *ompC* and, under conditions of phosphate starvation, also by the *phoE* gene.

At least three physicochemical characteristics of a molecule are known

to influence the rate at which it traverses these porin channels. The narrow diameter of the OmpF (1.16 nm) and OmpC (1.08) porins results in a marked influence of solute size on permeability. In addition, hydrophobicity of a solute retards passage through the porins. Penicillins have a more hydrophobic nucleus than do cephalosporins and are also less able to traverse the gram-negative outer membrane. The electrical charge of a molecule is also important. OmpF and OmpC facilitate the passage of cationic solutes; this may explain the very high permeability of cephaloridine, which is a dipolar molecule, as well as the relative inactivity of doubly-negatively charged carbenicillin against E. coli. (The PhoE channel, which is expressed only when phosphate is limiting, preferentially accepts anions.)

Figure 1. Proposed structure of the envelope of gram-negative bacteria (4).

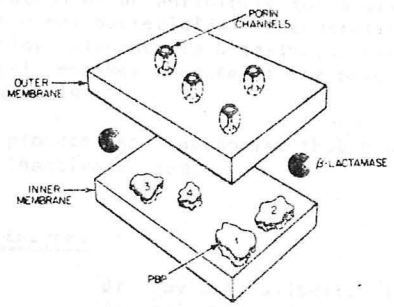


Bulky solutes such as the second-, and third-generation cephalosporins cefotaxime, ceftazidime, ceftizoxime, and cefuroxime diffuse relatively slowly through the porins, but the time necessary for equilibration of extracellular and periplasmic drug is still brief relative to the generation time of bacteria. Nikaido suggests that in terms of net antibacterial activity the increases in antibiotic resistance to beta-lactamases seen in the third-generation cephalosporins compensate for the decreased rate of influx of drug into the periplasmic space (9).

Cultivation of E. coli at 37°C or under conditions of relatively high

osmotic strength (approximately 300 mosmol/kg) favors the production of OmpC and the repression of the larger OmpF porin. Thus, OmpC may be the predominant porin expressed by E. coli in infected tissue. Nikaido has suggested that the reduction in solute diffusion represented by elimination of OmpF could be the limiting factor in two situations (Figure 2): 1. Beta-lactams with intrinsically slow rates of penetration because of bulk, hydrophobicity, or multiple negative charges, could be ineffectual regardless of the intracellular level of beta-lactamase activity. 2. In the presence of high levels of periplasmic beta-lactamase, even highly diffusible enzyme-susceptible drugs might be inactivated more rapidly than they could enter the cell. An OmpC-deficient mutant of Salmonella typhimurium harboring an R plasmid coding for a TEM beta-lactamase has been recovered from a patient treated with cephalexin for a renal abscess. This strain proved to be broadly resistant to beta-lactam antibiotics when grown in the presence of 0.9% NaCl, under which conditions the only access of the drugs to the periplasmic space was presumably a small number of residual OmpF channels (9).

Figure 2. Schematic diagram of gram-negative cell wall. PBP represents penicillin-binding protein (6).



At Parkland Memorial Hospital routine antimicrobial susceptibility testing is performed with a microdilution method (Microdilution MIC Test Panels, Micro-Media Systems) using inocula of 1-2 x 10^5 bacteria/ml in broth containing serial dilutions of the drugs to be tested. These inocula are then incubated overnight at 35°C.

In Pseudomonas aeruginosa the porin channels are wider than in E. coli, with correspondingly little effect of solute size, hydrophobicity, and charge upon diffusibility. However, the majority of these channels are believed to be functionally closed, restricting solute access to the periplasmic space and limiting the influx of potential substrate for that organism's chromosomally coded beta-lactamase. Accordingly, resistance to enzymatic hydrolysis rather than rapidity of diffusion may be the most important attribute of antipseudomonal beta-lactams (4, 9, 10).

Penicillin-binding proteins vary in number and size between bacterial genera, although similarities exist in the penicillin-binding proteins of groups of related organisms (1-4). At least seven major penicillin-binding proteins are known in E. coli, for example, and progress has been made in determining their individual enzymatic specificities. Penicllin-binding proteins vary in their affinity for a given beta-lactam. Since these proteins vary between bacterial species and between strains of a given species, concomitant variations in susceptibility to a given beta-lactam antibiotic are to be expected (11, 12). Mutant penicillin-binding proteins have been incriminated in the development of intrinsic beta-lactam resistance in several bacterial pathogens. In the gonococcus, intrinsic (chromosomal) resistance depends on alterations in the penicillin-binding proteins that result in decreased affinity for antibiotic (3, 12, 13). Intrinsically resistant strains of Streptococcus faecalis, S. faecium, and Staphylococcus aureus can be shown to have acquired a new penicillin-binding protein. In the pneumococcus, relative penicillin resistance is associated with decreased affinity of penicillin-binding proteins, whereas high-level resistance is accompanied by the acquisition of a new penicillin-binding protein (13).

Tolerance is defined as a great discrepancy between the minimal inhibitory concentration of an antibiotic for a given bacterial strain and the corresponding minimal bactericidal concentration (MBC). Some strains of S. aureus that display tolerance to beta-lactam drugs have been shown to be deficient in autolytic enzymes because of the persistence of an excess of an inhibitor of autolysis (4).

Many bacteria produce beta-lactamases that hydrolyze penicillins and cephalosporins and inactivate them.

Beta-lactamases as Enzymes

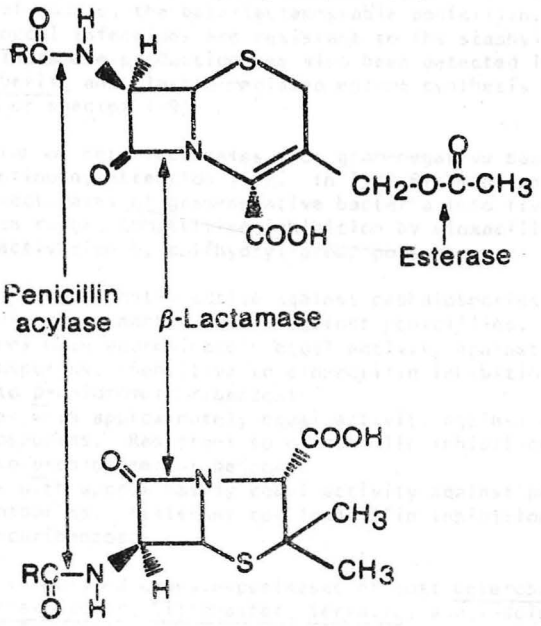
> Dr. David N. Gilbert. Is there any hope for a logical categorization or classification of the β-lactamases?
>
> Dr. Richard B. Sykes. Richmond and I classified them in 1965; then we found enzymes that did not fit. When cefoxitin was first marketed, enzymes that hydrolyzed cefoxitin were not recognized. I have enzymes today that will hydrolyze cefoxitin as fast as they hydrolyze cephaloridine. There is a continual evolution; hence, any classification will be continuously out-of-date (14).

As early as 1940 Abraham and Chain reported that extracts of E. coli could inactivate penicillin and coined the term "penicillinase" to represent the responsible enzyme (15). Although the side chains of penicillin and related compounds can be attacked by various acylases and esterases, clinically relevant enzymatic inactivation of these antibiotics is the result of beta-lactamase activity.

Beta-lactamases are bacterial enzymes that hydrolyze the beta-lactam bond, opening the azetidinone ring and inactivating the antibiotic (Figure

3). Numerous species of gram-positive and gram-negative bacteria are known to synthesize beta-lactamases (17). The terminology of beta-lactamases has

Figure 3. Interaction of cephalosporins (top) and penicillins (bottom) with hydrolyzing enzymes (16).



undergone evolution over several decades. Following the lead of Abraham and Chain, "penicillinase" was the generic term for over a decade. In 1965 the Enzyme Commission codified this usage in "penicillin amido β-lactam hydrolase" [EC 3.5.2.6]. With the recognition in the 1960's of enzymes hydrolyzing cephalosporins, the term "cephalosporinase" came into being. In 1972 "cephalosporinase" was given the classification EC 3.4.2.8. However, at the same time the Enzyme Commission introduced "β-lactamase I" for penicillinase and "β-lactamase II" for cephalosporinase. These terms have not met with favor, and the most current classification should see all beta-lactamases consolidated as EC 3.5.2.6 (16).

A replicon is a genetic unit that is capable, as a whole, of self-replication. The chromosome of a bacterium is a repiicon. Plasmids are small bacterial replicons that are independent of the chromosome. Under certain circumstances a plasmid and the genetic information that it carries

may be transferred from a donor to a recipient bacterial cell of the same or another species in a process known as conjugation (18).

Four types of staphylococcal beta-lactamases, designated A though D, are differentiated serologically. These are coded for by plasmid-borne genes (19). Staphylococcal beta-lactamases are functionally true penicillinases. Cephalosporins and, of course, the beta-lactam-stable penicillins used in the therapy of staphylococcal infections are resistant to the staphylococcal enzymes (16). Beta-lactamase production has also been detected in strains of S. faecalis and S. uberis, and plasmid-mediated enzyme synthesis has been described in the former species (19).

The classification of beta-lactamases from gram-negative bacteria has been a subject of continuing attention (16). In 1973 Richmond and Sykes (20) classified the beta-lactamases of gram-negative bacteria into five classes on the basis of substrate range, competitive inhibition by cloxacillin, and susceptibility to inactivation by sulfhydryl-group poisons:

Class I: Enzymes predominantly active against cephalosporins.
Class II: Enzymes predominantly active against penicillins.
Class III: Enzymes with approximately equal activity against penicillins and cephalosporins. Sensitive to cloxacillin inhibition and resistant to p-chloromercuribenzoate.
Class IV: Enzymes with approximately equal activity against penicillins and cephalosporins. Resistant to cloxacillin inhibition and sensitive to p-chloromercuribenzoate.
Class V: Enzymes with approximately equal activity against penicillins and cephalosporins. Resistant to cloxacillin inhibition and to p-chloromercuribenzoate.

The chromosomally mediated cephalosporinases of most Enterobacteriaceae, including E. coli, Enterobacter, Citrobacter, Serratia, and indole-positive Proteus, and those of Pseudomonas characteristically belong to Class I. Production of class I enzymes may be either constitutive (unaffected by the presence or absence of substrate in the environment) or inducible (derepressed upon exposure of the organism to substrate). Certain chromosomally mediated Proteus penicillinases comprise class II. The Class III enzymes include important plasmid-mediated beta-lactamases. Klebsiella penicillinases belong to Class IV and are invariably constitutive. Class V enzymes, which include oxacillin-, and carbenicillin-hydrolyzing enzymes, have been shown to be carried on R plasmids (Table 1) (16, 19, 20).

Table 1. Richmond-Sykes beta-lactamases classes.

