

# SERINE $\beta$ -LACTAMASES AND PENICILLIN-BINDING PROTEINS

*Jean-Marie Ghuysen*

Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B6, B-4000  
Sart Tilman (Liège 1), Belgium

**KEY WORDS:** bacterial wall peptidoglycan,  $\beta$ -lactam antibiotics, emergence of resistance,  
enzyme catalysis, serine peptidases, divergent evolution, molecular modeling,  
protein engineering

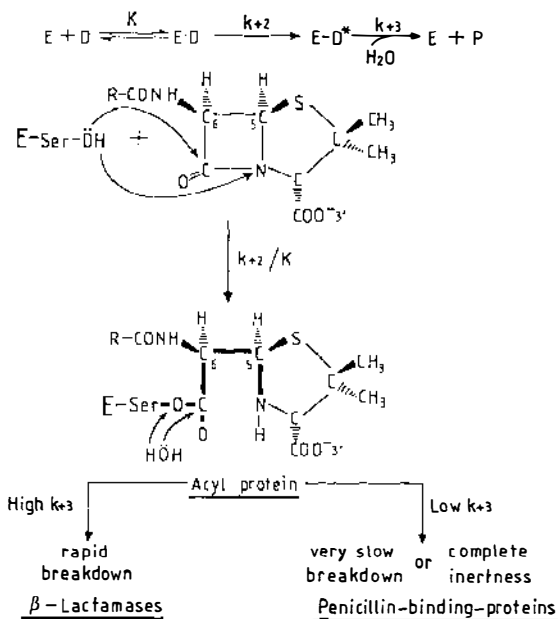
---

## CONTENTS

<i>The Penicillin-Interactive, Active-Site Serine Protein Family</i> .....	37
<i>The Low-Mr PBPs</i> .....	45
<i>The <math>\beta</math>-Lactamases and the Penicillin Sensory-Transducer BLAR</i> .....	47
<i>The Atomic-Level Enzyme-Ligand Interactions</i> .....	50
<i>The High-Mr PBPs of Class A and B</i> .....	54
<i>Intrinsic Resistance by Emergence of Altered High-Mr PBPs</i> .....	58

### *The Penicillin-Interactive, Active-Site Serine Protein Family*

In the bacterial world, hundreds, perhaps thousands, of distinct proteins catalyze rupture of the lactam amide bond of penicillin and transfer the penicilloyl moiety to an essential serine, forming a serine ester-linked acyl derivative (Figure 1). The reaction is analogous to the catalyzed rupture of a peptide bond by the serine peptidases of the trypsin and subtilisin families. It



*Figure 1* The penicillin-interactive, active-site serine proteins. Reactions with  $\beta$ -lactam carbonyl donors. E = protein; D =  $\beta$ -lactam carbonyl donor; E·D = Michaelis complex; E·D\* = acyl protein; P = reaction product (penicilloate); K = dissociation constant;  $k_{+2}$  and  $k_{+3}$  = first-order rate constants. The acyl enzyme mechanism was proposed in 1965 (112). Involvement of an essential serine was demonstrated in 1976 for reaction with the *Streptomyces* R61 penicillin-binding protein (36) and in 1979–1981 for reaction with several  $\beta$ -lactamases (17, 20, 35, 71). The concept of a penicillin-interactive, active-site serine protein family was put forward in 1988 (66).

involves initial binding of the ligand to the enzyme (K), enzyme acylation ( $k_{+2}$ ) and, in a third step, enzyme deacylation ( $k_{+3}$ ). A common substrate requirement is that a negative charge must occur at the 3' position in the penicillins and the 4' position in the cephalosporins (this group is usually a carboxylate, but a sulfonate is found in the monobactams), and the acyl side chain borne by the  $\beta$ -lactam ring must be on the  $\beta$  face in a *cisoid* position relative to the thiazolidine or dihydrothiazine ring. As a result of these requirements, the scissile amide bond in the  $\beta$ -lactam ring is equivalent to a carboxy-terminal peptide bond extending between two carbon atoms with the D configuration

Given the endocyclic nature of the scissile bond, acylation of the active-site serine generates a bulky leaving group that remains part of the acyl enzyme through the C5-C6 bond (Figure 1) and thus cannot diffuse away. As a result of this occupancy, only water has access to the active site and can attack the acyl enzyme, release the penicilloyl moiety, and concomitantly recover the

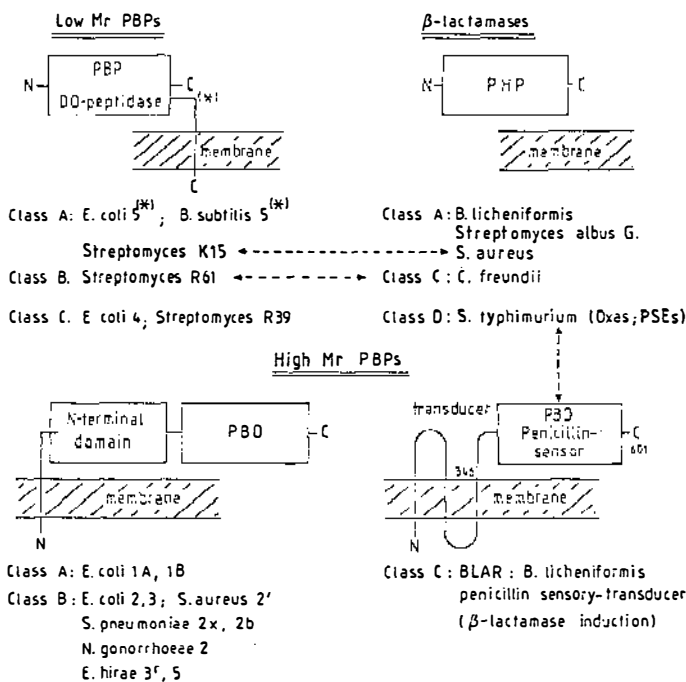
enzyme. This mechanism is general. However, with the  $\beta$ -lactamases, water has a facile access to the active site and is an excellent attacking nucleophile. The  $\beta$ -lactamases turn over rapidly and hydrolyze effectively the  $\beta$ -lactam substrates into biologically inactive metabolites. With the penicillin-binding proteins (PBPs), the acyl enzyme is almost hydrolytically inert. The higher the  $k_{+2}/K$  and the smaller the  $k_{+3}$  are, the lower the antibiotic concentration required to immobilize completely a given PBP as acyl enzyme at the steady-state of the reaction (46, 78).

The fact that the acyl enzymes are sufficiently stable to be analyzed with SDS gel electrophoresis has led to the development of a convenient procedure for the detection of the individual PBPs as radioactively labeled proteins (104). All the bacteria possess multiple PBPs. Each bacterial species has its own assortment of PBPs. The PBPs occur in a small number of copies per cell, from a few hundred to one or two thousand. Their affinities for penicillin and other  $\beta$ -lactam antibiotics vary widely, and their molecular masses range from 25,000 to 100,000.

The penicillin-interactive proteins fall into three groups: the  $\beta$ -lactamases, the low-Mr PBPs, and the high-Mr PBPs. The  $\beta$ -lactamases and the low-Mr PBPs are monofunctional catalytic entities. The active-site serine resides close to the amino terminus of the protein. The high-Mr PBPs possess a several-hundred-amino acid domain that is fused to the amino terminus of the penicillin-binding domain per se, which is assumed to start 60 residues upstream from the essential serine. Pair-wise comparison of amino acid sequences leads to the conclusion that each of these groups falls into several classes (Figure 2). Members of a given class are related in the primary structure. However, proteins that lack relatedness may fulfill similar functions. Whether they belong to class A, C, or D, the  $\beta$ -lactamases are  $\beta$ -lactam antibiotic-hydrolyzing enzymes. Conversely, similarity in the primary structure does not necessarily reflect similarity in the physiological function. The *Streptomyces* K15 low-Mr PBP of class A is related to the staphylococcal  $\beta$ -lactamase of class A, and the *Streptomyces* R61 low-Mr PBP of class B is related to the *Citrobacter freundii*  $\beta$ -lactamase of class C by a low but significant similarity index of about 7. The carboxy-terminal, penicillin-binding domain of the class C high-Mr PBP BLAR of *Bacillus licheniformis* is related to the Oxa-2  $\beta$ -lactamase of class D by a high-similarity index of 40.

Divergent evolution implies that proteins of the same family evolved from a common ancestor. Depending on the evolutionary distance, they may have acquired very different amino acid sequences and distinct functionalities and specificities while conserving the same polypeptide scaffolding. The case of a divergent evolutionary relationship between the penicillin-interactive, active-site serine proteins and domains is supported by a bulk of experimental data and predictional studies.

The three-dimensional structures are known for the class A  $\beta$ -lactamases of



**Figure 2** The penicillin-interactive, active-site serine proteins. Groups, classes, and membrane topology. PBP/PBD, penicillin-binding protein/domain; (\*), membrane-bound; PHP, penicillin-hydrolyzing protein. Many β-lactamases other than those listed are of known primary structure. ↔ indicates similarity in the primary structure. The algorithm used for homology searches (66) generates the best alignment for each pair of amino acid sequences and expresses the extent of similarity by a score. This score is the sum of the individual scores obtained for each pair of amino acids minus a penalty of 8 for each gap introduced. Pair-wise comparison of members of a given class generates scores that are at least 5 but may be as high as 50 standard deviations above that expected from a run of 20 randomized pairs of proteins with the same amino acid compositions as the two proteins under consideration. This standard deviation defines a similarity index. Figure 7 shows pair-wise comparison of the amino-terminal and carboxy-terminal domains of the high-Mr PBPs of class A and B.