Class	Specificity	Gene	Control	Distribution
I	cephalosporins	chromosome	constitutive or inducible	Enterobacteriaceae Pseudomonas
II	penicillins	chromosome	constitutive	Proteus
III	broad spectrum	plasmid	constitutive	multiple taxa
IV	broad spectrum	chromosome	constitutive	Klebsiella
V	penicillins including cloxacillin	plasmid	constitutive	Pseudomonas Enterobacteriaceae

A given beta-lactamase can be characterized in terms of its substrate profile (20). This is the relative rate of hydrolysis of various beta-lactams shown by the enzyme in comparison with its activity against a reference substrate (usually penicillin G). The rate of hydrolysis of the standard is conventionally set at 100. Thus a substrate profile of penicillin G, 100; ampicillin, 180; carbenicillin, 45; and cloxacillin, 0; indicates an enzyme that hydrolyzes ampicillin rather more, and carbenicillin rather less rapidly than penicillin G and that is inactive against cloxacillin. The Richmond-Sykes scheme subdivides each of the five enzyme classes into one or more enzyme types on the basis of detailed substrate profiles (Table 2) (20).

Table 2. Classification of beta-lactamases from gram-negative bacteria by relative activity and substrate profile.

Enzyme class	Enzyme type	Substrate profile					
		Penicillin	Ampicillin	Carben-icillin	Cloxacillin	Cepha-loridine	Cephalexin
I	a	100	0	0	ND	8000	620
	b	100	0	0	ND	350	80
	c	100	150	ND	ND	2000	ND
	d	100	10	0	0	600	80
II	a	100	180	45	ND	<20	0
	b	100	160	ND	0	<20	0
III	a	100	180	10	0	140	<10
IV	a	100	120	10	<10	150	0
	b	100	125	45	20	50	<10
	c	100	170	50	20	70	0
V	a	100	950	ND	200	120	ND
	b	100	300	ND	200	50	ND
	c	100	100	60	0	20	<10
	d	100	180	80	0	40	<10

For the sake of uniformity, the comparative studies of enzyme activities on which the Richmond-Sykes classification are based are carried out under standard conditions of pH and temperature. Typical assay conditions are pH 5.9 and 30°C (20). It must be stressed that these conditions may be very different both from those at which the activity of a particular enzyme is optimal and from those encountered in infected tissue. Thus, despite their utility in classifying bacterial beta-lactamases, substrate profiles should not be extrapolated uncritically to infer the potential activity of a given enzyme against the corresponding antibiotics *in vivo*.

The Richmond-Sykes scheme and to its component classes are still frequently cited by workers in the field, but this system no longer suffices for a comprehensive classification of all the known beta-lactamases produced by gram-negative bacteria (19).

Beta-lactamases are also now categorized on the basis of isoelectric

focusing in polyacrylamide or agarose (19), as described by Matthew and colleagues. This sensitive technique has revealed the almost universal presence of beta-lactamase in gram-negative organisms. The isoelectric focusing patterns, substrate profile, and susceptibility to inhibitors of 11 plasmid-coded beta-lactamases from gram-negative bacteria have been utilized in another classification scheme (Table 3).

Table 3. Comparative nomenclature of selected plasmid-coded beta-lactamases from gram-negative bacteria.

Matthew nomenclature	Enzyme	Richmond and Sykes nomenclature
broad-spectrum penicillinases	TEM-1 (Temoniera)	IIIa
	TEM-2	IIIa
	SHV-1 (sulfhydryl-variable)	IV
	HMS-1 (Hedges, Matthew, and Smith)	
oxacillinases	OXA-1 (oxacillin-hydrolyzing)	Va
	OXA-2	V
	OXA-3	V
carbenicillinases	PSE-1 (Pseudomonas-specific)	V
	PSE-2	V
	PSE-3	V
	PSE-4	V

Inasmuch as carbenicillin-hydrolyzing enzymes have been found in many genera of Enterobacteriaceae and not solely in Pseudomonas, some workers prefer the designation CARB to PSE. In addition to the enzymes listed in Table D, additional TEM (ROB-1, LCR-1), OXA (OXA-4, OXA-5, OXA-6, OXA-7), and CARB (AER-1) beta-lactamases have recently been described (19).

A given bacterial species may serve as the recipient of more than one type of plasmid-borne beta-lactamase (19). Twelve different plasmid-coded beta-lactamases have been found in strains of P. aeruginosa, and multiple different enzymes have also been detected in E. coli (ten types), P. mirabilis (seven), Klebsiella (five), Salmonella (five), Providencia (four), and Shigella (three). Furthermore, a single bacterial strain may produce two or more plasmid-coded beta-lactamases simultaneously in addition to synthesis of a chromosomal enzyme.

Transposons are plasmid segments capable of moving from one replicon to another. At least 11 plasmid-determined beta-lactamases from gram-negative bacteria are known to be encoded by transposons. These include representatives of the TEM (TEM-1, TEM-2, SHV-1), OXA (OXA-1, OXA-4, OXA-5, OXA-6), and CARB (PSE-1, PSE-2, PSE-4, AER-1) groups (19). Some

beta-lactamase transposons also bear genes conferring resistance to streptomycin and sulfonamides.

The nearly ubiquitous chromosomally coded beta-lactamases of gram-negative bacilli are often specific for bacterial species and even subspecies. The chromosomal beta-lactamases of P. vulgaris and Klebsiella hydrolyze penicillin G, ampicillin, and carbenicillin as well as cephalosporins. Most other species of Enterobacteriaceae as well as Pseudomonas, Acinetobacter, and Bacteroides produce chromosomal cephalosporinases with little activity against these penicillins. Many of these cephalosporinases are inducible enzymes, and some have been observed to become stably derepressed as a result of mutations (19).

A chromosomal beta-lactamase of K. pneumoniae is believed to be identical to the plasmid-borne SHV-1 enzyme. In general, however, the chromosomal enzymes appear to be distinct from those coded by plasmid-borne genes (19).

Three evolutionarily distinct classes of beta-lactamase have been delineated on the basis of amino acid and nucleotide sequence studies (19). Class A includes penicillinases isolated from S. aureus PC1 and Bacillus licheniformis 749c as well as the TEM-1 beta-lactamase. These proteins are similar in amino acid sequence and molecular weight (approximately 29,000 daltons) and in the presence of serine at the active site. An evolutionary origin for the class A beta-lactamases is suggested by homology between their active site and that of a penicillin-binding protein of Bacillus, D-alanine carboxypeptidase. The suggestion is that beta-lactamases may be derived from enzymes involved in peptidoglycan synthesis. The metalloprotein beta-lactamase of B. cereus is the prototype of class B. Class C enzymes comprise chromosomally determined beta-lactamases of E. coli, Shigella, Klebsiella, Serratia, Salmonella, and Pseudomonas. The class C beta-lactamases are larger (approximately 39,000 daltons) than the unrelated proteins in class A but also have serine at the active site of the enzyme.

Testing for Bacterial Beta-lactamase Production

This was the most unkindest cut of all.

William Shakespeare, Julius Caesar

Several methods are currently in use in clinical microbiology laboratories for the identification of bacterial strains that produce beta-lactamase (21, 22). The technical limitations of each test should be borne in mind (20).

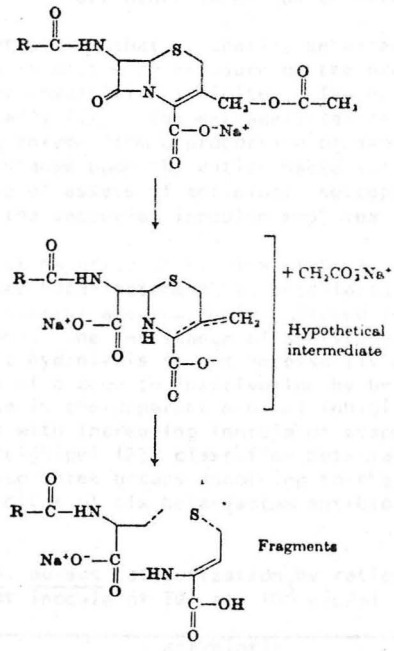
Hydrolysis of a penicillin by beta-lactamase yields the corresponding penicilloic acid. Since there is a stoichiometric conversion of antibiotic to acid, the enzyme activity can be assayed quantitatively or qualitatively by monitoring the pH of a mixture of bacteria and substrate (20). This may conveniently be done with an indicator dye.

Another sensitive measure of the production of penicilloic acid relies on the reduction of iodine to iodide by this reaction product. Since iodine

(but not iodide) forms a blue complex with starch, decoloration of a mixture of starch and iodine can be used as evidence of penicilloic acid production in a mixture of bacteria and penicillin (20). This iodometric assay for beta-lactamase may of course be falsely negative if the enzyme produced by the bacterial strain in question is susceptible to inactivation by iodine.

It is obvious that penicillin substrates are unsuitable for the detection of Class I enzymes, the so-called cephalosporinases. In contrast to the situation with penicillin, however, there is no stoichiometric relationship between cephalosporin hydrolysis and production of cephalosporanic acid. Instead, hydrolysis of the azetidinone ring of a cephalosporin typically induces secondary chemical changes, including rearrangements in the adjacent dihydrothiazine ring and spontaneous fragmentation of the molecule (Figure 4). Thus the pH and iodometric assays

Figure 4 Possible reaction sequence catalyzed by beta-lactamases with cephalosporins as substrates (20).



used for so-called penicillinases are inappropriate for measuring cephalosporinase activity. Changes in the absorbance spectrum of a cephalosporin may accompany the secondary changes in resonance in the

dihydrothiazine nucleus that occur with cleavage of the beta-lactam bond. This phenomenon has been exploited to detect cephalosporinases. In the chromogenic cephalosporin assay hydrolysis of a yellow cephalosporin results in the accumulation of a red reaction product (20).

The Role of Beta-lactamases in Bacterial Resistance to Antibiotics

> Maid of Athens, ere we part,
> Give, oh give me back my heart!
> Or, since that has left my breast,
> Keep it now, and take the rest.
>
> George Gordon Byron, Lord Byron, Maid of Athens

> Except the blind forces of Nature, nothing moves in this world which is not Greek in its origin.
>
> Sir Henry James Sumner Maine, Rede Lecture, 1875

In gram-positive organisms that synthesize beta-lactamase, production of the enzyme is typically inducible by exposure of the organism to a beta-lactam substrate or competitive inhibitor. The enzyme is then transported extracellularly (17). The extracellular release of antibiotic-inactivating enzyme from a proportion of exposed cells can thus potentially confer resistance upon the entire bacterial population. Accordingly, the outcome of assays of antibiotic susceptibility in vitro are markedly influenced by the bacterial inoculum employed (20).

At present, the vast majority of strains of S. aureus encountered in clincial practice produce beta-lactamase, so beta-lactamase-resistant penicillins and cephalosporins are routinely employed in the treatment of staphylococcal infections. The resistance of antistaphylococcal beta-lactam antibiotics to enzymatic hydrolysis is not necessarily absolute, however. Relative susceptibility of a drug to inactivation by beta-lactamases may be reflected in an increase in the apparent minimal inhibitory concentration (MIC) of the antibiotic with increasing inocula of staphylococci in the test system. Chapman and Steigbigel (23) classified beta-lactamase producing strains of S. aureus into three groups according to the magnitude of the inoculum effect on the MIC's of six beta-lactam antibiotics (Table 4). All

Table 4. S. aureus categorization by ratio of MIC's at inocula of 10^7 and 10^3 cfu/ml.

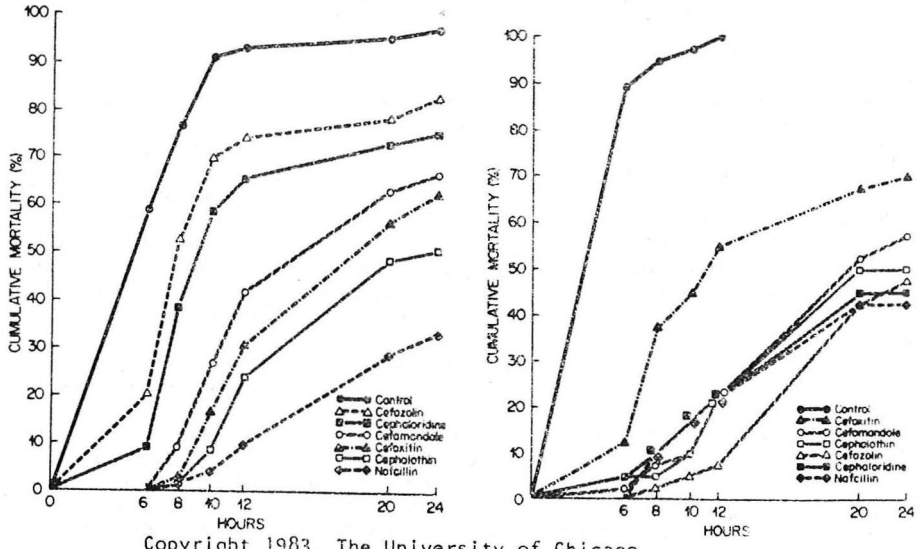
Group	Antibiotic					
	Nafcillin	Cefoxitin	Cephalothin	Cefamandole	Cefazolin	Cephaloridine
I	1	1-2	2-3.5	10-16	35->40	25-42
IA	1-1.3	1-2	2-2.8	8.6-10	15-20	25-65
II	1	1	1	2-4	1	5.8-15

strains showed negligible inoculum effects on the MIC's of nafcillin and

cefoxitin. Strains in group I exhibited an inoculum effect with cephalothin, a greater effect with cefamandole, and a marked influence of inoculum size on MIC's of cefaloridine and cefazolin. Group Ia strains were similar to those in group I except for a lesser inoculum effect when tested with cefazolin. Group II strains exhibited no inoculum effect with cephalothin and cefazolin, a small effect with cefamandole, and smaller increase in cephaloridine MIC with inoculum than seen in the other two groups. Extracts of group I and group IA stains had detectable beta-lactamase activity that could be enhanced by prior exposure of the bacteria to subinhibitory concentrations of cefazolin. Extracts of uninduced group II strains had no significant beta-lactamase activity, but enzyme activity in cefazolin-induced cells was even higher than for similarly treated strains in groups I and IA.

The same investigators studied the efficacy of these six antibiotics in the treatment of mice infected by intraperitoneal injection of group I or group II strains of S. aureus (23). The drugs were administered in doses chosen to yield roughly equivalent ratios of peak serum level to MIC (for an inoculum of 100,000 cfu/ml) for each antibiotic. In mice infected with group I strains survival was greatest in animals receiving nafcillin, followed, in order of decreasing efficacy, by cephalothin or cefoxitin, cefamandole, and cephaloridin or cefazolin. Among mice infected with group II strains, five of the six drugs showed comparable efficacy, but cefoxitin therapy was significantly inferior to the others (Figure 5). Survival of infected mice

Figure 5. Cumulative mortality as a function of antibiotic therapy among mice infected with S. aureus of group I (left) or group II (right) (23).



treated with cefazolin improved with increasing dose of the drug. The effect of cefazolin dose on outcome was most marked in infections with group I strains and least important with group II infections. The relevance of these studies to the therapy of naturally acquired staphylococcal infections in man remains to be established. However, poor results have been reported by some investigators in the therapy of serious staphylococcal disease with relatively beta-lactamase-susceptible cephalosporins such as cefamandole (24).

A role for extracellular beta-lactamase in the pathogenesis of colitis produced by Clostridium difficile toxin has been suggested. The Syrian hamster provides an experimental model for pseudomembranous colitis. Administration of ampicillin to these animals reproducibly provoked fatal C. difficile-associated ileocolitis despite the fact that isolates of this bacterium were uniformly susceptible to the antibiotic. Control animals lacked detectable beta-lactamase, C. difficile, and C. difficile cytotoxin in the cecum. Sequential monitoring of the cecal contents of ampicillin-treated animals revealed the appearance of detectable beta-lactamase activity as a precursor to rising titers of C. difficile and of its cytotoxin. Ampicillin concentrations in the cecal contents fell as beta-lactamase activity rose. The cecal filtrate was found to inactivate ampicillin, amoxicillin, azlocillin, cefazolin, cephalothin, cyclacillin, penicillin G, mezlocillin, ticarcillin, carbenicillin, cefoperazone, cefoxitin, moxalactam, and oxacillin but not ceftizoxime or cephalexin (25).

Sulbactam is a poorly absorbed antibiotic that also acts as a noncompetitive, irreversible beta-lactamase inhibitor. It has little antibacterial activity against C. difficile. Administration of sulbactam by itself induced fatal ileocolitis in Syrian hamsters. However, animals given concurrent ampicillin and sulbactam maintained detectable fecal concentrations of ampicillin and did not develop ileocolitis until after these drugs were discontinued (25).

The same investigators tested feces from patients for beta-lactamase activity. Fecal enzyme activity was detected in ten of 11 patients receiving ampicillin, two of three on cefoxitin, and one receiving oxacillin. No beta-lactamase was found in feces from three patients taking clindamycin, one receiving cefazolin, and six control subjects not on antibiotics. Two patients receiving ampicillin and one receiving clindamycin developed C. difficile pseudomembranous colitis. Fecal beta-lactamase activity was confirmed in only one of the two ampicillin patients with colitis, however the specimen tested in the other patient was obtained three days after discontinuation of the antibiotic (25).

Beta-lactamases in Mycobacterium are characteristically cell-bound and constitutive (20).

Strains of gram-negative bacteria are frequently encountered in which constitutive synthesis of beta-lactamase is mediated by structural genes borne on plasmids. The TEM-1 beta-lactamase, which has been found in many species of gram-negative bacteria (20), is the most prevalent plasmid-mediated beta-lactamase in ampicillin-resistant E. coli (19). This enzyme was named after an ampicillin-, and streptomycin-resistant strain of E. coli, designated TEM, isolated in Athens, Greece, in 1963 from a woman

named Temoniera (26). In addition to its presence in enteric bacteria, ampicillin resistance due to TEM-1 was reported in Haemophilus influenzae in 1972, in Neisseria gonorrhoeae in 1976, and in N. meningitidis in 1983 (19).

Many gram-negative species have chromosomal genes that code for inducible beta-lactamase. Regardless of the site of the structural gene for the enzyme, however, beta-lactamase produced by most species of gram-negative bacteria is thought to be largely retained within the cell. At least for plasmid-coded enzymes, the intracellular localization is typically considered to be the periplasmic space, which lies between the cell wall and the outer membrane. Detection of significant quantities of extracellular enzyme is thought to reflect spillage from disrupted bacterial cells (20).

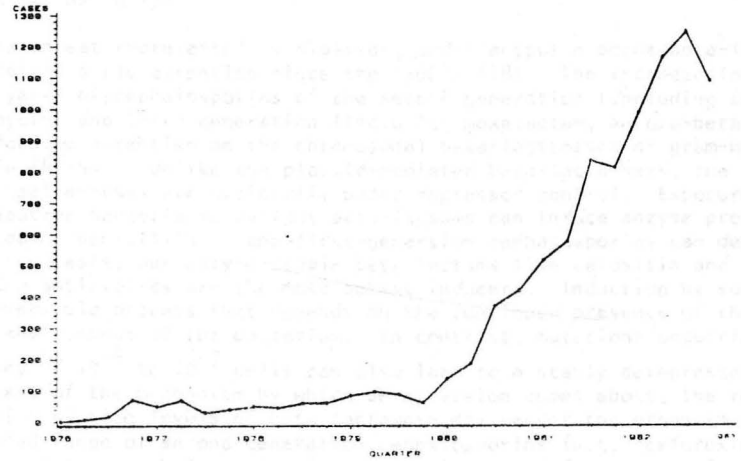
The chromosomal inducible beta-lactamase (type Id) of P. aeruginosa displays homology with the chromosomal ampC beta-lactamase gene product. The Id enzyme in Pseudomonas is exceptional in that appreciable quantities of the enzyme leak out into culture medium. When broth containing penicillin G is inoculated with P. aeruginosa, the bacterium is not killed, but bacterial replication is delayed until enzymatic hydrolysis of the antibiotic in the medium is complete. The MIC of penicillin G for this organism can be greatly reduced by blocking the Id enzyme with methicillin or cloxacillin. Difficulties experienced in obtaining mutants of P. aeruginosa deficient in this enzyme suggest that it may play some essential role in the metabolism of the cell apart from inactivating beta-lactam antibiotics (4).

Just as little extracellular beta-lactamase activity is normally detectable in supernates of cultures of gram-negative bacteria, these organisms appear to possess a barrier to the intracellular access of beta-lactam drugs. This phenomenon, designated the crypticity factor, is reflected in the increase in the apparent enzyme activity per unit weight of bacteria upon disruption of the bacterial cells (20).

In contrast to the situation with gram-positive organisms, then, beta-lactamase production by a given gram-negative bacterial cell protects only itself. However, relatively low levels of enzyme production may suffice to inactivate the small proportion of ambient antibiotic that actually penetrates into the bacterium (20).

The clinical relevance of beta-lactamase production by baterial pathogens is exemplified by the emergence of penicillinase-producing N. gonorrhoeae (PPNG) (27). The first reports of PPNG were made in the United States and England in 1976. Strains in this country resembled PPNG from East Asia, in which the beta-lactamase gene is on a 4.4-megadalton plasmid that can be mobilized by an accompanying 24.5-megadalton plasmid. Strains in the United Kingdom were like PPNG from West Africa, carrying a 3.2-megadalton self-mobilizing plasmid coding for beta-lactamase (28). Since tetracycline resistance is common in PPNG strains in the United States, PPNG infections must be treated with relatively costly parenteral drugs (29), including spectinomycin (30, 31), cefoxitin (32), and third-generation cephalosporins (33-35). Pharyngeal infections are treated with trimethoprim-sulfamethoxazole (36). After initial success in containing PPNG to a few cities experiencing high rates of importation, the incidence of PPNG infections in the United States increased dramatically in 1981 (Figure 6) (37, 38).

Figure 6. Reported PPNG cases by quarter, United States (including Guam, Puerto Rico, Virgin Islands, and the Pacific Trust Territories), January 1976--December 1982 (37).



Centers for Disease Control, 1983. Morbidity and Mortality Weekly Report.