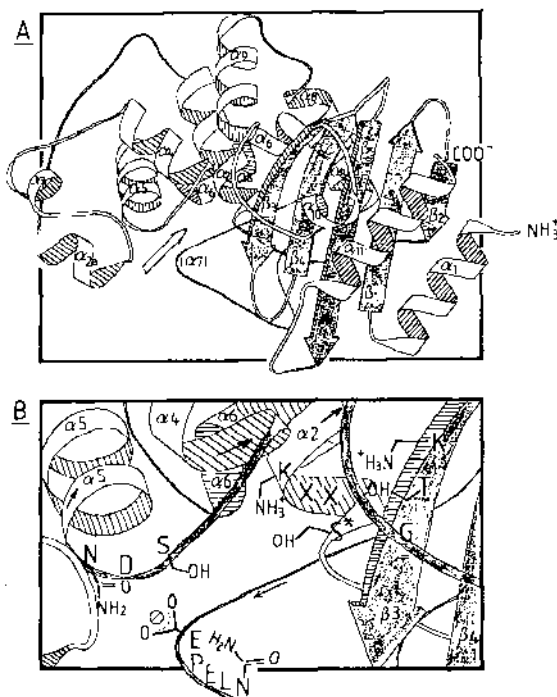
*Staphylococcus aureus* (56), *Streptomyces albus* G (25, 62), and *B. licheniformis* (82); the class C β-lactamase of *C. freundii* (89); and the class B low-Mr PBP of *Streptomyces* R61 (70, 72). They have a unique signature in the form of several amino acid groupings that occur along their amino acid sequences (66, 103) (Figure 3): the tetrad active-site serine-X-X-lysine (S\*XXK), where X is a variable amino acid; the triad serine or tyrosine-X-asparagine [S(Y)XN]; a peptide segment (D/E) that contains two dicarboxylic amino acids; and the triad lysine or histidine-threonine or serine-glycine [K(H)T(S)G]. As a result of the folding of the polypeptide chain, these four

Class	Low-Mr PBPs	NH <sub>3</sub> <sup>+</sup>	S*XXX	S(Y)XN	D/E	K(H)T(S)G	COO <sup>-</sup>	Ref.	
A	<i>E. coli</i> 5	D30	42 S73LTK	62 S199 GN	41 D183, D204	37 K242 TG	158 403	15	
	<i>B. subtilis</i> 5	?	? S36MTK	63 S100 AN	43 E144, D179	47 K227 TG	181 412	115	
	<i>Streptomyces</i> K15	V30	38 S64TTK	57 S125 GC	41 D169, D172	69 K242 TG	46 291	(*)	
B	<i>Streptomyces</i> R61	A32	60 S93VTK	93 Y190 SN	63 D254, E100	69 H329 TG	74 406	30	
	<i>E. coli</i> 4	A21	40 S627DK	240 S306 DN	38 D342, D355	61 K427 TG	57 477	83	
C	<i>Actinomadura</i> R39	R50	47 S98NMK	245 S347 NN	44 D394, D411	46 K458 TG	77 538	(*)	
	<b>β-Lactamases</b>								
A	<i>S. aureus</i>	K25	37 S63TSK	54 S121 DN	33 E157 TELN	65 K225 SG	59 281	19	
	<i>Streptomyces albus</i> G	G40-S47	41 S89VFK	60 S150 DN	35 E189PELN	65 K257 TG	52 312	23	
	<i>B. licheniformis</i>	S35-E45	40 S86TK	54 S144 DN	35 E180PELN	67 K248 TG	56 307	87	
C	<i>C. freundii</i>	A21	42 S84VSK	82 Y170 AN	64 D212 AEAY	65 K335 TG	45 381	79	
D	Oxa-2	Q23	44 S68TFK	74 Y146 GN	12 E161	48 K210 TG	62 275	22	
<b>High-Mr PBPs</b>									
A	<i>E. coli</i> 1A	M1	463 S465NFK	55 S524 KN	32 E559	156 K716 TG	131 850	14	
	<i>E. coli</i> 1B	M1	508 S510 LAK	58 S572 MN	26 D604, E620	77 K698 TG	143 844	14	
B	<i>E. coli</i> 2	M1	328 S30TVK	53 S387 AD	34 E424, D447	96 K544 SG	86 633	5	
	<i>E. coli</i> 3	M1	305 S30TVK	48 S359 SN	34 E396, D409	84 K494 TG	91 588	86	
	<i>S. aureus</i> 2'	M1	403 S405TK	55 S464 DN	25 E492, D511	87 K599 SG	68 670		
	<i>N. gonorrhoeae</i> 2	M1	308 S310ATK	48 S362 SN	34 E399, E414	82 K497 TG	81 581	100	
	<i>S. pneumoniae</i> 2x	M1	335 S337MK	54 S395 SN	34 E432, D441	106 K547 SG	200 750	74	
	<i>S. pneumoniae</i> 2b	M1	383 S385VFK	53 S442 SN	32 E475, E483	130 K614 TG	62 679	28	
	<i>E. hirae</i> 5	M1	420 S420TFK	54 S480 DN	25 E508, E542	74 K617 TG	58 678	33	
	C	<i>B. licheniformis</i> BLAR	M1	400 S402YK	70 Y476 GN	11 D490	48 K539 TG	59 601	73

Figure 3 The penicillin-interactive, active-site serine proteins. Location of the motifs S\*XXX, S(Y)XN, D/E, and K(H)T(S)G along the amino acid sequences. The amino acid numbering refers to the protein precursors. The low-Mr PBPs and the β-lactamases are manufactured with a cleavable signal peptide. Cleavage may produce a mature protein that has a ragged amino terminus (NH<sub>3</sub><sup>+</sup>). The amino acid sequence of the *Streptomyces albus* G β-lactamase (23) has been corrected by deleting Gly128 and substituting the dyad Gln141-Leu142 by Met (4, 67). In the ABL numbering scheme for the β-lactamases of class A (4), S\* of the S\*XXX motif is at position 70, S of the SDN motif is at position 130, E\* of the E/D motif is at position 166, and K of the KT(S)G motif is at position 234. The *E. coli* PBP1B is a mixture of two major proteins resulting from the use of alternative sites for the initiation of translation. The numbering refers to the largest form. (\*), Unpublished data (see text). Reference 86 should read 85.

motifs are brought close to each other, generating an active-site at the junction between an all-α domain and an α-β domain whose five-stranded β-sheet is protected by additional α-helices on both faces (Figure 4). In this structure, the serine of the S\*XXX motif is at the amino terminus of helix α2 of the all-α domain and occupies a central position in the cavity. The S(Y)XN motif connects helices α4 and α5 of the all-α domain and forms one side of the cavity. The D/E motif resides at the entrance of the cavity. The K(H)T(S)G motif on the innermost β3 strand of the β-sheet forms the other side of the cavity. Using the *S. aureus* β-lactamase as reference, one finds that the differences in length in the *S. albus* G β-lactamase, the *C. freundii* β-lactamase, and the *Streptomyces* R61 PBP are associated with surface loops, small helices, and β-strands that occur away from the active site and that connect conserved structures (Figure 5). In this proposed scheme, the conserved secondary structures have the same numbering.

Superimposition experiments lead to the result that the β3 strand is sim-



**Figure 4** Structure of the *Streptomyces albus* G  $\beta$ -lactamase of class A. Polypeptide folding (A) and disposition of the conserved motifs (B): The active site is indicated by the white arrow in A and is threefold enlarged in B. Adapted from References 25, 62, 67.

ilarly situated with respect to the S\*XXX motif in the three-dimensional structures. However, the Y(A or S)N motif in the  $\beta$ -lactamase of class C and the PBP is about 4–5 Å farther away from the K(H)T(S)G motif than the corresponding SDN motif in the  $\beta$ -lactamases of class A. As a consequence of this displacement, the phenolic oxygen of the tyrosine of the Y(A or S)N motif and the  $\gamma$ -OH of the serine of the SDN motif are similarly disposed with respect to the  $\gamma$ -OH of the active-site serine of the S\*XXX motif. Superimposition experiments also highlight noticeable differences at the level of the D/E motif. Site-directed mutagenesis (49) and molecular modeling (J. Lamotte-Brasseur, G. Dive, O. Dideberg, J.-M. Frère, & J.-M. Ghuyesen, in preparation) support the view that the glutamic acid E\* of the sequence E\*XELN (Figures 3 and 4) of the class A  $\beta$ -lactamases is essential and that a water molecule bound to this glutamic acid acts as proton abstractor-donor in catalysis. The corresponding sequences DAEAY in the *C. freundii*  $\beta$ -lactamase and DSTEQ in the *Streptomyces* R61 PBP (Figure 3) have a spatial

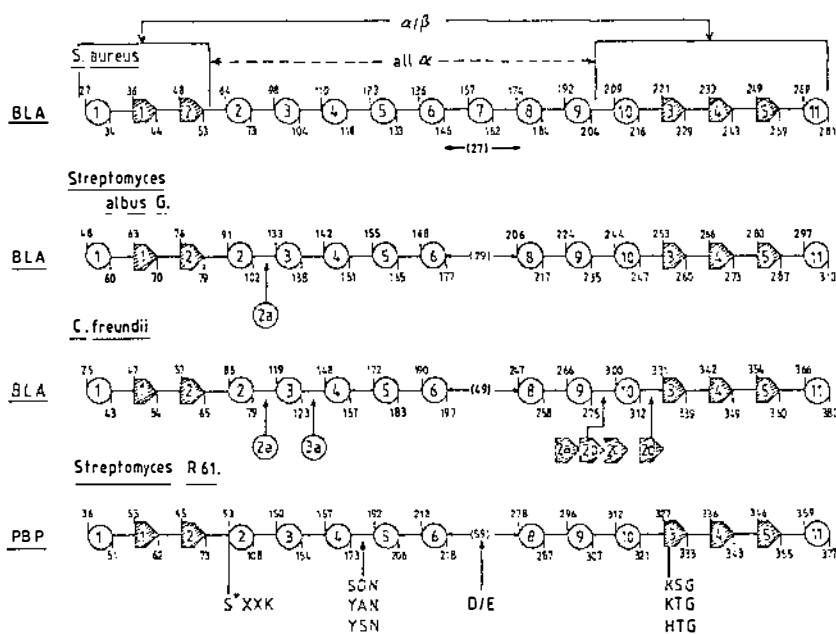


Figure 5 Occurrence of the conserved motifs and secondary structures along the amino acid sequences of the *S. aureus*  $\beta$ -lactamase of class A (56), the *Streptomyces albus G*  $\beta$ -lactamase of class A (25, 62, 67), the *C. freundii*  $\beta$ -lactamase of class C (89), and the *Streptomyces R61* PBP (70, 72). Circles,  $\alpha$ -helix; pointing boxes,  $\beta$ -strand. BLA =  $\beta$ -lactamase. The amino acid numbering is that of the protein precursors.

disposition that is not compatible with such a role in catalysis. Yet alterations of the D/E motif of the *C. freundii*  $\beta$ -lactamase [DAEAY  $\rightarrow$  DAKAY and DAEAY  $\rightarrow$  (E, K or T)AEAY] modify the specificity profile of the enzyme and extend its hydrolytic activity to oxymino-cephalosporins (117, 118).

All the  $\beta$ -lactamases, the low-Mr PBPs, and the penicillin-binding domains of the high-Mr PBPs possess, along their amino acid sequences, the same four motifs as those found in the penicillin-interactive proteins of known three-dimensional structure (Figure 3). Identification of the S\*XXK, S(Y)XN, and K(H/R)T(S/G) motifs (66, 103) is not, most often, an issue of controversy [HTG occurs in the *Streptomyces R61* PBP (30) and RSG in the PSE-4 carbenicillinase (10)], and analysis, at the molecular level, of protein mutants (see below) leads to the conclusion that these motifs are elements of the active sites. Given that several dicarboxylic amino acids occur approximately at the expected distances in the primary structures, identification of the D/E motif is hypothetical unless the importance of the predicted motif is demonstrated

experimentally. As shown by site-directed mutagenesis, Asp444 in the high-Mr PBP2 of *Escherichia coli* is probably the homolog of the glutamic acid E\* of the  $\beta$ -lactamases of class A (H. Adachi, M. Ishiguro, S. Imajo, T. Ohta, & H. Matsuzawa, personal communication).