The epidemic of PPNG infections in the United States is thought to have arisen by dissemination of imported strains of resistant gonococci rather than by transmission of R plasmids between strains of N. gonorrhoeae (39). However, the possibility that commensal Neisseira species might serve as alternative hosts for gonococcal R plasmids has been studied in vitro. Conjugation of N. gonorrhoeae bearing a 4.4-megadalton beta-lactamase plasmid and a 24.5-megadalton conjugal plasmid was attempted with a variety of recipient species. Some, but not all strains of N. cinerea, N. flava, N. mucosa, N. subflava, and N. perflava/sicca were able to serve as recipients for the R plasmid. The plasmid was found to be stable in N. flava and N. cinerea, less so in N. subflava, and unstable in N. mucosa and N. perflava/sicca. A transconjugant of N. cinerea was found to transfer the 4.4-, and 24.5-megadalton plasmids back into N. gonorrhoeae. No transfer of the beta-lactamase plasmid to strains of the commensals N. flavescens, N. lactamica, and Branhamella catarrhalis was observed. Attempts to transfer the R plasmid to 13 representatives of N. meningitidis serogroups A, B, C, D, X, Z, and W135 were also unsuccessful (40).

Recognition in 1976 of the apparently coincidental emergence of two different strains of PPNG in East Asia and West Africa prompted study of the molecular basis for beta-lactamase production in the two strains. DNA hybridization studies revealed that the East Asian plasmid, comprising 7.4 kilobases, was found to be a combination of the 5.3-kilobase West African plasmid and a 2.1-kilobase insertion. Both plasmids contained approximately 40% of a transposon designated TnA, including the bla gene, which encodes for the TEM-1 beta-lactamase (41). Thus the R plasmids in the classic PPNG

strains from East Asia and West Africa are very closely related. Furthermore, they derive their capacity to synthesize beta-lactamase production from the same gene that was first recognized in a strain of E. coli in Athens in 1963.

The threat represented by plasmid-, and transposon-borne beta-lactamases has received ample attention since the 1960's (18). The introduction in recent years of cephalosporins of the second generation (including cefoxitin, a cefamycin) and third generation (including moxalactam, an oxa-beta-lactam) has refocused attention on the chromosomal beta-lactamases of gram-negative bacteria (42-44). Unlike the plasmid-mediated beta-lactamases, the chromosomal enzymes are ordinarily under repressor control. Exposure of gram-negative bacteria to various beta-lactams can induce enzyme production. Cefamandole, penicillin G, and first-genertion cephalosporins can derepress enzyme synthesis, but enzyme-stable beta-lactams like cefoxitin and certain new penem antibiotics are the most potent inducers. Induction by substrate is a reversible process that depends on the continued presence of the inducer in the environment of the bacterium. In contrast, mutations occurring at a frequency of 10^{-6} to 10^{-7} cells can also lead to a stably derepressed state. Regardless of the mechanism by which derepression comes about, the resultant production of high levels of beta-lactamase may render the organism resistant to a broad range of second-generation cephalosporins (e.g., cefuroxime), third-generation cephalosporins (e.g., moxalactam, cefotaxime, ceftriaxone, ceftazidime), antipseudomonal penicillins (e.g., carbenicillin, ticarcillin, mezlocillin, piperacillin, azlocillin), and even the monobactams (e.g., aztreonam).

One of the most striking examples of the importance of induction of chromosomal beta-lactamase in gram-negative bacteria has been provided by the experience with cefamandole (44). The principal attractiveness of cefamandole was supposed to be its extended bacterial spectrum, ecompassing genera that are routinely resistant to ampicillin and first-generation cephalosporins, particularly Enterobacter and Serratia. Derepression of chromosomal beta-lactamase is believed to be important in reports of discrepant results among various tests of cefamandole susceptibility, cefamandole treatment failures in experimental and naturally acquired infections due to ostensibly susceptible organisms, and the rapid emergence of resistant strains in vitro and in vivo upon exposure to this drug. These disturbing phenomena have characteristically been associated with bacteria in the extended spectrum of cefamandole, including Enterobacter, Serratia, C. freundii, indole-positive Proteus, and Providencia, as well as P. aeruginosa. Since the principal advantage of cefamandole is supposed to be its extension of the cephalosporin spectrum to include Enterobacter and Serratia, the utility of this antibiotic is open to doubt, as relected in the following case report:

> H. L. J. (PMH 108255) was a 28-year-old junkyard cashier with hemoglobin SC who was admitted to Parkland Memorial Hospital on May 19, 1982, with a three-day history of fever to 40°C, diaphoresis, anorexia, a productive cough, vomiting, and generalized pains. He took amphetamines intravenously and had a history of hepatitis and syphilis. The right kidney has been nonfunctional since childhood. Physical examination revealed an agitated, uncooperative black man

in evident pain. His rectal temperature was 39.7°C, and his respiratory rate was 18/min; his blood pressure and pulse changed from 140/90 mm Hg and 104/min to 130/80 mm Hg and 120 as he sat up. There was a presystolic gallop. The admission urinalysis revealed 1+ proteinuria and 11-20 leukocytes per high-power field with rare bacteria. The hemoglobin was 13.1 g/dl, the hematocrit was 40.6, and there was a leukocytosis of 21,000 cells/ul (78% segmented neutrophils, 8% bands, 8% lymphocytes, 2% eosinophils, and 2% monocytes). The sedimentation rate was 21 mm/h. The bilirubin was 1.5 mg/dl, the aspartate aminotransferase 202 IU/l, and the amylase 147 U/l.

The patient was initially considered to be suffering from sickle pain crisis. However, on the second hospital day four of four blood cultures obtained in the emergency room were reported to be growing gram-negative rods, and therapy with tobramycin and ticarcillin was initiated. The isolate was identified as E. cloacae susceptible to aminoglycosidic aminocyclitols and to carbenicillin (MIC of 2 ug/ml), cefamandole (2 ug/ml), cefoxitin (16 ug/ml), piperacillin (2 ug/ml), moxalactam (1 ug/ml), and cefotaxime (8 ug/ml). A bone scan performed on the third hospital day showed increased uptake in the shoulders, humeri, left femur, and knees interpreted as most consistent with infarction. However, by the sixth hospital day the patient's initially generalized discomfort had given way to pain localized to the right shoulder. No abnormality was appreciated on a radiograph of the right shoulder, but when the examination was repeated on the seventh hospital day, a cortical defect was evident on the anteromedial aspect of the right humerus.

One day later a Craig needle biopsy of the right humerus yielded chocolate-brown bloody material that was positive for E. cloacae with the following sensitivities: carbenicillin (16 ug/ml), cefamandole (8 ug/ml), cefoxitin (not inhibited), piperacillin (2 ug/ml), moxalactam (1 ug/ml), and cefotaxime (8 ug/ml). Because of continued fever and purulent drainage from the humerus, on the tenth hospital day the bone was incised and drained with opening of a cortical window. Meanwhile, ticarcillin and tobramycin were discontinued and therapy with cefamandole was begun. Within three days, the patient had become afebrile. However, copious pus obtained at surgery subsequently yielded E. cloace that was even less susceptible to beta-lactams: carbenicillin (64 ug/ml), cefamandole (not inhibited), cefoxitin (not inhibited), piperacillin (128 ug/ml), moxalactam (8 ug/ml), and cefotaxime (128 ug/ml). The patient found that he could enhance drainage from the wound by doing pushups each day. A wound isolate on the seventeenth hospital day had an antibiogram similar to that of the organism recovered at surgery. When the eivdence of resistance in vitro was noted on hospital day 24, cefamandole was discontinued and tobramycin was reinstituted and continued for 66 more days. A wound isolate obtained one day after resumption of tobramycin was still more resistant to beta-lactams: carbenicillin (256 ug/ml), cefamandole (not inhibited), cefoxitin (not inhibited), piperacillin (256 ug/ml), moxalactam (16 ug/ml), and cefotaxime

(not inhibited). On the twenty-ninth hospital day the patient sustained a pathologic fracture of the right humerus while reaching for a bottle of cola, and his fever recurred. On hospital day thirty-five, operative drainage of the humerus was repeated. A bone culture yielded a light growth of E. cloacae: carbenicillin (64 ug/ml), cefamandole (not inhibited), cefoxitin (not inhibited), piperacillin (256 ug/ml), moxalactam (16 ug/ml), cefotaxime (128 ug/ml), and cefoperazone (128 ug/ml). Ticarcillin was added to the tobramycin for 33 days beginning on the thirty-eighth day, by which time the patient had again become afebrile. Moxalactam was added to the tobramycin for the last 13 days of antibiotic therapy. On August 18 the patient was discharged afebrile with a serum creatinine of 1.1 mg/dl. At the time of his readmission for pulmonary tuberculosis on February 22, 1983, there was no allusion to any residual problem with the right arm apart from a notation of "mild" right-sided weakness on the neurologic examination.

Presumably, many strains of gram-negative bacteria that are isolated from untreated patients are not actively synthesizing chromosomal beta-lactamase and are susceptible to inhibition upon abrupt exposure to high concentrations of antibiotic in vitro. Thus, the clinical microbiology laboratory may report them to be susceptible to a drug like cefamandole. In the patient, however, a subpopulation of bacteria may survive initial exposure to the antibiotic long enough for enzyme induction to occur, leading to the accumulation of sufficient enzyme to inactivate the drug. If these induced strains are subsequently isolated from the patient and submitted to testing in vitro, their true antibiotic resistance potential may become evident.

Induction of broad-spectrum beta-lactam resistance in vivo is particularly characteristic of certain drug-pathogen combinations. Emergence of resistance, relapses, and treatment failures have been reported in infections with P. aeruginosa in association with carbenicillin, ticarcillin, or piperacillin therapy. This problem has been particularly frequent with Pseudomonas infections outside the urinary tract. Derepression of chromosomal beta-lactamase is also considered to be important in unsatisfactory results reported in the treatment of Enterobacter and Serratia infections with cefoxitin or cefamandole and in the therapy of Pseudomonas infections with cefotaxime and moxalactam. Similar reports exist regarding newer cephalosporins, including ceftazidime, ceftriaxone, and ceftizoxime, and the monobactam aztreonam (43, 44).

Sykes and Bush have systematically analyzed the stability of selected third-generation cephalosporins in the presence of enzymes from beta-lactamase-producing gram-negative bacilli and have examined the influence of bacterial inoculum size on the MIC's of third-generation cephalosporins for these beta-lactamase-positive strains (45). Among the plasmid-coded enzymes, the broad-spectrum beta-lactamases including TEM-1 and the oxacillinase OXA-2 showed little activity against three aminothiazole oxime compounds (cefotaxime, ceftizoxime, ceftazidime) and moxalactam, but hydrolysis of cefoperazone was comparable to that of the first-generation compound cephaloridine (Table 5). Of four carbenicillinases tested, PSE-2 was active against cefotaxime and ceftizoxime as well as cefoperazone, but ceftazidime and moxalactam were poor substrates.

Table 5. Relative efficiency* of plasmid-mediated beta-lactamases from gram-negative bacteria in hydrolysis of selected cephalosporins (45).

	Beta-lactamase		
Antibiotic	Broad-spectrum (TEM-1, TEM-2, SHV-1)	Oxacillinase (OXA-2)	Carbenicillinase (PSE-1, PSE-2, PSE-3, PSE-4)
Cephaloridine	100	100	100
Cefotaxime	0.07 - 0.14	6	0.03 - 40
Ceftizoxime	0.03 - 0.2	5	<0.01 - 60
Ceftazidime	<0.01 - 0.04	<0.5	<0.01 - <3
Cefoperazone	60 - 150	250	6 - 1900
Moxalactam**	<0.1	<0.1	<0.1
Cefsulodin			5 - 20

*Relative efficiency = $\frac{100(\text{efficiency of hydrolysis of substrate})}{(\text{efficiency of hydrolysis of cephaloridine})}$

**Relative V_{max} = $\frac{(V_{max}/\text{ul enzyme}) \text{ of substrate}}{(V_{max}/\text{ul enzyme}) \text{ of cephaloridine}}$

Marked differences among bacterial genera were observed in the activity of chromosomal beta-lactamases against three aminothiazole oxime cephalosporins and cefoperazone (Table 6) (45). Enzyme from E. coli was

Table 6. Relative efficiency* of chromosomal beta-lactamases from gram-negative bacteria in hydrolysis of selected cephalosporins (45).

	Genus					
Antibiotic	Klebsiella	Proteus	Serratia	Entero-bacter	Providencia	Bacteroides
Cephaloridine	100	100	100	100	100	100
Ceftazidime	0.01	<0.1	<0.2	0.04 - 0.2	1.3	4
Cefotaxime	5	0.6	5	0.02 - 5	3	30
Ceftizoxime	0.05	3**	7	3 - 20	2	12
Cefoperazone	30	>1	30	5 - 7	5	40

*Relative efficiency = $\frac{100(\text{efficiency of hydrolysis of substrate})}{(\text{efficiency of hydrolysis of cephaloridine})}$

**Nonlinear kinetics.

inactive against all four substrates. Strains of Klebsiella producing the K1 enzyme hydrolyzed cefoperazone and, to some extent, cefotaxime. The inducible enzymes produced by P. vulgaris and Morganella morganii hydrolyzed cefotaxime and ceftizoxime slowly; hydrolysis of cefoperazone displayed

nonlinear kinetics. All four drugs were hydrolyzed to some degree by chromosomal enzymes from P. stuartii and P. rettgeri. All Enterobacter strains produce an inducible chromosomal beta-lactamase designated E1, which is relatively effective against ceftizoxime and cefoperazone. The constitutive P99 Enterobacter enzyme had activity against all four drugs but is less prevalent. Chromosomal enzyme from S. marcescens was inactive against ceftazidime. The chromosomal beta-lactamase of P. aeruginosa is inhibited by the drugs used in this study. Neither the aminothiazole oxime cephalosporins nor cefoperazone were stable in the presence of B. fragilis beta-lactamase.

The results of analyses of enzyme substrate profiles were paralelled by the data on drug MIC's for groups of 20 beta-lactamase producing strains of selected genera of gram-negative bacilli (45) (Table 7). Twenty strains of E. coli bearing R plasmids were uniformly susceptible to the aminothiazole oxime compounds cefotaxime, ceftizoxime, and ceftazidime, but the MIC's of cefoperazone required to inhibit 50% or 90% of the strains rose markedly with increasing bacterial inocula. Although some inoculum effect was observed with each compound, the most Klebsiella strains were also susceptible to the aminothiazole oxime cephalosporins, although not to cefoperazone. Cetazidime was the most active drug against 20 strains of P. vulgaris and M. morganii analyzed together. An inoculum effect was seen with each drug but was most pronounced with cefotaxime and with cefoperazone, the least active antibiotic. Ceftazidime was the most active compound against strains of Providencia; cefoperazone was least active and showed the greatest inoculum effect. Many of the 20 strains of Enterobacter tested, which frequently carried plasmids coding for beta-lactamases, were resistant to all four antibiotics tested. Ceftazidime was the most effective drug against S. marcescens, whereas cefoperazone was relatively inactive. Most strains were susceptible to cefotaxime and ceftizoxime, but some inoculum effect was seen with both these drugs. Ceftazidime, cefsulodin, and cefoperazone showed good activity against plasmidless strains of P. aeruginosa. However, strains bearing carbenicillinase plasmids, particularly PSE-2, tended to be resistant to cefsulodin and even more so to cefoperazone, while retaining susceptibility to ceftazidime. Cefoxitin, which is highly resistant to B. fragilis beta-lactamase, was markedly superior to cefotaxime, ceftizoxime, cefoperazone, and ceftazidime in inhibiting this anaerobe.

A distinction has been reported in the beta-lactam resistance evoked in Enterobacter depending upon whether the organism is exposed to cefoxitin or to cefamandole. Cefoxitin is relatively stable to beta-lactamase but has little intrinsic activity against Enterobacter. Exposure of Enterobacter to cefoxitin typically results in induction of beta-lactamase production that is reversible upon removal of the antibiotic from the environment of the bacterium. In contrast, cefamandole is more readily hydrolyzed by beta-lactamase but also has good antibacterial activity against Enterobacter. This antibiotic is a weak inducer of beta-lactamase, and the majority of resistant strains that emerge upon exposure to cefamandole are stably derepressed mutants (46).

Induction of chromosomal beta-lactamases may create a problem even when the inducer is resistant to hydrolysis by the enzyme. As noted above, it is such enzyme-resistant drugs, such as cefoxitin, that tend to be the best inducers. Thus combination therapy using cefoxitin plus another beta-lactam

presents the risk of antagonism through inactivation of the second antibiotic by beta-lactamase elicited by the enzyme-stable drug (43). The recently introduced acylampicillins mezlocillin, piperacillin, and azlocillin are all susceptible to beta-lactamase. Piperacillin, in comparison to carbenicillin, is a relatively weak inducer of beta-lactamase. However, the combination of cefoxitin with piperacillin or mezlocillin is antagonistic against some strains of P. aeruginosa, S. marcescens, Enterobacter, and indole-positive Proteus, although not usually against Klebsiella or E. coli (47). It should be noted that the first four taxa are uniformly resistant to first-generation cephalosporins, whereas the latter two are not.

Since there is no theoretical reason why such antagonism must be limited solely to beta-lactam combinations that include cefoxitin, the suggestion has been made that the use of two beta-lactams simultaneously should be avoided in the absence of clinical evidence of therapeutic superiority of such a regimen (43). In fact, antagonism between acylampicillins and cephalothin or cefamandole and, less commonly, between acylampicillins and cefotaxime or moxalactam has also been reported (47).

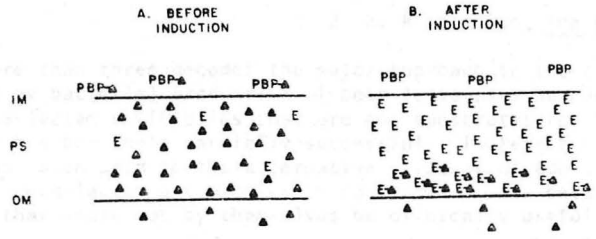
In addition to specific induction of beta-lactamase production by exposure of bacteria to beta-lactam drugs, nonspecific enzyme induction in vitro has also been reported. A strain of E. cloacae isolated from a patient in Germany was found to display high-level resistance to most beta-lactam drugs. Beta-lactamase activity in cell-free supernates of cultures of this strain in isosensitest broth (Oxoid) was markedly increased by adding a subinhibitory concentration of cefoxitin to the medium. However, enzyme induction was also observed when the medium was supplemented with heat-inactivated serum or with pleural fluid, cerebrospinal fluid, or urine. Furthermore, the highest levels of beta-lactamase activity were detected in supernates of cultures of the organism in another medium, Schaedler's broth, without supplementation with beta-lactams or body fluids. This report raises the possibility that induction of beta-lactamase production might occur in vivo even without exposure of the organism to antibiotics (48).

Induction of chromosomal beta-lactamases is thought to confer resistance to cefamandole because the antibiotic is enzymatically hydrolyzed. However, there is an apparent paradox in the resistance of gram-negative bacteria such as Enterobacter, Serratia, and Pseudomonas to certain beta-lactam antibiotics that do not appear to be substrates of the beta-lactamases produced by these organisms. Some workers have suggested that, after induction, sufficiently large amounts of enzyme might accumulate in the periplasmic space to neutralize antibiotic molecules nonhydrolytically simply by blocking their access to the bacterial penicillin-binding proteins attached to the cytoplasmic membrane (42-44, 49) (Figure 7).

Evidence that such nonhydrolytic trapping of antibiotic by beta-lactamase may occur has been sought in a model employing a susceptible strain of E. coli grown in broth. Addition of a cephalosporin to the broth lysed the bacteria. Lysis by cephalothin was prevented by concomitant addition of a crude extract of E. cloacae having beta-lactamase activity and capable of hydrolyzing the antibiotic. However, lysis of E. coli by ceftriaxone, cefotaxime, and moxalactam, which are relatively resistant to hydrolysis, was also prevented by beta-lactmase, albeit only at relatively high concentrations of enzyme. Oxacillin, which was resistant to hydrolysis

by the beta-lactamase, did not by itself lyse E. coli. However, addition of oxacillin to cultures containing both a cephalosporin and beta-lactamase reversed the protective effect exerted by the enzyme. These results suggest that the bactericidal effect of beta-lactams like ceftriaxone can be nonhydrolytically blocked by beta-lactamase through formation of a reversible enzyme-antibiotic complex that is susceptible to competitive inhibition by excess oxacillin (50). In this experimental system, gram-negative bacteria are exposed to high extracellular concentrations of beta-lactamase and antibiotic. The relevance of this model to the interaction of enzyme and drug in the periplasmic space of bacteria that are present in infected tissues remains to be established.

Figure 7. Model of mechanisms responsible for resistance to substrate and nonsubstrate beta-lactam antibiotics produced by induction of beta-lactamase. A. Before induction little enzyme (E) is present in the periplasmic space (PS). Thus drug molecules (Δ) can pass freely through the outer membrane (OM) across the periplasmic space to their target penicillin-binding proteins (PBP) on the inner membrane (IM). B. After induction the high levels of enzyme in the periplamic space either hydrolyze substrate drugs or bind nonsubstrate drugs, preventing their access to target proteins (43).



Nikaido has recently commented that the kinetics of beta-lactam antibiotic entry into the periplasmic space and of beta-lactamase production are consistent with the hypothesis that low-level drug resistance is mediated by nonhydrolytic trapping (51). He argues, however, that trapping is unlikely to explain the frequent observation of the emergence of bacterial strains with very high MIC's of beta-lactams that are ostensibly refractory to hydrolysis by the enzymes that these strains produce. His alternative suggestion is that the low V_{max} of beta-lactamases from certain bacteria to beta-lactam antibiotics to which the organisms are resistant is an artefact of the assay conditions for the enzyme. According to Nikaido, these enzymes are conventionally assayed at substrate concentrations far in excess of those that are biologically relevant. With 0.1 uM substrate, hydrolysis of second-, and third-generation cephalosporins conventionally regarded as enzyme-stable is found to proceed at rates comparable to those observed with a first-generation drug like cefazolin (Table 7).

Table 7. Susceptibility of selected cephalosporins to hydrolysis by beta-lactamase (51).

Antibiotic	Relative V_{max}	V (molecules/cell/sec)	MIC (ug/ml) calculated	MIC (ug/ml) observed
Cefazolin	100	34,600	114	250
Cefoperazone	0.12	12,800	295	125
Cefoxitin	0.002	14,000	76	125
Cefotaxime	0.001	5,620	73	250
Ceftazidime	0.002	430	12	125

Beta-lactamase Inhibitors in Antibacterial Chemotherapy

> These bonds, in their own nature but weak, may nevertheless be made to hold, by the danger, though not by the difficulty of breaking them.
>
> Thomas Hobbes, Leviathan

> One Ring to rule them all, One Ring to find them,
> One Ring to bring them all and in the darkness bind them....
>
> J. R. R. Tolkien, The Lord of the Rings

For more than three decades the major approach to the clinical problem represented by bacterial production of beta-lactamases has been the search for new beta-lactam antibiotics that are not substrates for these enzymes. This effort has been only partially successful. In recent years increasing attention has been paid to the alternative strategy of administering enzyme-susceptible beta-lactam antibiotics in conjunction with beta-lactamase inhibitors that would not by themselves be clinically useful antibiotics (52).