The penicillin-interactive, active-site serine proteins and domains exhibit endless variations. Predictional studies suggest that the 275 amino acid  $\beta$ -lactamases of class D, which are the smallest known  $\beta$ -lactamases, have a shorter connection between the YDN and KTG motifs and that the homologous 477-amino acid PBP4 of *E. coli* (83) and 538-amino acid PBP of *Actinomyces* R39 (B. Granier, C. Duez, S. Lepage, J. Van Beeumen, S. Englebort, et al, in preparation), which are the largest known low-Mr PBPs/DD-peptidases, have an additional 180- to 190-amino acid insert located between the S\*XXK and SDN motifs and occurring on the surface of the protein. Deletion by genetic engineering of a substantial part of this insert from PBP4 produces a truncated protein that still binds penicillin (H. Mottl & W. Keck, personal communication). Several  $\beta$ -lactamases and low-Mr PBPs are susceptible to thiol reagents. The *Streptomyces* K15 PBP has two cysteines. One of them occurs in the active-site as a result of the replacement of the usual S(Y)XN motif by a SDC motif, and the other occurs at the tenth position downstream from the lysine of the KTG motif, i.e. probably on strand  $\beta$ 4 (P. Palomeque-Messia, M. Leyh-Bouille, M. Nguyen-Distèche, C. Duez, S. Houba, et al, in preparation). Derivatization of one of these cysteines by para-chloromercuribenzoate greatly decreases but does not abolish the enzymatic activities of the PBP (77).

The wall peptidoglycan is an essential polymer of the bacterial cell. Its structure and biosynthesis are well-known (Figure 6). The peptidoglycan precursors are lipid-transported disaccharide (N-acetylglucosaminyl-N-acetylmuramyl)-L-Ala- $\gamma$ -D-Glu-L-Xaa-D-Ala-D-Ala pentapeptide units. Depending on the bacterial species, L-Xaa is a diamino acid whose  $\omega$  amino group is either free or substituted by additional amino acids. These precursors, the end products of the cytoplasmic and membrane cycles of the biosynthetic pathway, are transported onto the outer face of the plasma membrane for final peptidoglycan assembly, a process that requires a glycan chain elongation and interpeptide crosslinking machinery.

The penicillin-interactive, active-site serine proteins fulfill multiple functions. The low-Mr PBPs and the high-Mr PBPs of class A and B are involved, in one way or another, in peptidoglycan assembly. They are the targets of penicillin action. The  $\beta$ -lactamases are defensive enzymes that the bacteria synthesize to protect their PBPs from the deleterious effects of penicillin.  $\beta$ -Lactamase synthesis is not always constitutive. The high-Mr PBP BLAR of class C acts as a penicillin sensory-transducer involved in the specific inducibility of  $\beta$ -lactamase synthesis.



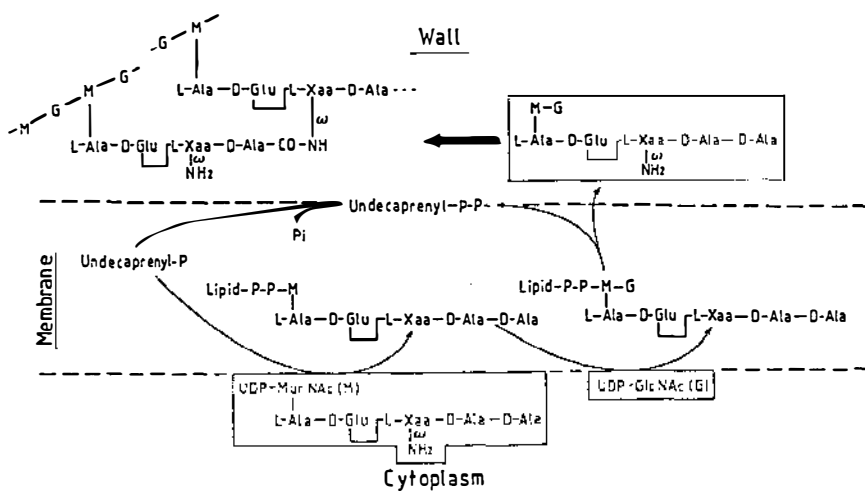


Figure 6 Bacterial wall peptidoglycan assembly from lipid-transported disaccharide-pentapeptide units. The final steps (heavy arrow) are made by transglycosylation and peptide crosslinking.

### The Low-Mr PBP<sub>s</sub>

The low-Mr PBP<sub>s</sub> are manufactured with a cleavable signal peptide. Some low-Mr PBP<sub>s</sub> do not possess transmembrane segments. The *E. coli* PBP<sub>4</sub> and the *Streptomyces* K15 PBP somehow interact with the membrane surface and are isolated as membrane components. Overproducing strains produce a high proportion of the PBP<sub>s</sub> in a water-soluble form. The PBP of *Streptomyces* R61 and that of *Actinomadura* R39 are excreted in the medium during growth. When compared to the excreted protein, the 406-amino acid precursor of the *Streptomyces* R61 PBP possesses both a cleavable peptide signal and a cleavable 26-amino acid carboxy-terminal extension (30). Should it not be removed during protein maturation, this carboxy-terminal extension might function as a stop transfer sequence.

All the other known low-Mr PBP<sub>s</sub> are membrane-bound (97) (Figure 2). The polypeptide chain does not terminate approximately 60 residues downstream from the KT(S)G motif as observed with the soluble PBP<sub>s</sub>, but has an ~50- to 100-amino acid carboxy-terminal extension (Figure 3), the end of which contains a signal-like peptide segment that serves as membrane anchor. As a consequence, the bulk of the protein is on the outer face of the membrane. Catalytically active, water-soluble derivatives that lack the membrane anchor can be produced by proteolytic treatment of isolated membranes (33, 123) and by engineering the encoding genes (34, 61).

The low-Mr PBP<sub>s</sub> are DD-peptidases. They catalyze acyl transfer reactions



is quantitatively converted into the transpeptidated product Ac<sub>2</sub>-L-Lys-D-Ala-CONH-X. This PBP functions as a strict transpeptidase.

Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala and  $\beta$ -lactam antibiotics are competing substrates of the low-Mr PBPs/DD-peptidases. The  $\beta$ -lactam antibiotics, however, are suicide substrates or mechanism-based inactivators: they immobilize the essential serine in the form of a stable ester-linked acyl enzyme (Figure 1). Yet the inertness of the acyl (benzylpenicilloyl) PBP is not absolute. Slow attack of the penicilloyl moiety by water generates penicilloate, which is the degradation product of  $\beta$ -lactamase action on penicillin. Alternatively, a slow and rate-limiting intramolecular rearrangement of the penicilloyl moiety can rupture the bond between C5 and C6 (Figure 1) and release the leaving group (3, 37–39, 80, 121). As a result of the vacancy thus created in the active site, the newly formed acyl (phenylacetyl) enzyme undergoes immediate breakdown. Attack by water gives rise to phenylacetyl-glycine; attack by an amino compound NH<sub>2</sub>-X gives rise to the transpeptidated product phenylacetyl-glycyl-CONH-X.

Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala is an analog of the carboxy-terminal tripeptide moiety of the peptidoglycan precursors (Figure 6). As with the reactions that they catalyze, the low-Mr PBPs/DD-carboxypeptidases help control the extent of peptidoglycan crosslinking (8a). They hydrolyze the carboxy-terminal D-alanyl-D-alanine peptide bond of peptidoglycan precursors and, in those bacterial species where peptidoglycan crosslinking is mediated by a carboxy-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkage, they hydrolyze bonds previously made through transpeptidation. All the bacteria possess one or several low-Mr PBPs. *Streptococcus pneumoniae* PBP3-negative mutants are severely affected in cell division and show highly altered morphology (99a). A 10-fold overproduction of the *E. coli* PBP5 causes conversion of the rod-shaped cells into round cells (106). The sporulation-specific PBP5a is temporally and spatially regulated in *B. subtilis*. It is detected in large amounts only during spore peptidoglycan synthesis from stage II to stage V (16, 113).

### *The $\beta$ -Lactamases and the Penicillin Sensory-Transducer BLAR*

The  $\beta$ -lactamases are secretory proteins. They are excreted in the growth medium (gram-positive bacteria) or they accumulate in the periplasm (gram-negative bacteria). The  $\beta$ -lactamase III of *Bacillus cereus* and that of *B. licheniformis* contain a diacylglyceride that is thioether-linked to the amino terminal cysteine and a fatty acid that is amide-linked to the same cysteine (57). This hydrophobic moiety functions as the anchor of the protein to the plasma membrane. In fact, these  $\beta$ -lactamases have membrane-bound and secretory forms.

The only known function of the  $\beta$ -lactamases is hydrolysis of the  $\beta$ -lactam antibiotics.  $\beta$ -Lactamases show hysteretic kinetics (99). A unique conformation is induced in the enzymes by each of several closely related  $\beta$ -lactam substrates, and the enzymes can adjust to unfavorable modifications in the substrate.

The  $\beta$ -lactamases lack DD-peptidase activity and thus do not interfere with wall peptidoglycan metabolism. However, they catalyze acyl transfer reactions on noncyclic depsipeptides (2, 91–93) with, for some of them, complex steady-state kinetics comparable to those shown by the low-Mr PBPs/DD-peptidases. Like the PBPs, the  $\beta$ -lactamases may give rise to long-lived acyl enzymes (42) and long-lived acyl enzymes may undergo various types of intramolecular rearrangements (18). Rupture of the bond between S1 and C5 in the acyl enzyme formed by reaction with the penam sulfones and 6- $\beta$ -bromo(iodo)penicillanate or rupture of the bond between O1 and C5 in clavulanate is an integral part of the  $\beta$ -lactamase inactivation process. The established common feature is that of a branched pathway in which  $\beta$ -elimination leads to a hydrolytically inert acyl enzyme and, sometimes, to further modification of some residues of the active site.

The  $\beta$ -lactamases are dispensable enzymes, while the low-Mr PBPs/DD-peptidases fulfill functions in wall peptidoglycan metabolism. A possible mechanism that may explain the emergence of  $\beta$ -lactamases is the excretion of one or several low-Mr PBPs/DD-peptidases by bacteria exposed to lactam antibiotics. Further improvement of this detoxication mechanism results in the conversion of these water-soluble PBPs into  $\beta$ -lactam-hydrolyzing enzymes (69). Hence, the presumed features of evolution are catalysis of deacylation of the acyl enzyme formed with cyclic amide compounds, i.e. the  $\beta$ -lactam antibiotics, and loss of productive binding of planar acyclic amides, i.e. loss of DD-peptidase activity. Productive binding of planar acyclic esters has no physiological significance; it is conserved to varying extent.

Emergence of new  $\beta$ -lactamases can be achieved by altering a limited number of amino acids in the direct environment of the active site. A key mutation in the decreased cephalosporinase activity of the *S. albus* G  $\beta$ -lactamase of class A is the conversion of the SDN motif into SDS (62, 63). This single change has little effect on the hydrolytic activity towards good penicillin substrates but results in a drastically decreased hydrolytic activity towards the cephalosporins. Yet the presence of a SDN or SDS motif in the  $\beta$ -lactamases of class A does not, by itself, determine the specificity profile of the enzyme. The  $\beta$ -lactamase III of *B. cereus* possesses a SDS motif (57). It does not exhibit as strong a preference for penicillins as does the *S. albus* G  $\beta$ -lactamase SDS mutant.