Bush and Sykes have classified beta-lactamase inhibitors on the basis of their mechanisms of action (53). A reversible inhibitor (I) forms a fully reversible complex with the enzyme (E):

$$E + I \rightleftharpoons E \cdot I$$

Reversible inhibition may be either competitive or noncompetitive. A competitive inhibitor binds to the enzyme at the same site that binds the substrate. Thus, competitive inhibition is characteristically counteracted by the presence of an excess of substrate. Penicilloates, which lack a carbonyl group, bind to Bacillus cereus beta-lactamase I but cannot undergo hydrolysis and are thus examples of competitve inhibitors (53).

Certain antibiotics, including isoxazolyl penicillins (e.g., cloxacillin), cefoxitin, and aztreonam, behave like competitive beta-lactamase inhibitors in some systems. However, these compounds can be shown to undergo slow enzymatic hydrolysis and are therefore distinguished by Bush and Sykes as competitive substrates (53).

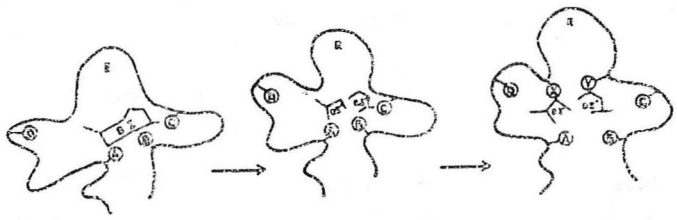
Noncompetitive inhibitors are believed to bind to the enzyme without occupying the entire active site. Resultant conformational change in the enzyme is thought to be responsible for loss of catalytic activity. Noncompetitive inhibition is recognized by its persistence in the presence of excess substrate (53).

Irreversible inhibitors inhibit beta-lactamase by binding covalently to the enzyme. Removal of excess inactivator does not restore catalytic activity:

$$E + I \rightleftharpoons E'I \longrightarrow E\text{-}I^{*} \text{ (inactive)}$$

Three categories of irreversible inhibitors have been delineated. Amino acid modifiers like p-chloromercuribenzoate, which reacts with cysteine residues in some enzymes, inactivate beta-lactamase by binding to one or more amino acids at the active site or elsewhere. Active-site-directed inhibitors are compounds that resemble normal substrate but that contain a reactive moiety that mediates covalent binding to the enzyme after initial binding at the active site. Suicide inhibitors bind at the active enzyme site and are then catalytically converted to reactive molecules that attach covalently to the enzyme (Figure 8).

Figure 8. The active site of a beta-lactamase in the presence of a suicide inactivator (53).



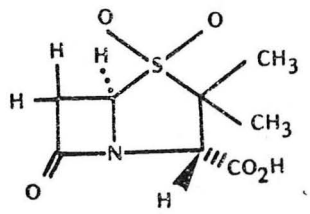
One candidate for an example of suicide inhibitors is the antibiotic moxalactam. This drug resists inactivation by a broad range of gram-negative bacterial beta-lactamases (54), although it is hydrolyzed by the PSE-2 and PSE-3 carbenicillinases (55). The drug does appear to inhibit certain beta-lactamases (55, 56), and the suggestion has been made that the inactivation is mediated by suicide inhibition (56), although this contention has been questioned (53).

Two examples of drugs clearly proven to inactivate beta-lactamase by suicide inhibition are sulbactam and clavulanic acid (53).

Sulbactam (CP-45,899) is a penicillanic acid sulfone (Figure 9) that has intrinsic antibacterial properties as well as beta-lactamase inhibitory

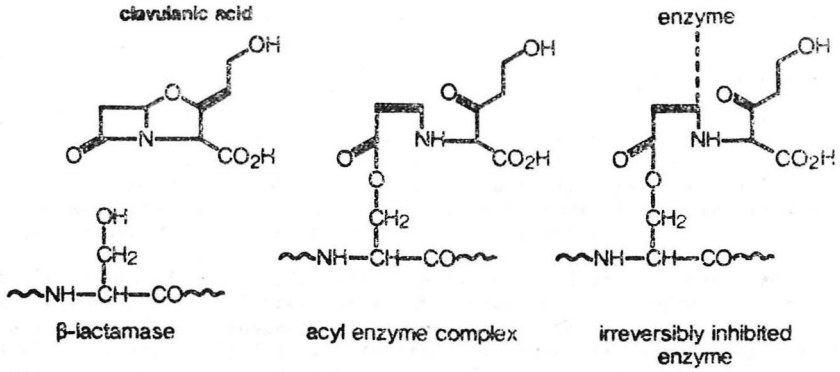
activity (57, 58). Combinations including this drug have not to date been marketed in the United States.

Figure 9. Sulbactam (57).



Clavulanic acid is a beta-lactam drug (Figure 10) with negligible intrinsic antibacterial activity that can, however, irreversibly bind and inactivate certain beta-lactamases (59-62).

Figure 10. Mode of action of clavulanic acid (61).



The combination of amoxcillin trihydrate with potassium clavulanate has recently been marketed in the United States as Augmentin (Beecham). The approved indications (59, 63-64) include otitis media (66-67), sinusitis (68), and infections of the lower respiratory tract (69-74), skin and soft tissues (75-79), and urinary tract (80-88).

The antibacterial spectrum of amoxicillin includes S. pyogenes, S. pneumoniae, and other streptococci, and strains of Staphylococcus, Neisseria gonorrhoeae, H. influenzae, E. coli, and P. mirabilis that do not produce beta-lactamase. The addition of potassium clavulanate extends the in-vitro spectrum of the antibiotic to include beta-lactamase-positive strains of B. catarrhalis, N. gonorrhoeae (89), H. influenzae, E. coli (62), Proteus, and K. pneumoniae as well as B. fragilis. The combination also inhibits beta-lactamase-producing strains of S. aureus and S. epidermidis that are susceptible to methicillin (59, 63).

Methicillin-resistant staphylococci, in which resistance is mediated by mutations in the penicillin-binding proteins, are resistant to amoxicillin with or without potassium clavulanate. P. aeruginosa and many strains of Serratia and Enterobacter are also resistant to the combination (59, 63).

Synergistic activity of amoxicillin and clavulanate in vitro has been reported in beta-lactamase-producing strains of H. ducreyi, the etiologic agent of chancroid (90).

Amoxicillin inhibited only four of 15 strains of M. tuberculosis grown in broth and was bactericidal against none. However, the combination of amoxicillin and clavulanate was bactericidal against 14 of the 15 strains at amoxicillin concentrations of 4 ug/ml or less and clavulanate concentrations of 2 ug/ml or less (91). The addition of clavulanate to amoxicillin or cephalothin also reduced the MIC of these two drugs against M. fortuitum (92).

Legionella pneumophila, which produces a beta-lactamase that is chiefly active against cephalosporins (93, 94), is also susceptible to amoxicillin-clavulanate in vitro (59, 94, 95), as are several other species of Legionella (95). However, there is no evidence that this combination is efficacious in the therapy of Legionnaires' disease (95). Infection with L. pneumophila responds poorly to therapy with a number of drugs, including penicillins and aminoglycosidic aminocyclitols (96-98), to which the organism is susceptible in vitro and in ovo (99-103). L. pneumophila is a facultative intracellular pathogen (104-106), and the haven afforded by this intracellular niche may be the most important determinant of response to antimicrobial therapy in infections with this organism (107). At present, amoxicillin-clavulanate cannot be recommended for the therapy of legionellosis.

The efficacy of clavulanic acid in antimicrobial therapy has been studied in an experimental model in which subcutaneous abscesses are induced in mice by injection of suspensions of selected bacteria. The combination of clavulanic acid and penicillin was superior to penicillin monotherapy in the treatment of abscesses caused by S. aureus, E. coli, or K. pneumoniae as well as by B. fragilis alone or in mixed infections with S. pyogenes, S. faecalis, S. aureus, E. coli, or K. pneumoniae (Table 8). Clavulanic acid alone had no therapeutic effect. If similar results are shown to be attainable in the treatment of naturally acquired abscesses in man, the combination of clavulanic acid with a beta-lactam drug could provide an alternative to regimens employing toxic aminoglycosidic aminocyclitols in the therapy of mixed infections with obligate anaerobes and enteric bacteria (108).

Table 8. Effect of antimicrobial therapy on abscess size in mice infected subcutaneously with selected bacteria (108).

Bacteria	Therapy	
	Penicillin	Penicillin + Clavulanic acid
B. fragilis	1.10*	0.30
S. pyogenes	0.02	0.001
S. faecalis	0.02	0.02
S. aureus	0.88	0.06
E. coli	1.00	0.17
K. pneumoniae	0.96	0.01
B. fragilis and		
S. pyogenes	0.76	0.02
S. faecalis	0.54	0.02
S. aureus	1.14	0.22
E. coli	1.20	0.18
K. pneumoniae	1.00	0.06

*Ratio of abscess diameters in treated animals to abscess diameters in untreated animals infected with the same organisms.

Amoxicillin and clavulanate are rapidly absorbed and can be taken with meals. Both compounds are widely distributed in body tissues outside the central nervous system (59, 109-111).

The combination is marketed as tablets of 250 or 500 mg amoxicillin plus 125 mg clavulanate. It is important to note that two 250-mg tablets contain twice the clavulanate of a singe 500-mg tablet. The combination is also available as an oral suspension in two concentrations: 125 mg amoxicillin and 31.25 clavulanate per 5 ml and 250 mg amoxicillin plus 62.5 mg clavulanate per 5 ml. Thus, the ratio of amoxicillin to clavulanate in both oral suspensions is the same as that in the 500-mg tablet. The ususal adult dose, expressed in terms of the amoxicillin content, is 250 mg q8h. Severe infections may be treated with 500 mg q8h. The recommended pediatric dose is 20-40 mg/kg/day in three divided doses (59).

Both amoxicillin and clavulanate are chiefly eliminated by renal excretion. Probenecid retards the renal elimination of amoxicillin but not of clavulanate. Both substances accumulate in patients with renal insufficiency (59, 111). The half-life of amoxicillin was 7.6 h in 3 patients with a mean creatinine clearance of 11 ml/min in comparison with 1.2 h in 23 patients with a mean creatinine clearance of 100 ml/min. The half-life of clavulanate was 2.0 h and 1.5 h, respectively, in the same two groups (111). Since amoxicillin-clavulanate is available only in fixed-dose combinations, use of the combination in patients with advanced renal disease would be quite complicated, since accumulation of the drugs would ordinarily not occur in parallel.

The various adverse reactions seen with administration of amoxicillin are all known or presumed to occur when it is combined with clavulanate. Furthermore, the addition of clavulanate increases the attack rate of

diarrhea seen when amoxicillin alone is prescribed (59, 64).

The Medical Letter regards amoxicillin-clavulanate as "a reasonable choice for treatment of bite wounds [112], otitis media, sinusitis, and some lower respiratory tract and urinary tract infections," but adds cautionary notes regarding the relatively high attack rate of gastrointestinal side effects and the availability of cheaper alternative therapies, including trimethoprim-sulfamethoxazole (59). There is obvious potential for irrational overuse of the combination, as for example in a proposal that it might be useful in the treatment of streptococcal pharyngitis (113).

References

1. Tomasz A. From penicillin-binding proteins to the lysis and death of bacteria: a 1979 view. Rev Infect Dis 1979;1:434-67.

2. Tomasz A. Penicillin-binding proteins in bacteria. Ann Intern Med 1982;96:502-4.

3. Dougherty TJ. Intrinsic resistance: penicillin target alterations and effects on cell wall synthesis. In: Leive L, Schlessinger D, eds. Microbiology--1984. Washington: American Society for Microbiology, 1984:398-401.

4. Sabath LD. Biochemical and physiologic basis for susceptibility and resistance of Pseudomonas aeruginosa to antimicrobial agents. Rev Infect Dis 1984;6:S643-56.

5. Sparling PF. Antibiotic resistance in Neisseria gonorrhoeae. Med Clin N Amer 1972;56:1133-44.

6. Jacoby GA. Introduction. In: Leive L, Schlessinger D, eds. Microbiology--1984. Washington: American Society for Microbiology, 1984:379-80.

7. Nikaido H, Rosenberg EY, Foulds J. Porin channels in Escherichia coli: studies with β-lactams in intact cells. J Bacteriol 1983;153:232-40.

8. Nikaido H, Rosenberg EY. Porin channels in Escherichia coli: studies with liposomes reconstituted from purified proteins. J Bacteriol 1983;153:241-52.

9. Nikaido H. Outer membrane permeability and β-lactam resistance. In: Leive L, Schlessinger D, eds. Microbiology--1984. Washington: American Society for Microbiology, 1984:381-4.

10. Yoshimura F, Nikaido H. Permeability of Pseudomonas aeruginosa outer membrane to hydrophilic solutes. J Bacteriol 1982;152:636-42.

11. Sarubbi FA Jr, Blackman E, Sparling PF. Genetic mapping of linked antibiotic resistance loci in Neisseria gonorrheae. J Bacteriol 1974;120:1284-92.

12. Dougherty TJ. Peptidoglycan biosynthesis in Neisseria gonorrhoeae strains sensitive and intrinsically resistant to β-lactam antibiotics. J Bacteriol 1983;153:429-35.

13. Tomasz A, Zighelboim-Daum S, Handwerger S, Liu H, Qian H. Physiology and genetics of intrinsic beta-lactam resistance in pneumococci. In: Leive L, Schlessinger D, eds. Microbiology--1984. Washington: American Society for Microbiology, 1984:393-7.

14. Sykes RB, Bush K. Interaction of new cephalosporins with β-lactamases and β-lactamase-producing gram-negative bacilli. Rev Infect Dis 1983;5:S356-67.

15. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. Nature 1940;146:837.

16. Sykes RB. The classification and terminology of enzymes that hydrolyze β-lactam antibiotics. J Infect Dis 1982;145:762-5.

17. Citri N, Pollock MR. The biochemistry and function of β-lactamase (penicillinase). Adv Enzymol 1966;28:237-323.

18. Falkow S. Infectious multiple drug resistance. London: Pion Limited, 1975.

19. Medeiros AA. Classification and distribution of beta-lactamases. In: Leive L, Schlessinger D, eds. Microbiology--1984. Washington: American Society for Microbiology, 1984:385-90.

20. Richmond MH, Sykes RB. The β-lactamases of gram-negative bacteria and their possible physiological role. Adv Microb Physiol 1973;9:31-88.

21. Thornsberry C, Gavan TL, Gerlach EH. New developments in antimicrobial agent susceptibility testing. In: Sherris JC, ed. Cumitech 6. Washington: American Society for Microbiology, 1977:1-13.

22. Anhalt JP, Sabath LD, Barry AL. Special tests: bactericidal activity, activity of antimicrobics in combination, and detection of β-lactamase production. In: Lennette EH, ed. Manual of clinical microbiology. Washington: American Society for Microbiology, 1980:478-84.

23. Chapman SW, Steigbigel RT. Staphylococcal β-lactamase and efficacy of β-lactam antibiotics: in vitro and in vivo evaluation. J Infect Dis 1983;147:1078-89.

24. Landesman SH, Corrado ML, Tillotson JR. Cefamandole in Staphylococcus aureus endocarditis. In: American Society for Microbiology. Program and abstracts, 20th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington: American Society for Microbiology, 1980:abstract 279.

25. Intestinal β-lactamase activity in ampicillin-induced, Clostridium difficile-associated ileocecitis. J Infect Dis 1983;147:227-35.

26. Datta N, Knontomichalou P. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature 1965;208:239-41.

27. Jaffe HW, Biddle JW, Johnson SR, Weisner PJ. Infections due to penicillinase-producing Neisseria gonorrhoeae in the United States: 1976-1980. J Infect Dis 1981;144:191-7.

28. Johnson SR, Anderson BE, Biddle JW, Perkins GH, DeWitt WE. Charactrization of concatemeric plasmids of Neisseria gonorrheae. Infect Immun 1983;40:843-6.

29. McCormack WM. Penicillinase-producing Neisseria gonorrhoeae--a retrospective. N Engl J Med 1982;307:438-9.

30. McCormack WM, Finland M. Spectinomycin. Ann Intern Med 1976;84:712-6.

31. Holloway WJ. Spectinomycin. Med Clin N Amer 1982;66:169-73.

32. Berg SW, Kilpatrick ME, Harrison WO, McCutchan JA. Cefoxitin as a single-dose treatment for urethritis caused by penicillinase-producing Neisseria gonorrhoeae. N Engl J Med 1979;301:509-11.

33. Handsfield HH. Treatment of uncomplicated gonorrhea with cefotaxime. Rev Infect Dis 1982;4:S448-52.

34. Tupasi TE, Crisologo LB, Torres CA, Calubrian OV. Comparison of spectinomycin, cefuroxime, thiamphenicol, and penicillin G in the treatment of uncomplicated gonococcal infections in women. J Infect Dis 1982;145:583.

35. Kerbs SB, Stone JR Jr, Berg Sw, Harrison WO. In vitro antimicrobial activity of eight new β-lactam antibiotics against penicillin-resistant Neisseria gonorrhoeae. Antimicrob Agents Chemother 1983;23:541-4.

36. Centers for Disease Control. Sexually transmitted diseases treatment guidelines 1982. Morbidity and Mortality Weekly Report 1982;31(2S).

37. Centers for Disease Control. Statistical tables, graphs, maps, and narratives for notifiable diseases in the United States. Morbidity and Mortality Weekly Report 1983;31(54):13-95.

38. Centers for Disease Control. Penicillinase-producing Neisseria gonorrhoeae--Los Angeles. Morbidity and Mortality Weekly Report 1983;32:181-3.

39. Handsfield HH, Sandström EG, Knapp JS, Perine PL, Ehittington WL, Sayers DE, Holmes KK. Epidemiology of penicillinase-producing Neisseria gonorrhoeae infections. Analysis by auxotyping and serogrouping. N Engl J Med 1982;306:950-4.

40. Genco CA, Knapp JS, Clark VL. Conjugation of plasmids of Neisseria gonorrhoeae to other Neisseria species: potential reservoirs for the β-lactamase plasmid. J Infect Dis 1984;150:397-401.

41. Dickgiesser N, Bennett PM, Richmond PM. Penicillinase-producing Neisseria gonorrhoeae: a molecular comparison of 5.3-kb and 7.4-kb β-lactamase plasmids. J Bacteriol 1982;151:1171-5.

42. Pechère J-C, Levesque R. β-lactamases: clnical and genetic perspectives. J Antimicrob Chemother 1983;12:529-34.

43. Sanders CC, Sanders WE Jr. Emergence of resistance during therapy with the newer β-lactam antibiotics: role of inducible β-lactamases and implications for the future. Rev Infect Dis 1983;5:639-48.

44. Sanders CC. Alterations in beta-lactamase control as a resistance mechanism. In: Leive L, Schlessinger D, eds. Microbiology--1984. Washington: American Society for Microbiology, 1984:391-2.

45. Sykes RB, Bush K. Interaction of new cephalosporins with β-lactamases and β-lactamase-producing gram-negative bacilli. Rev Infect Dis 1983;5:S356-67.

46. Gootz TD, Sanders CC, Goering RV. Resistance to cefamandole: derepression of β-lactamases by cefoxitin and mutation in Enterobacter cloacae. J Infect Dis 1982;146:34-42.

47. Drusano GL, Schimpff SC, Hewitt WL. The acylampicillins: mezlocillin, piperacillin, and azlocillin. Rev Infect Dis 1984;6:13-32.

48. Cullmann W, Dick W, Dalhoff A. Nonspecific induction of β-lactamase in Enterobacter cloacae. J Infect Dis 1983;148:765.

49. Sanders CC. Inducible β-lactamases and non-hydrolytic resistance mechanisms. J Antimicrob Chemother 1984;13:1-3.

50. Gutmann L, Williamson R. A model system to demonstrate that β-lactamase-associated antibiotic trapping could be a potential means of resistance. J Infect Dis 1983;148:316-21.

51. Nikaido H. Role of outer membrane barrier in bacterial resistance to antibiotics. Hoechst-Roussel Award Lecture. Twenty-fourth Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, D.C. October 9, 1984.

52. Neu HC. Introduction. Postgraduate Medicine: Custom Communications Sep/Oct 1984;3.

53. Bush K, Sykes RB. β-Lactamase inhibitors in perspective. J Antimicrob Chemother 1983;11:97-107.

54. Goto S. In vitro and in vivo antibacterial activity of moxalactam, an oxa-β-lactam antibiotic. Rev Infect Dis 1982;4:S501-10.

55. Richmond MH. Susceptibility of moxalactam to β-lactamase. Rev Infect Dis 1982;4:S522-6.

56. Labia R. Moxalactam: an oxa-β-lactam antibiotic that inactivates β-lactamases. Rev Infect Dis 1982;4:S529-35.

57. English AR, Retsema JA, Girard AE, Lynch JE, Barth WE. CP-45,899, a beta-lactamase inhibitor that extends the antibacterial spectrum of beta-lactams: initial bacteriological characterization. Antimicrob Agents Chemother 1978;14:414-9.

58. Greenwood D, Eley A. In-vitro evaluation of sulbactam, a penicillanic acid sulphone with β-lactamase inhibitory properties. J Antimicrob Chemother 1983;11:97-107.

59. Anonymous. Amoxicillin-clavulanic acid (Augmentin). Med Lett Drugs Ther 1984;26:99-100.

60. Neu HC. Beta-lactamases: a perspective on the contribution of these enzymes to bacterial resistance. Postgraduate Medicine: Custom Communications Sep/Oct 1984;7-21.

61. Rolinson GN. History and mode of action of augmentin. Postgraduate Medicine: Custom Communications Sep/Oct 1984;23-8.