A key step in the increased cephalosporinase activity of the R-TEM  $\beta$ -lactamases of class A is alteration of the  $\beta$ 3 strand. The first mutant of this

type, obtained through selective pressure on the *E. coli* host cells (51), has one single change just after the KSG motif (KSGA  $\rightarrow$  KSGT) (66). R-TEM  $\beta$ -lactamase mutants from clinical isolates exhibiting high hydrolyzing capacity towards third-generation cephalosporins have more than one alteration (21, 101). Thus, for example, the TEM-5 enzyme, which confers resistance to ceftazidime, has two changes in the  $\beta$ 3 strand (KSGAGE  $\rightarrow$  KSGTGTK) and one additional change upstream from the D/E motif (DRWE\*  $\rightarrow$  DSWE\*, where E\* is the essential glutamic acid). The TEM-4 enzyme, which confers resistance to both cefotaxime and ceftazidime, has one change in the  $\beta$ 3 strand (KSGAG  $\rightarrow$  KSGAS), one change at position 26 upstream from the SDN motif (VEY  $\rightarrow$  VKY; note that the 25-amino acid stretch that extends between this tyrosine and the SSN motif controls the specificity profile of some high-Mr PBPs, see below) and two other changes close to the amino (L19  $\rightarrow$  F) and carboxy (T261  $\rightarrow$  M) terminus of the mature protein.

Some  $\beta$ -lactamases are produced in maximal amounts only in the presence of  $\beta$ -lactam antibiotics. The locus *blaR*, required for the induction of  $\beta$ -lactamase synthesis in *B. licheniformis*, encodes the 601-amino acid BLAR protein (125). This high-Mr PBP of class C (Figure 2) acts as penicillin sensory-transducer. A likely organization of BLAR (68) is that the 346-amino acid amino-terminal region, i.e. the transducer, has the same membrane topology as that found in some chemotactic transducers, with an extracellular domain responsible for signal reception and a cytosolic domain responsible for the generation of an intracellular signal. The 255-amino acid carboxy terminal region of BLAR, i.e. the penicillin sensor, extends on the outer face of the plasma membrane and is fused to the transducer by means of an additional transmembrane segment; this fusion is a direct consequence of the absence of periplasm in *B. licheniformis*.

The penicillin sensor of BLAR can be produced in *E. coli* as a periplasmic, stable water-soluble low-Mr PBP (68). This PBP, which now has the amino acid numbering M1 . . . R255, possesses the four markers S\*56TTK, Y130GN, D144, and K193TG. It is very similar, in the primary structure, to the  $\beta$ -lactamases of class D, in particular to the *Salmonella typhimurium* Oxa-2  $\beta$ -lactamase (homology index  $>$  40). It lacks detectable  $\beta$ -lactamase, DD-peptidase, or esterase activity. Its only function is to bind penicillin. In all likelihood, conformational changes induced by penicilloylation of the active-site serine give rise to a signal that, in the intact BLAR, is transmitted to the extracellular domain of the transducer and, from this, to the intracellular domain.

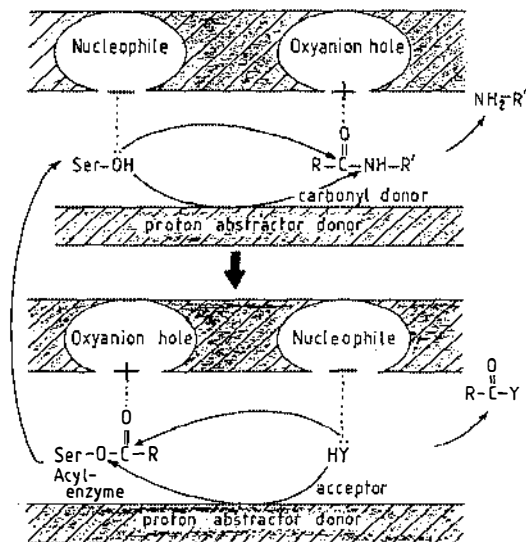
$\beta$ -Lactamase inducibility is a more refined defensive mechanism than  $\beta$ -lactamase constitutivity. The BLAR penicillin sensor might have evolved from a class-D  $\beta$ -lactamase. The presumed features are loss of deacylation of

acyl enzyme, no acquisition of DD-peptidase activity, and fusion to a trans-membrane transducer.

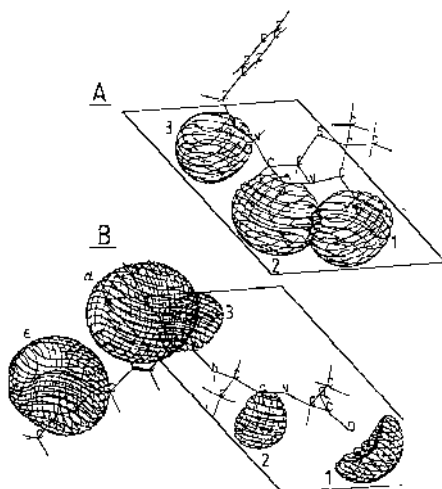
### *The Atomic-Level Enzyme-Ligand Interactions*

Serine peptidase-catalyzed rupture of a peptide bond is carried out by several enzyme reagents that fulfill the required functions of an electrophile or oxyanion hole, a proton abstractor-donor, and a nucleophile (Figure 8). In each of the serine peptidases of the trypsin and subtilisin families, two backbone NH groups, one of which belongs to the active-site serine, create the oxyanion hole environment; a histidine adjacent to the essential serine (and in interaction with a buried aspartic acid) is involved in proton abstraction-donation; and one side of the active site generates a nucleophilic suction-pump in the immediate vicinity of the essential serine (75). Reaction-rate enhancement, when compared to that of the analogous uncatalyzed reaction, depends on the goodness of fit of the ligand in the active site and on its correct positioning with respect to the enzyme functional groups.

Geometry optimization, superimposition searches, and conformational analysis of lactam compounds belonging to different chemical families (penicillins, 6-epoxy-spiroenicillins, cephalosporins, thienamycin,  $\gamma$ -lactams,



**Figure 8** Serine peptidase-catalyzed rupture of a peptide bond. The oxyanion hole polarizes the C = O bond; the nucleophile enhances the reactivity of the serine hydroxyl or the acceptor HY; and the proton abstractor-donor achieves nucleophilic attack.



**Figure 9** Coplanarity of the binding entity of benzylpenicillin (A) and the most stable, extended conformer of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala (B). Electrostatic potential isocontours at  $-10$  kcal/mol. 1, COOH; 2, CO of the scissile CONH bond (i.e. the electrophilic center); 3, CO of the exocyclic carbonyl (penicillin) or the L-Lys-D-Ala peptide bond (tripeptide). Distances and dihedral angles are given in Reference 76. The increased volume of well 2 of penicillin when compared to well 2 of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala expresses the increased intrinsic reactivity of a  $\beta$ -lactam amide bond when compared to an acyclic peptide bond. The two additional wells seen in the tripeptide are generated by the acetyl carbonyl groups that substitute the  $\alpha$  and  $\epsilon$  lateral chains of lysine. The views are shown along the plane of the scissile amide bond. The  $\alpha$  and  $\epsilon$  side chains of lysine are above and below the reference plane, respectively.

lactivicin) have led to the concept of a productive binding entity (76). To confer activity to these compounds (either as substrates of the  $\beta$ -lactamases or inactivators of the PBP<sub>s</sub>), the triad formed by the carbon atom of the carboxylate, the carbonyl of the scissile amide bond (i.e. the electrophilic center C = O) and the oxygen atom of the exocyclic carbonyl (or COH in thienamycin) must fulfill rather strict spatial requirements—one of which is an almost coplanar disposition of these four atoms (Figure 9A). In the most stable extended conformer of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala, the triad formed by the carboxylate, the C = O of the scissile D-Ala-D-Ala peptide bond, and the C = O of the L-Lys-D-Ala peptide bond has a spatial disposition comparable to that of the equivalent triad of the biologically active lactam compounds (Figure 9B). These functional groups generate a typical electronic property, and this electronic distribution gives rise to an electrostatic potential of defined shape and volume. Isosterism, however, is partial. The Ac<sub>2</sub>-L-Lys moiety of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala and the side-chain of the lactam ring in the antibiotics are oriented differently (79). The spatial disposition adopted by the Ac<sub>2</sub>-L-Lys

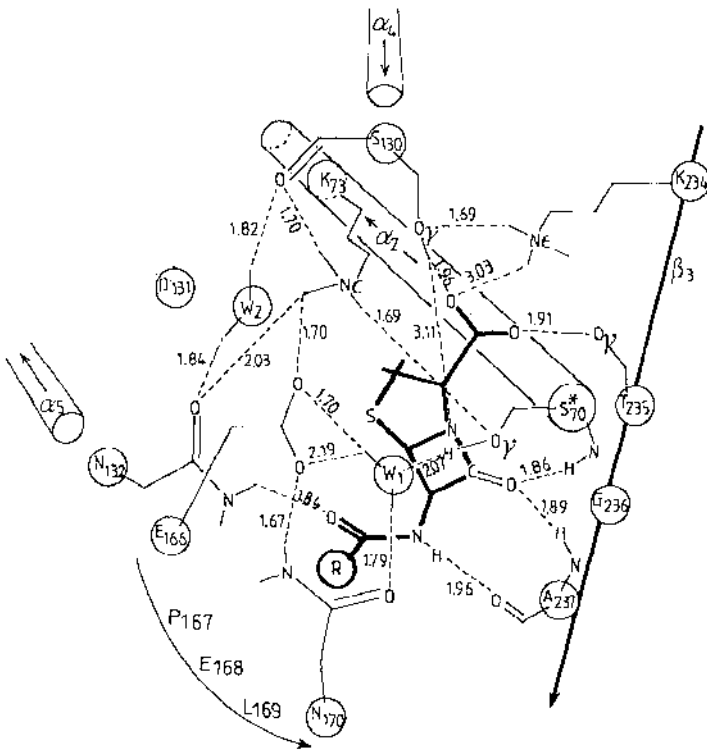
moiety relative to the D-alanyl-D-alanine dipeptide in the tripeptide can be visualized using the negative electrostatic wells generated by the carbonyl groups of the two acetyl substituents (Figure 9B).

The active site of the  $\beta$ -lactamases of class A and C and that of the *Streptomyces* R61 PBP/DD-peptidase are dense hydrogen bonding networks (Figure 10A). One important feature of the enzyme-ligand interactions (lactam compound- $\beta$ -lactamase; lactam compound-PBP; Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala-PBP) is an antiparallel hydrogen bonding between the innermost  $\beta$ 3 strand of the  $\beta$ -sheet and the binding entity of the ligand (Figure 10B) (25, 56, 62, 67, 70, 72, 82, 89). The carbonyl oxygen of the scissile bond forms hydrogen bonds to the backbone NH group of the active-site serine itself and to the backbone NH of the alanine (the class A  $\beta$ -lactamase), serine (the class C  $\beta$ -lactamase), or threonine (the PBP) immediately downstream from the K(H)TG motif of the  $\beta$ -strand, in a way reminiscent of the oxyanion-hole hydrogen bonds found in the peptidases of the trypsin family. The NH and CO of the exocyclic amide bond borne by the lactam ring or the equivalent groupings of the L-Lys-D-Ala peptide bond of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala are in hydrogen bond interactions with the backbone carbonyl of the same alanine, serine, or threonine of the  $\beta$ -strand and with the side chain of the asparagine of the S(Y)XN motif, respectively. The carboxylate (sulfonate in the monobactams) head of the ligand interacts with the OH group of the threonine of the K(H)TG motif and is oriented towards the lysine or histidine of the same motif.