62. Reading C, Farmer T. The inhibition of periplasmic β-lactamase in E. coli by clavulanic acid and other β-lactamase inhibitors. Postgraduate Medicine: Custom Communications Sep/Oct 1984;163-8.

63. Clocombe B, Beale AS, Boon RJ, et al. Antibacterial activity in vitro and in vivo of amoxicillin in the presence of clavulanic acid. Postgraduate Medicine: Custom Communications Sep/Oct 1984;29-49.

64. Croydon P. Worldwide clinical review of augmentin. Postgraduate Medicine: Custom Communications Sep/Oct 1984;71-8.

65. Jaffe AC, O'Brien CA, Lemon E, Capretta TM, Aronoff SC, Blumer JL. Clinical experience with augmentin in a pediatric outpatient setting. Postgraduate Medicine: Custom Communications Sep/Oct 1984;199-211.

66. Bluestone CD. Are beta-lactamase-inhibiting antimicrobial agents needed for treatment of otitis media? Postgraduate Medicine: Custom Communications Sep/Oct 1984;111-20.

67. Bluestone CD. Augmentin therapy for acute otitis media in children: summary of presentations from four clinical trials. Postgraduate Medicine: Custom Communications Sep/Oct 1984;137-9.

68. Wald ER, Reilly JS, Casselbrant MC, Chiponis DM. Treatment of acute sinusitis in children: augmentin vs cefaclor. Postgraduate Medicine: Custom Communications Sep/Oct 1984;133-6.

69. Gooch WM III, Congeni BL, Swenson E, Snellman LW III. Oral augmentin in the management of pediatric pneumonia. Postgraduate Medicine: Custom Communications Sep/Oct 1984;187-90.

70. Penn RL, Manocha KL, Burford JG, George RB. Prospective comparison of augmentin and cefaclor as therapy for lower respiratory infections. Postgraduate Medicine: Custom Communications Sep/Oct 1984;251-5.

71. Plouffe JF. Augmentin treatment of lower respiratory tract infections. Postgraduate Medicine: Custom Communications Sep/Oct 1984;183-5.

72. Seidenfeld JJ. A double-blind trial of amoxicillin and clavulanic acid versus cefaclor in adult patients with lower respiratory tract infection. Postgraduate Medicine: Custom Communications Sep/Oct 1984;177-81.

73. Tan JS, File TM Jr. Double blind efficacy and safety study of augmentin versus cefaclor in the treatment of lower respiratory tract infection. Postgraduate Medicine: Custom Communications Sep/Oct 1984;129-31.

74. Wallace RJ Jr. The increasing role of β-lactamase-producing organisms as causative agents of respiratory tract disease: the decline and fall of penicillin? Postgraduate Medicine: Custom Communications Sep/Oct 1984;121-8.

75. Aten EM, Neu HC. A multi-center double-blind comparative study of amoxicillin/clavulanic acid and cefaclor in the treatment of skin and skin structure infections. Postgraduate Medicine: Custom Communications Sep/Oct 1984;147-55.

76. Bromberg K, Worku M, Gullans CR, Sierra MF. Augmentin (amoxicillin/potassium clavulanate) for the therapy of pediatric skin and soft tissue infections. Postgraduate Medicine: Custom Communications Sep/Oct 1984;235-8.

77. Hansen R. A double-blind efficacy and tolerance study of amoxicillin-clavulanic acid and cefaclor in the treatment of skin and skin structure infections. Postgraduate Medicine: Custom Communications Sep/Oct 1984;239-43.

78. Quinn EL, Cardenas JV, Pohlod D, et al. Treatment of skin and soft tissue infections with amoxicillin/clavulanic acid or cefaclor. Postgraduate Medicine: Custom Communications Sep/Oct 1984;97-104.

79. Risser WL, Jaffe AC, Bromberg K, et al. A review of treatment of skin and soft tissue infections in children with amoxicillin-clavulanic acid combination or cefaclor. Postgraduate Medicine: Custom Communications Sep/Oct 1984;245-50.

80. Dambro N, Friedman AD, Alexander ER, Harrison HR. Augmentin therapy for urinary tract infections in children. Postgraduate Medicine: Custom Communications Sep/Oct 1984;263-6.

81. Faro S, Pastorek J III, Aldridge K. Open efficacy and tolerance study of augmentin in the treatment of urinary tract infections. Postgraduate Medicine: Custom Communications Sep/Oct 1984;195-8.

82. Fleisher G, Scott P, Wilmott C, Campos J. Amoxicillin combined with clavulanic acid for the treatment of urinary tract infections in children. Postgraduate Medicine: Custom Communications Sep/Oct 1984;219-24.

83. Iravani A, Richard GA. Clinical experience with the combination of amoxicillin-potassium clavulanate in the treatment of urinary tract infections in college coeds. Postgraduate Medicine: Custom Communications Sep/Oct 1984;87-95.

84. Lerner SA, Lyon ES, Wald VC. The treatment of urinary tract infections with amoxicillin/potassium clavulanate (augmentin). Postgraduate Medicine: Custom Communications Sep/Oct 1984;225-8.

85. Madsen PO, Dørflinger T, Frimodt-Møller P. Augmentin and cefaclor in treatment of complicated urinary tract infections. A comparative study. Postgraduate Medicine: Custom Communications Sep/Oct 1984;191-3.

86. Snavely SR, Harms JL, Hinthorn DR, Liu Chien. Efficacy of amoxicillin-potassium clavulanate in the treatment of urinary tract infections. Postgraduate Medicine: Custom Communications Sep/Oct 1984;213-7.

87. Stein GE, Gurwith M, Gurwith D. Treatment of acute urinary tract infections with amoxicillin-clavulanic acid. Postgraduate Medicine: Custom Communications Sep/Oct 1984;229-33.

88. Tolkoff-Rubin NE, Rubin RH, Weber DJ. Augmentin in the treatment of urinary tract infection. Postgraduate Medicine: Custom Communications Sep/Oct 1984;79-85.

89. Khan WN, Rodriguez WJ, Ahmad S. Comparative inhibition of penicillinase-producing Neisseria gonorrhoeae by ten antibiotics. Postgraduate Medicine: Custom Communications Sep/Oct 1984;159-62.

90. Girouard YC, Maclean IW, Ronald AR, Albritton WL. Synergistic antibacterial activity of clavulanic acid and amoxicillin against β-lactamase-producing strains of Haemophilus ducreyi. Antimicrob Agents Chemother 1981;20:144-5.

91. Cynamon MH, Palmer GS. In vitro activity of amoxicillin in combination with clavulanic acid against Mycobacterium tuberculosis. Antimicrob Agents Chemother 1983;24:429-31.

92. Cynamon MH, Palmer GS. In vitro susceptibility of Mycobacterium fortuitum to amoxicillin or cephalothin in combination with clavulanic acid. Antimicrob Agents Chemother 1983;23:935-7.

93. Thornsberry C, Kirven LA. β-Lactamase of the Legionnaires' bacterium. Current Microbiology 1978;1:51-4.

94. Fu KP, Neu HC. Inactivation of β-lactam antibiotics by Legionella pneumophila. Antimicrob Agents Chemother 1979;16:561-4.

95. Jones RN, Thornsberry C. Beta-lactamase studies of eight Legionella species: antibacterial activity of augmentin compared to newer cephalosporins, erythromycin and rifampin. Postgraduate Medicine: Custom Communications Sep/Oct 1984;259-62.

96. Beaty HN, Miller AA, Broome CV, Goings S, Phillips CA. Legionnaires' disease in Vermont, May to October 1977. JAMA 240:127-31.

97. Tsai TF, Finn DR, Plikaytis BD, McCauley W, Martin SM, Fraser DW. Legionnaires' disease: Clinical features of the epidemic in Philadelphia. Ann Intern Med 1979;90:509-17.

98. Kirby BD, Snyder KM, Meyer RD, Finegold SM. Legionnaires' disease: report of sixty-five nosocomially acquired cases and review of the literature. Medicine (Baltimore) 1980:59:188-205.

99. Thorsberry C, Baker CN, Kirven LA. In vitro activity of antimicrobial agents on Legionnaires disease bacterium. Antimicrob Agents Chemother 1978;18:403-8.

100. Lewis VJ, Thacker WL, Shepard CC, McDade JE. In vivo susceptibility of the Legionnaires disease bacterium to ten antimicrobial agents. Antimicrob Agents Chemother 1978:13:419-22.

101. Edelstein PH, Meyer RD. Susceptibility of Legionella pneumophila to twenty antimicrobial agents. Antimicrob Agents Chemother 1980;18:403-8.

102. Bacheson MA, Friedman HM, Benson CE. Antimicrobial susceptibility of intracellular Legionella pneumophila. Antimicrob Agents Chemother 1981:20:691-2.

103. Pasculle AW, Dowling JN, Weyant RS, Sniffen JM, Cordes LG, Gorman GW, Feeley JC. Susceptibility of Pittsburgh pneumonia agent (Legionella micdadei) and other newly recognized members of the genus Legionella to nineteen antimicrobial agents. Antimicrob Agents Chemother 1981;20:793-9.

104. Horwitz MA, Silverstein SC. Legionnaires' disease bacterium (Legionella pneumophila) multiplies intracellularly in human monocytes. J Clin Invest 1980;66:441-50.

105. Horwitz MA, Silverstein SC. Interaction of the Legionnaires' disease bacterium (Legionella pneumophila) with human phagocytes. II. Antibody promotes binding of L. pneumophila to monocytes but does not inhibit intracellular multiplication. J Exp Med 1981;153:398-406.

106. Horwitz MA, Silverstein SC. Activated human monocytes inhibit the intracellular multiplication of Legionnaires' disease bacteria. J Exp Med 1981;154:1618-35.

107. Horwitz MA, Silverstein SC. Intracellular multiplication of Legionnaires' disease bacteria (Legionella pneumophila) in human monocytes is reversibly inhibited by erythromycin and rifampin. J Clin Invest 1983;71:15-26.

108. Brook I, Coolbaugh JC, Walker R. Antibiotic and clavulanic acid treatment of sucutaneous abscesses caused by Bacteroides fragilis alone or in combination with aerobic bacteria. J Infect Dis 1983;148:156-9.

109. Daigneault EA, Ferslew KE, Roseman JM, Aten EM. Comparison of the pharmacokinetics and urinary excretion of clavulanic acid after oral administration of augmentin using high performance liquid chromatographic and microbiological assays. Postgraduate Medicine: Custom Communications Sep/Oct 1984;169-75.

110. Grizzard MB. Steady-state plasma levels of clavulanic acid in pediatric patients treated with augmentin. Postgraduate Medicine: Custom Communications Sep/Oct 1984;141-5.

111. Jackson D, Cooper DL, Filer CW, Langley PF. Augmentin: absorption, excretion and pharmacokinetic studies in man. Postgraduate Medicine: Custom Communications Sep/Oct 1984;51-70.

112. Goldstein EJC, Reinhardt JR, Murray PM, Finegold SM. Animal and human bite wounds: a comparative study, augmentin vs penicillin +/- dicloxacillin. Postgraduate Medicine: Custom Communications Sep/Oct 1984;105-10.

113. Aronovitz GH. Safety and efficacy of augmentin in the treatment of streptococcal pharyngitis. Postgraduate Medicine: Custom Communications Sep/Oct 1984;257-8.