Optimal positioning of the electrophilic center of the ligand relative to the  $\gamma$ -OH of the active-site serine also depends much on the appendages borne by the binding entity. Structural variations in the acyl side chain of the lactam ring, or the R-L-Xaa moiety of the R-L-Xaa-D-alanyl-D-alanine-terminated peptides, cause wide variations in the value of the second-order rate constant ( $k_{+2}/K$ ) of protein acylation. These effects are both protein and ligand specific (41, 45). In the emerging picture, the *Streptomyces* R61 PBP possesses a subsite that specifically interacts with the side chain borne by the lactam ring and another subsite that specifically interacts with the  $\epsilon$  side chain of the L-lysine residue of the tripeptide Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala. As a corollary, these side-chains are not interchangeable (9). Also according to the picture, the  $\alpha$  side chain of Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala extends to the exterior of the enzyme cavity and, thereby, binding of glycan-substituted pentapeptides to the active-site would be mediated mainly by the C-terminal tripeptide moiety.

Though we are learning much, microbiologists still seek answers to questions regarding the distinction between the PBPs and the  $\beta$ -lactamases in terms of stability vs lability of the acyl enzymes formed by reaction with the lactam antibiotics, the inability of the  $\beta$ -lactamases to perform acyl transfer reactions on acyclic carbonyl donor peptides, and the mechanism of





**Figure 10** Hydrogen bonding interactions between penicillin and the active site of the *Streptomyces albus* G  $\beta$ -lactamase of class A (25, 62, 67; J. Lamotte-Brasseur, G. Dive, O. Dideberg, J.-M. Frère, J. M. Ghuysen, in preparation). S\*70 = active-site serine. The amino acid numbering follows the ABL scheme (4). S\*70K73 is S89K92 in the precursor (Figure 3). Similarly, S130DN132 is S153DN155; E166PELN170 is E189PELN193; K234TGA2375 is K257TGA260. Two water molecules W1 and W2 are shown. Distances are given in Å. The acyl side chain of penicillin is represented by CO-(R). W1, W2, E166, and S130 would be involved in proton abstraction-donation. Attack of the carbonyl carbon of the  $\beta$ -lactam scissile bond by the O $\gamma$  of S\*70 occurs on the well-exposed  $\alpha$  face. Similar hydrogen bonding networks are proposed for the *S. aureus* (56) and *B. licheniformis* (82)  $\beta$ -lactamases of class A, the *C. freundii*  $\beta$ -lactamase of class C (89), and the *Streptomyces* R61 PBP (70, 72).

transpeptidation by the PBP<sub>s</sub>/DD-peptidases. Recent advances, however, have shed light on the mechanism of proton abstraction-donation.

Site-directed mutagenesis (49) and molecular modeling (J. Lamotte-Brasseur, G. Dive, O. Dideberg, J.-M. Frère, & J. M. Ghuysen, in preparation) support the view that the essential glutamic acid E\* of the E\*XELN sequence, i.e. the E/D motif, activates, via a bound water molecule, the essential serine of the class A  $\beta$ -lactamases (Figure 10) and that the serine of the SDN motif may be involved in proton shuttle. This serine has a homolog

in the tyrosine of the YAN motif in the  $\beta$ -lactamases of class C, but the spatial disposition of the D/E motif in these  $\beta$ -lactamases is not compatible with the role of a general base in catalysis. Superimposition of class C *C. freundii*  $\beta$ -lactamase onto trypsin, using the active-site serine and the oxyanion hole as reference points, results in the phenolic oxygen of the tyrosine of the YAN motif in the  $\beta$ -lactamase being  $< 0.5 \text{ \AA}$  from the N $\epsilon$ 2 position of the essential histidine in trypsin, indicating that the tyrosine, as its anion, might act in a way similar to the essential histidine in trypsin (89). The constellation of two positively charged lysines around this tyrosine would sufficiently lower its pK<sub>a</sub> value.

### *The High-Mr PBPs of Class A and B*

The high-Mr PBPs of class A and B have two functional domains that are fused to each other and that operate in a concerted manner (Figure 2). An amino terminal domain several hundred amino acids long is linked on its carbonyl side to the penicillin-binding domain per se. The penicillin-binding domain is assumed to start 60 residues upstream from the S\*XXK motif and to terminate 60 residues downstream from the KT(S)G motif. In most high-Mr PBPs, but not in all, this domain bears a tail in the form of a  $\sim 100$ -amino acid carboxy terminal extension (Figure 3).

The high-Mr PBPs are not synthesized as preproteins but have a highly hydrophobic region close to the amino terminus that acts as a noncleaved signal-like segment, both to translocate the bulk of the protein to the periplasm and to anchor the protein in the plasma membrane (1, 6, 12, 31, 32, 96). The *E. coli* PBP3 has been suggested to be a lipoprotein (84) because the sequence L26LCGC30 is similar to the consensus sequence for the modification and processing of bacterial lipoproteins. However, only a small fraction of PBP3 appears to be lipid-modified, at least under conditions of protein overexpression. The PBP3 undergoes maturation by eliminating a cleavable 10-amino acid carboxy-terminal stretch, from I578 to S588, in a way reminiscent of that observed with the low-Mr PBP of *Streptomyces* R61. Short hydrophobic regions upstream from Leu558 in the carboxy terminal tail are important for the functioning and/or stability of the protein (52, 84).

The simple mode of membrane insertion of the high-Mr PBPs allows the production of water-soluble forms through proteolytic treatment of isolated membranes (32, 96). Genetic constructs aimed at removing the membrane anchor from high-Mr PBPs also yield truncated proteins that are sufficiently stable to be isolated (1, 6, 31). They may require high salt concentrations to remain soluble and to retain their penicillin-binding capacity. A gene fusion that removes the amino terminal domain of the *E. coli* PBP3 and links the carboxy-terminal domain to the amino-terminal of the  $\beta$ -galactosidase leads to a chimeric protein that binds penicillin but is unstable (55). Expression of that portion of the gene that encodes only the carboxy-terminal domain of the

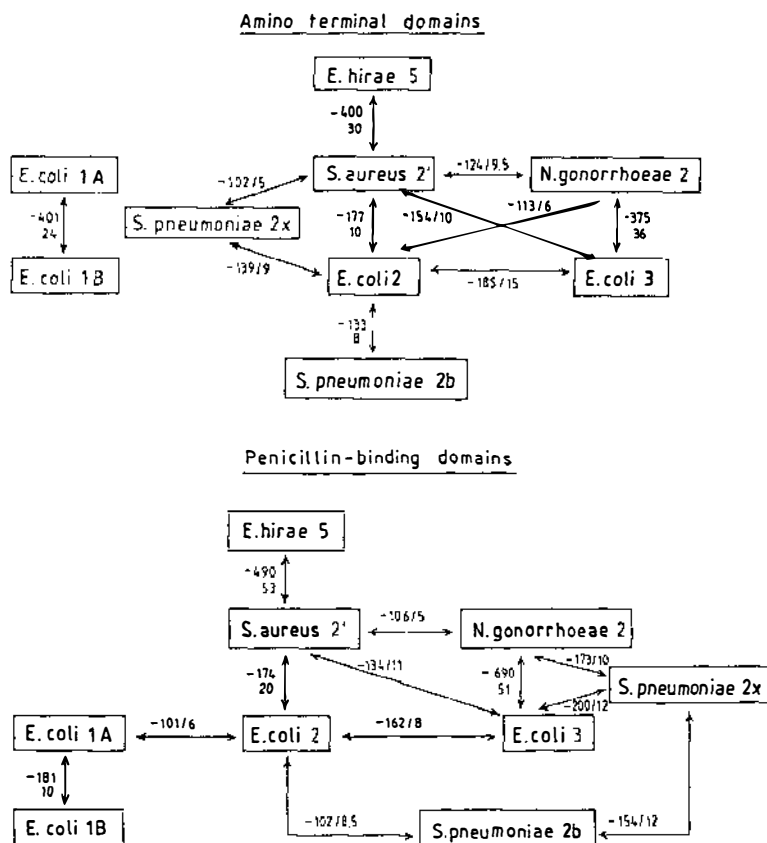


Figure 11 Search of homology between the amino acid sequences of the amino-terminal domains and penicillin-binding, carboxy-terminal domains of the high-Mr PBPs of class A and B. Comparison scores (negative values) and significance in standard deviations (positive values) are shown. For more details, see the legend of Figure 2. The *E. coli* PBPs 1A and 1B are of class A. The other PBPs are of class B.

PBP yields the expected polypeptide as evidenced by immunological tests and penicillin binding, but this polypeptide is very unstable. Similar observations have been made with other high-Mr PBPs. In addition, no simple in vitro assays are available that would allow one to monitor the enzymatic activities related to peptidoglycan synthesis of the high-Mr PBPs. To all appearances, all high-Mr PBPs lack activity on Ac<sub>2</sub>-L-Lys-D-Ala-D-Ala, the standard substrate analog of the low-Mr PBPs.

The distinction between the two classes A and B (Figures 2 and 11) derives from homology searches. The *E. coli* PBPs 1A and 1B of class A and the *E. coli* PBPs 2 and 3 of class B have received much attention.

The 850-amino acid PBPIA (encoded by *ponA* in the 73.5-min region of

the chromosome map) and the 799- to 844-amino acid PBP1B (encoded by *ponB* in the 3.3-min region of the map) are bifunctional, peptidoglycan-synthesizing enzymes (59, 85, 107, 108). Their amino terminal domain is a penicillin-insensitive transglycosylase that catalyzes glycan chain elongation, and their penicillin-binding carboxy-terminal domain is a transpeptidase that catalyzes peptidoglycan crosslinking. Their bifunctionality is revealed by the reaction products that they generate upon incubation with the lipid-linked precursor N-acetylglucosaminyl-N-acetylmuramyl (D-alanyl-D-alanine-terminated pentapeptide) diphosphoryl-undecaprenol. The overall reaction, however, proceeds with a low turnover number, suggesting that the in vitro conditions poorly mimic the in vivo situation. PBP1A and PBP1B show differences in reaction conditions for optimal activity. They may compensate for each other according to the cell culture conditions. Selective inactivation of the transpeptidase domain of both PBP1A and PBP1B by derivatization of the essential serine by  $\beta$ -lactam antibiotics causes cell death by cell lysis. Deletion of both *ponA* and *ponB* genes has also that effect.

The interplay between the two catalytic domains of PBP1B is well documented. Penicillin inhibits peptide crosslinking and, under certain conditions, greatly increases formation of uncrosslinked peptidoglycan, suggesting that the uncoupling of the two reactions stimulates the transglycosylase activity. Moenomycin, which inhibits the transglycosylase (120), strongly inhibits or prevents peptide crosslinking, suggesting that transpeptidation requires prior or concomitant glycan chain elongation. Studies with monoclonal antibodies also suggest that transpeptidation depends on the product of transglycosylation and that PBP1B has only one type of pentapeptide (donor)/tetrapeptide (acceptor) transpeptidase activity (24). Membrane components act as effectors of the catalyzed reaction and strongly stimulate the polymerization reaction.

The 633-amino acid PBP2 (encoded by *pbpA* at the 14-min region of the map) and the 588-amino acid PBP3 [encoded by *pbpB* (also called *ftsI*) at the 2-min region] of class B fulfill essential functions in cell morphogenesis, in connection with other membrane proteins. The 14-min region contains, in addition to the PBP2-encoding *pbpA* gene, other genes required for cell-wall elongation and maintenance of the rod shape of the cell. In particular, *rodA* encodes the 370-amino acid integral membrane protein RodA (81), and *dacA* encodes the low-Mr PBP5 (15). Inactivation of *dacA* does not cause growth defects, but a 10-fold overproduction of PBP5 results in spherical cells. Inactivation of PBP2 by the amidinopenicillin mecillinam causes *E. coli* to grow and divide as round-coccal cells. Mutations in either *pbpA* or *rodA* also have that effect and confer resistance to mecillinam.

The 2-min region of the chromosome map contains, in addition to the PBP3-encoding *pbpB* gene, other genes required for the synthesis of the

peptidoglycan precursors (*murE*, *murF*, *murG*, *murC*, *ddl*) and for cell division and septum formation (*ftsW*, *ftsQ*, *ftsA*, *ftsZ*) (109). Selective inactivation of PBP3 either by mutation of *pbpB* or by reaction with, for example, the monobactam aztreonam, leads to filamentous growth of the cells. FtsW is an intrinsic membrane protein very similar to RodA (similarity index: 40). Mutations in FtsW also cause defects in cell division (58). The 420-amino acid FtsA protein is thought to participate in the construction of the septum (98, 116). It must be synthesized during a short period just before division for cell division to be completed. High-level expression of FtsA inhibits cell septation. The 383-amino acid FtsZ protein (124) probably regulates the frequency of cell division. It is the target of the SOS-induced inhibitor SfiA (64). Overexpression of FtsZ leads to a hyperdivision activity displayed as the minicell phenotype (122). The relative levels of FtsZ and trigger factor, an abundant cytosolic 543-amino acid protein that might act as a chaperone, probably control initiation of cell division (50).

The pair RodA-PBP2 is involved in formation of the rod-shape of the cell (81). The pair FtsW-PBP3 may be involved in cell division (58), but interaction between RodA and PBP3 has also been suggested (8). The complex RodA-PBP2 appears to be linked to the ribosomes via the *lov* gene product and to the cyclic AMP (cAMP)-receptor protein (CAP) complex via the adenylate cyclase-encoding *cya* gene and the CAP-encoding *crp* gene (11, 90). The cAMP-CAP complex is involved in the transcriptional regulation of many operons. Mutations in either *cya* or *crp* confer high-level resistance to mecillinam, associated with a slow growth rate. All these data suggest that the intrinsic membrane proteins RodA and FtsW (and perhaps FtsA and FtsZ) link PBP2 and PBP3 at the surface of the cell to structures, chaperones, and/or enzymes in the cytosol, and that correct functioning of these networks is essential for cell morphogenesis.

A connection between PBPs and intracellular components is a feature that is not unique to *E. coli*. In the course of sporulation, the PBP profile of *Bacillus subtilis* undergoes profound remodeling with a sporulation-specific increase in vegetative PBPs and a de novo synthesis of PBPs (114). SpoVE, which is extremely similar in the primary structure to the pair RodA-FtsW of *E. coli* (58, 65), seems to be involved in this process. Mutations in the *spoVE* locus cause a blockage at stage V of sporulation (95), and *spoVE* mutants are grossly deficient in cortex, which consists of a sporulation-specific peptidoglycan. *B. subtilis* also contains homologs of the *E. coli* cell division genes *ftsA* and *ftsZ*. Expression of these *B. subtilis* homologs in *E. coli* results in filamentation and cell death (7).

The use of *E. coli* strains overproducing PBP2 and RodA (60) or PBP3 and FtsW (M. Matsuhashi, personal communication) supports the view that these PBPs catalyze transpeptidation reactions. Assignment of a peptidoglycan

transpeptidase activity to the penicillin-binding, carboxy-terminal domain of the high-Mr PBPs is also supported by the study, at the molecular level, of the resistance to  $\beta$ -lactam antibiotics acquired through emergence of altered PBPs with a much decreased affinity for the drugs (see below). A remodeling of the penicillin-binding–transpeptidase site that results in a decreased affinity for the drug should presumably affect the specificity profile of the enzyme for the carbonyl donor and/or amino acceptor peptides involved in transpeptidation and, thereby, should cause structural modifications of the wall peptidoglycan. The analysis of the peptidoglycans of penicillin-sensitive and penicillin-resistant pneumococci has confirmed the prediction (43).

The question of which enzymatic function the amino terminal domains of the high-Mr PBPs of class B perform is still a matter of controversy. These domains (at least some of them) possess several conserved amino acid groupings not found in the transglycosylase domain of the *E. coli* PBPs 1A and 1B of class A (96). These groupings might be the signature of an active site whose function would be different from that of a transglycosylase. One should remember that PBPs are involved, directly or indirectly, in the average glycan chain length of the peptidoglycan, in the extent of peptidoglycan O-acetylation, and in the amounts of peptidoglycan-attached lipoprotein. None of these observations has received a satisfactory explanation.

### *Intrinsic Resistance by Emergence of Altered High-Mr PBPs*

Assuming that a given high-Mr PBP is the most sensitive killing target of a lactam antibiotic, conversion of this PBP into an altered PBP whose penicillin-binding domain has acquired a decreased affinity for the drug, but has retained the capacity of performing its physiological (transpeptidase) function, causes an increased resistance of the bacterial cell to this particular antibiotic.

Alterations affecting amino acids located away from the active site may be neutral or they may stabilize the protein in response to changes occurring within or close to the active site. Study of an altered PBP2X of a *S. pneumoniae* laboratory mutant with a decreased affinity for cefotaxime shows that such distal alterations may be of real significance (74). By reference to the PBP2X of the cefotaxime-sensitive strain, the two mutations G597  $\rightarrow$  D and G601  $\rightarrow$  V, occurring about 50 residues downstream from the KTG motif, are responsible for the first level of resistance to the drug. One may hypothesize that these amino acid alterations cause changes in the conformation and thereby, changes in the specificity profile of the penicillin-binding domain.

The acquisition of resistance through alterations affecting the active site is well documented. Many  $\beta$ -lactamases of class A have a tyrosine approximate-

ly 25 residues upstream from the SDN motif (4). Alterations of the equivalent peptide stretches Tyr334-S359SN of the *E. coli* PBP3, Tyr337-S362SN of the *Neisseria gonorrhoeae* PBP2, and Tyr419-S447SN of the *S. pneumoniae* PBP2B profoundly modify the specificity profile of these PBPs. Cephalexin, at the minimum inhibitory concentration (MIC), kills *E. coli* by inactivating PBP3. *E. coli* laboratory mutants have been obtained whose PBP3 has a much decreased affinity for cephalexin and other cephalosporins but not for penicillins and aztreonam (53, 54). These PBP3 mutants have either a Val344  $\rightarrow$  Gly substitution (occurring approximately in the middle of the Tyr334-S359SN sequence) or an Asn361  $\rightarrow$  Ser substitution (converting the SSN motif into a SSS motif). This latter mutant has some degree of functional impairment that causes the formation of *E. coli* cells with pointed polar caps (110). Maximal level of resistance to cephalexin, however, is achieved by combining four changes: the conversion SSN  $\rightarrow$  SSS, the substitution Glu349  $\rightarrow$  Lys (which also occurs approximately in the middle of the Tyr  $\rightarrow$  SSS stretch), and the substitutions Val530  $\rightarrow$  Ile and Tyr541  $\rightarrow$  Ser (about 50 to 40 residues upstream from the carboxy terminus of the 388-amino acid protein). Penicillin kills *N. gonorrhoeae* by inactivating PBP2. The PBP2 of clinical isolates that have a decreased affinity for penicillin differs from the PBP2 of the penicillin-sensitive strains only in the insertion of an additional aspartic acid (Asp345A) between Arg345 and Asp346, thus affecting the Tyr337-S362SN sequence (13). However, a *penA* gene containing only the Asp345A codon insertion does not transform a penicillin-sensitive gonococcus to as high a level of resistance to penicillin as the *penA*<sup>R</sup> genes from other non-lactamase-producing, chromosomally mediated resistant gonococcal strains. The PBP2 of these strains contains not only the inserted Asp345A but numerous other amino acid changes in the penicillin-binding domain. Similarly, a *S. pneumoniae* PBP2B mutant obtained from clinical isolates exhibiting a >1000-fold increased resistance to penicillin has 17 amino acid substitutions (27). Seven of these changes are within the Tyr231-S249SN sequence and another change affects the amino acid immediately downstream from the SSN motif.

Acquired resistance may emerge through recombinational events that recruit portions of homologous genes from related species and result in the creation of mosaic genes encoding hybrid PBPs (26, 29, 102, 105). Such horizontal gene transfers occur between species that are naturally transformable such as *Neisseria* and *Streptococcus* spp. The gene encoding the penicillin-resistant PBP2 of *Neisseria meningitidis* consists of regions almost identical to the corresponding regions of the penicillin-sensitive strains and of two regions that are very different from them. These two blocks of altered sequence have resulted from the replacement of meningococcal sequences with the corresponding sequences of the *penA* gene of *Neisseria flavescens*.

The resulting altered form of PBP2, when compared to that of the penicillin-sensitive *N. meningitidis* strain, contains 44 amino acid substitutions and 1 amino acid insertion. *N. gonorrhoeae* also produces altered forms of PBP2, whose part of the *penA* gene has been replaced by the corresponding region of the *N. flavescens* gene.

Methicillin-resistant *S. aureus* strains have imported an inducible high-Mr PBP2' of low affinity for the drug (100). The PBP2'-encoding *mecA* gene may have evolved through recombination of a PBP gene of some bacterium and a  $\beta$ -lactamase inducible gene. The promoter and close upstream region of *mecA* are similar to those of the staphylococcal  $\beta$ -lactamase inducible gene, and the *mecR* region has two main open reading frames, one of which encodes a putative protein that has strong similarity in the primary structure to the penicillin sensory-transducer BLAR of *B. licheniformis* (111; B. Berger-Bächi, personal communication). The *mecA* gene is linked to the *aadD* gene that encodes the 4',4'' adenylyltransferase responsible for tobramycin resistance (119). The two linked genes are carried on a moving element and may have emerged in *S. aureus* through transposition.

The staphylococcal PBP2' has two homologs, PBP5 (32) and PBP3R (96), in the penicillin-resistant *Enterococcus hirae* strains. Complete derivatization of these PBPs by penicillin or cefotaxime requires conditions under which serum albumin is partially acylated. These PBPs can take over the functions needed for wall peptidoglycan assembly under conditions in which all the other PBPs are inactivated. Though they have conserved the characteristic motifs of the penicillin-interactive proteins, their active site has acquired a configuration that almost completely discriminates between penicillin and D-alanyl-D-alanine-terminated peptides, the ultimate achievement in intrinsic penicillin resistance.

#### ACKNOWLEDGMENTS

It gives me great pleasure to acknowledge the contribution of the colleagues who, at the University of Liège, carried out the work described here: Drs. P. Charlier, J. Coyette, D. Dehareng, O. Dideberg, G. Dive, C. Duez, J. Dusart, J.-M. Frère, B. Joris, J. Lamotte-Brasseur, M. Leyh-Bouille, M. Nguyen-Distèche, and C. Piron-Fraipont. I also thank Dr. J. Van Beeumen of the University of Ghent, Belgium, and Drs. J. Kelly and J. Knox of the University of Connecticut, Storrs, USA. The work at Liège has been supported by the *Fonds de la Recherche Scientifique Médicale*, the *Fonds National de la Recherche Scientifique*, the Belgian Government, the Government of the Walloon Region, and the *Fonds de Recherche de la Faculté de Médecine*, Université de Liège.



## Literature Cited

1. Adachi, H., Ohta, T., Matsuzawa, H. 1987. A water-soluble form of penicillin-binding protein 2 of *Escherichia coli* constructed by site-directed mutagenesis. *FEBS Lett.* 226:150-54
2. Adam, M., Damblon, C., Plaiton, B., Christiaens, L., Frère, J. M. 1990. Chromogenic depsipeptide substrates for  $\beta$ -lactamases and penicillin-sensitive DD-peptidases. *Biochem. J.* 270:525-29
3. Adriaens, P., Meesschaert, B., Frère, J.-M., Vanderhaeghe, H., Degelaen, J., et al. 1978. Stability of D-5,5-dimethyl- $\Delta^2$ -thiazolidine-4-carboxylic acid in relation to its possible occurrence as a degradation product of penicillin by the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R61 and the membrane-bound DD-carboxypeptidase from *Bacillus stearothermophilus*. *J. Biol. Chem.* 253:3660-65
4. Ambler, R. P., Frère, J.-M., Ghuysen, J.-M., Jaurin, B., Levesque, R. C., et al. 1991. A standard numbering scheme for the class A  $\beta$ -lactamases. *Biochem. J.* In press
5. Asoh, S., Matsuzawa, H., Ishino, F., Strominger, J. L., Matsuhashi, M., et al. 1986. Nucleotide sequence of the *pppA* gene and characteristics of the deduced amino acid sequence of penicillin-binding protein 2 of *Escherichia coli* K12. *Eur. J. Biochem.* 160:231-38
6. Bartholomé-De Belder, J., Nguyen-Distèche, M., Houba-Herlin, N., Ghuysen, J.-M., Maruyama, I. N., et al. 1988. Overexpression, solubilization and refolding of a genetically engineered derivative of the penicillin-binding protein 3 of *Escherichia coli* K12. *Mol. Microbiol.* 2:519-25
7. Beall, B., Lowe, M., Lutkenhaus, J. 1988. Cloning and characterization of *Bacillus subtilis* homologs of *Escherichia coli* cell division genes *ftsZ* and *ftsA*. *J. Bacteriol.* 170:4855-64
8. Begg, K. J., Spratt, B. G., Donachie, W. D. 1986. Interaction between membrane proteins PBP3 and RodA is required for normal cell shape and division in *Escherichia coli*. *J. Bacteriol.* 167:1004-8
- 8a. Begg, K. J., Takasuga, A., Edwards, D. H., Dewar, S. J., Spratt, B. G., et al. 1990. The balance between different peptidoglycan precursors determines whether *Escherichia coli* will elongate or divide. *J. Bacteriol.* 172:6697-6703
9. Bentley, P. H., Stachulski, A. V. 1983. Synthesis and biological activity of some fused  $\beta$ -lactam peptidoglycan analogues. *J. Chem. Soc. Perkin Trans.* 1:1187-92
10. Boissinot, M., Levesque, R. C. 1990. Nucleotide sequence of the PSE-4 carbenicillinase gene and correlations with the *Staphylococcus aureus* PCI  $\beta$ -lactamase crystal structure. *J. Biol. Chem.* 265:1225-30
11. Bouloc, P., Jaffé, A., D'Ari, R. 1989. The *Escherichia coli* *lov* gene product connects peptidoglycan synthesis, ribosomes and growth rate. *EMBO J.* 8:317-23
12. Bowler, L. D., Spratt, G. D. 1989. Membrane topology of penicillin-binding protein 3 of *Escherichia coli*. *Mol. Microbiol.* 3:1277-86
13. Brannigan, J. A., Tirodimos, I. A., Zhang, Q.-Y., Dowson, C. G., Spratt, B. G. 1990. Insertion of an extra amino acid is the main cause of the low affinity of penicillin-binding protein 2 in penicillin-resistant strains of *Neisseria gonorrhoeae*. *Mol. Microbiol.* 4:913-19
14. Broome-Smith, J. K., Edelman, A., Yousif, S., Spratt, B. G. 1985. The nucleotide sequences of the *ponA* and *ponB* genes encoding penicillin-binding proteins 1A and 1B of *Escherichia coli*. *Eur. J. Biochem.* 147:437-46
15. Broome-Smith, J. K., Ioannidis, I., Edelman, A., Spratt, B. G. 1988. Nucleotide sequences of the penicillin-binding protein 5 and 6 genes of *Escherichia coli*. *Nucleic Acids Res.* 16:1617
16. Buchanan, C. E., Neyman, S. L. 1986. Correlation of penicillin-binding protein composition with different functions of two membranes in *Bacillus subtilis* forespores. *J. Bacteriol.* 165:498-503
17. Cartwright, S. J., Coulson, A. E. W. 1980. Active-site of staphylococcal  $\beta$ -lactamase. *Phil. Trans. R. Soc. London B289:370-72*
18. Cartwright, S. J., Waley, S. G. 1983.  $\beta$ -Lactamase inhibitors. *Med. Res. Rev.* 3:341-82
19. Chan, P. T. 1986. Nucleotide sequence of the *Staphylococcus aureus* PCI  $\beta$ -lactamase gene. *Nucleic Acids Res.* 14:5940
20. Cohen, S. A., Pratt, R. F. 1980. In-

- activation of *Bacillus cereus*  $\beta$ -lactamase I by 6 $\beta$ -bromopenicillanic acid. Mechanism. *Biochemistry* 19:3996-4003
21. Collatz, E., Labia, R., Gutman, L. 1990. Molecular evolution of ubiquitous  $\beta$ -lactamases toward extended-spectrum enzymes active against newer  $\beta$ -lactam antibiotics. *Mol. Microbiol.* 4:1615-20
  22. Dale, J. W., Godwin, D., Mossakouska, D., Stephenson, P., Wall, S. 1985. Sequence of the Oxa-2  $\beta$ -lactamase: comparison with other penicillin-reactive enzymes. *FEBS Lett.* 191:39-42
  23. Dehottay, P., Dusart, J., De Meester, F., Joris, B., Van Beeumen, J., et al. 1987. Nucleotide sequence of the gene encoding the *Streptomyces albus* G  $\beta$ -lactamase precursor. *Eur. J. Biochem.* 166:345-50
  24. den Blaauwen, T., Aarsman, M., Nanninga, N. 1990. Interaction of monoclonal antibodies with the enzymatic domains of penicillin-binding protein 1b of *Escherichia coli*. *J. Bacteriol.* 172:63-70
  25. Dideberg, O., Chartier, P., Wéry, J. P., Dehottay, P., Dusart, J., et al. 1987. The crystal structure of the  $\beta$ -lactamase of *Streptomyces albus* G at 0.3 nm resolution. *Biochem. J.* 245:911-13
  26. Dowson, C. G., Hutchison, A., Brannigan, J. A., George, R. C., Hansman, D., et al. 1989. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Proc. Natl. Acad. Sci. USA* 86:8842-46
  27. Dowson, C. G., Hutchison, A., Spratt, B. G. 1989. Extensive re-modelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of *Streptococcus pneumoniae*. *Mol. Microbiol.* 3:95-102
  28. Dowson, C. G., Hutchison, A., Spratt, B. G. 1989. Nucleotide sequence of penicillin-binding protein 2B gene of *Streptococcus pneumoniae* strain R6. *Nucleic Acids Res.* 17:7518
  29. Dowson, C. G., Hutchison, A., Woodford, N., Johnson, A. P., George, R. C., et al. 1990. Penicillin-resistant *viridans* streptococci have obtained altered penicillin-binding protein genes from penicillin-resistant strains of *Streptococcus pneumoniae*. *Proc. Natl. Acad. Sci. USA* 87:5858-62
  30. Duez, C., Piron-Fraipont, C., Joris, B., Dusart, J., Urdea, M. S., et al. 1987. Primary structure of the *Streptomyces* R61 extracellular DD-peptidase. 1. Cloning into *Streptomyces lividans* and nucleotide sequence of the gene. *Eur. J. Biochem.* 162:509-18
  31. Edelman, A., Bowler, L., Broome-Smith, J. K., Spratt, B. G. 1987. Use of a  $\beta$ -lactamase fusion vector to investigate the organization of penicillin-binding protein 1B in the cytoplasmic membrane of *Escherichia coli*. *Mol. Microbiol.* 1:101-6
  32. El Kharroubi, A., Jacques, P., Piras, G., Coyette, J., Van Beeumen, J., et al. 1991. The penicillin-binding proteins 5 of *Enterococcus hirae* R40 and the penicillin-binding protein 2' of the methicillin-resistant *Staphylococcus aureus* are homologs. *J. Bacteriol.* In press
  33. El Kharroubi, A., Piras, G., Jacques, P., Szabo, I., Van Beeumen, J., et al. 1989. Active-site and membrane topology of the DD-peptidase/penicillin-binding protein n°6 of *Enterococcus hirae* (*Streptococcus faecium*) ATCC9790. *Biochem. J.* 262:457-62
  34. Ferreira, L. C. S., Schwarz, U., Keck, W., Chartier, P., Dideberg, O., et al. 1988. Properties and crystallization of a genetically engineered, water-soluble derivative of the penicillin-binding protein n°5 of *Escherichia coli* K12. *Eur. J. Biochem.* 171:11-16
  35. Fisher, J., Charnas, P. L., Bradley, S. M., Knowles, J. R. 1981. Inactivation of the RTEM  $\beta$ -lactamase from *Escherichia coli*. Interaction of penam sulfones with enzyme. *Biochemistry* 20:2726-31
  36. Frère, J.-M., Duez, C., Ghuyssen, J.-M., Vandekerkhove, J. 1976. Occurrence of a serine residue in the penicillin-binding site of the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R61. *FEBS Lett.* 70:257-60
  37. Frère, J.-M., Ghuyssen, J.-M., Degelaen, J., Loffet, A., Perkins, H. R. 1975. Fragmentation of benzylpenicillin after interaction with the exocellular DD-carboxypeptidase-transpeptidases of *Streptomyces* R61 and R39. *Nature* 258:168-70
  38. Frère, J.-M., Ghuyssen, J.-M., De Graeve, J. 1978. Fragmentation of penicillin catalysed by the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61. Isotopic study of hydrogen fixation on carbon 6. *FEBS Lett.* 88:147-50
  39. Frère, J.-M., Ghuyssen, J.-M., Vanderaeghe, H., Adnaens, P., Degelaen, J. 1976. Fate of thiazolidine ring during

- fragmentation of penicillin by exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61. *Nature* 260:451-54
40. Frère, J.-M., Ghuysen, J.-M., Perkins, H. R., Nieto, M. 1973. Kinetics of concomitant transfer and hydrolysis reactions catalysed by the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61. *Biochem. J.* 135: 483-92
  41. Frère, J.-M., Joris, B. 1985. Penicillin-sensitive enzymes in peptidoglycan biosynthesis. *CRC Crit. Rev. Microbiol.* 11:299-396
  42. Galleni, M., Amicosante, G., Frère, J.-M. 1988. A survey of the kinetic parameters of class C  $\beta$ -lactamases. *Biochem. J.* 255:119-22
  43. Garcia-Bustos, J., Tomasz, A. 1990. A biological price of antibiotic resistance: major changes in the peptidoglycan structure of penicillin-resistant pneumococci. *Proc. Natl. Acad. Sci. USA* 87: 5415-19
  44. Ghuysen, J.-M. 1977. The bacterial DD-carboxypeptidase-transpeptidase enzyme system. A new insight into the mode of action of penicillin. *E. R. Squibb Lectures on Chemistry of Microbial Products*, series ed. W. E. Brown. Tokyo: Univ. Tokyo Press. 162 pp.
  45. Ghuysen, J.-M., Frère, J.-M., Leyh-Bouille, M., Coyette, J., Dusart, J., et al. 1979. Use of model enzymes in the determination of the mode of action of penicillins and  $\Delta^3$ -cephalosporins. *Annu. Rev. Biochem.* 48:73-101
  46. Ghuysen, J.-M., Frère, J.-M., Leyh-Bouille, M., Nguyen-Distèche, M., Coyette, J. 1986. Active-site serine  $\alpha$ -alanyl-D-alanine-cleaving peptidases-catalysed acyl transfer reactions. Procedures for studying the penicillin-binding proteins of bacterial plasma membranes. *Biochem. J.* 235:159-65
  47. Ghuysen, J.-M., Leyh-Bouille, M., Campbell, J. N., Moreno, R., Frère, J.-M., et al. 1973. Structure of the wall peptidoglycan of *Streptomyces* R39 and the specificity profile of its exocellular DD-carboxypeptidase-transpeptidase for peptide acceptors. *Biochemistry* 12: 1243-51
  48. Ghuysen, J.-M., Reynolds, P. E., Perkins, H. R., Frère, J.-M., Moreno, R. 1974. Effects of donor and acceptor peptides on concomitant hydrolysis and transfer reactions catalysed by the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R39. *Biochemistry* 13:2539-47
  49. Gibson, R. S., Christensen, H., Waley, S. G. 1990. Site-directed mutagenesis of  $\beta$ -lactamase I. Single and double mutants of Glu166 and Lys73. *Biochem. J.* 272:613-19
  50. Guthrie, B., Wickner, W. 1990. Trigger factor depletion and overproduction causes defective cell division but does not block protein export. *J. Bacteriol.* 172:5555-62
  51. Hall, A., Knowles, J. R. 1976. Directed selective pressure on a  $\beta$ -lactamase to analyse molecular changes involved in development of enzyme function. *Nature London* 264:803-4
  52. Hara, H., Nishimura, Y., Kato, J. I., Suzuki, H., Nagasawa, H., et al. 1989. Genetic analysis of processing involving C-terminal cleavage in penicillin-binding protein 3 of *Escherichia coli*. *J. Bacteriol.* 171:5882-89
  53. Hedge, P. J., Spratt, B. G. 1985. Resistance to  $\beta$ -lactam antibiotics by remodelling the active site of an *E. coli* penicillin-binding protein. *Nature* 318: 478-80
  54. Hedge, P. J., Spratt, B. G. 1985. Amino acid alterations that reduce the affinity of penicillin-binding protein 3 of *Escherichia coli* for cephalixin. *Eur. J. Biochem.* 151:111-21
  55. Hedge, P. J., Spratt, B. G. 1988. A gene fusion that localises the penicillin-binding domains of penicillin-binding protein 3. *J. Bacteriol.* 170:5392-95
  56. Herzberg, O., Moul, J. 1987. Bacterial resistance to  $\beta$ -lactam antibiotics: crystal structure of  $\beta$ -lactamase from *Staphylococcus aureus* PCI at 2.5 Å resolution. *Science* 236:694-701
  57. Hussain, M., Pastor, F. I. J., Lampen, J. O. 1987. Cloning and sequencing of the *bla<sub>Z</sub>* gene encoding  $\beta$ -lactamase III, a lipoprotein of *Bacillus cereus* 569/H. *J. Bacteriol.* 169:579-86
  58. Ikeda, M., Sato, T., Wachi, M., Jung, H. K., Ishino, F., et al. 1989. Structural similarity among *Escherichia coli* FtsW and RodA proteins and *Bacillus subtilis* SpoVE protein, which function in cell division, cell elongation, and spore formation, respectively. *J. Bacteriol.* 171:6375-78
  59. Ishino, F., Mitsui, S., Tamaki, S., Matsuhashi, M. 1980. Dual enzymatic activities of cell wall peptidoglycan synthesis, peptidoglycan transglycosylase and penicillin-sensitive transpeptidase in purified preparations of *Escherichia coli* penicillin-binding protein 1A. *Biochem. Biophys. Res. Commun.* 97:287-93
  60. Ishino, F., Park, W., Tomioka, S.,

- Tamaki, S., Takase, I., et al. 1986. Peptidoglycan synthetic activities in membranes of *Escherichia coli* caused by overproduction of penicillin-binding protein 2 and RodA protein. *J. Biol. Chem.* 262:7024-31
61. Jackson, M. E., Pratt, J. M. 1988. Analysis of the membrane-binding domain of penicillin-binding protein 5 of *Escherichia coli*. *Mol. Microbiol.* 2:563-68
  62. Jacob, F., Joris, B., Dideberg, O., Dusart, J., Ghuysen, J.-M., et al. 1990. Engineering of a novel  $\beta$ -lactamase by a single point mutation. *Protein Eng.* 4:223:6427-37
  63. Jacob, F., Joris, B., Lepage, S., Dusart, J., Frère, J.-M. 1990. Role of the conserved amino acids of the "SDN" loop in a class A  $\beta$ -lactamase studied by site-directed mutagenesis. *Biochem. J.* 271:399-406
  64. Jones, C. A., Holland, I. B. 1985. Role of the SfiB (FtsZ) protein in division inhibition during the SDS response in *E. coli*. FtsZ stabilizes the inhibitor SfiA in maxicells. *Proc. Natl. Acad. Sci. USA* 82:6045-49
  65. Joris, B., Dive, G., Henriques, A., Piggot, P. J., Ghuysen, J.-M. 1990. The life-cycle proteins RodA of *Escherichia coli* and SpoVE of *Bacillus subtilis* have very similar primary structures. *Mol. Microbiol.* 4:513-17
  66. Joris, B., Ghuysen, J.-M., Dive, G., Renard, A., Dideberg, O. et al. 1988. The active-site serine penicillin-recognizing enzymes as members of the *Streptomyces* R61 DD-peptidase family. *Biochem. J.* 250:313-24
  67. Joris, B., Ledent, P., Dideberg, O., Fonzé, E., Lamotte-Brasseur, J., et al. 1991. A comparison of the sequences of class A  $\beta$ -lactamases and of the secondary structure elements of penicillin-recognizing proteins. *Biochem. J.* In press
  68. Joris, B., Ledent, P., Kobayashi, T., Lampen, J. O., Ghuysen, J.-M. 1990. Expression in *Escherichia coli* of the Met346-Arg601 carboxy terminal domain of the BLAR sensory transducer protein of *Bacillus licheniformis* as a water-soluble 26,000-Mr penicillin-binding protein. *FEMS Microbiol. Lett.* 70:107-14
  69. Kelly, J. A., Dideberg, O., Charlier, P., Wéry, J. P., Libert, M., et al. 1986. On the origin of bacterial resistance to penicillin. Comparison of a  $\beta$ -lactamase and a penicillin target. *Science* 231:1429-37
  70. Kelly, J. A., Knox, J. R., Zhao, H., Frère, J.-M., Ghuysen, J.-M. 1989. Crystallographic mapping of  $\beta$ -lactams bound to a D-alanyl-D-alanine peptidase target enzyme. *J. Mol. Biol.* 209:281-95
  71. Knott-Hunziker, V., Waley, S. G., Orlek, B. S., Sammes, P. G. 1979. Penicillinase active sites: labelling of serine-44 in  $\beta$ -lactamase I by 6 $\beta$ -bromopenicillanic acid. *FEBS Lett.* 99:59-61
  72. Knox, J. R., Pratt, R. F. 1990. Different modes of vancomycin and D-alanyl-D-alanine peptidase binding to cell wall peptide and a possible role for the vancomycin resistance protein. *Antimicrob. Agents Chemother.* 34:1342-47
  73. Kobayashi, T., Zhu, Y., Nicholls, N., Lampen, J. O. 1987. A second regulatory gene, BLAR1, encoding a potential penicillin-binding protein required for induction of  $\beta$ -lactamase in *Bacillus licheniformis*. *J. Bacteriol.* 169:3873-78
  74. Laible, G., Hakenbeck, R., Sicard, M. A., Joris, B., Ghuysen, J.-M. 1989. Nucleotide sequences of the *pbpX* genes encoding the penicillin-binding proteins 2X from *Streptococcus pneumoniae* R6 and a cefotaxime resistant mutant, C50G. *Mol. Microbiol.* 3:1337-48
  75. Lamotte-Brasseur, J., Dive, G., Dehareng, D., Ghuysen, J.-M. 1990. Electrostatic potential maps at the quantum chemistry level of the active sites of the serine peptidases,  $\alpha$ -chymotrypsin and subtilisin. *J. Theor. Biol.* 145:183-98
  76. Lamotte-Brasseur, J., Dive, G., Ghuysen, J.-M. 1991. Conformational analysis of  $\beta$ - and -lactam antibiotics. *Eur. J. Med. Chem.* In press
  77. Leyh-Bouille, M., Nguyen-Distèche, M., Bellefroid-Bourguignon, C., Ghuysen, J.-M. 1987. Effects of thiol reagents on the *Streptomyces* K15 DD-peptidase-catalysed reactions. *Biochem. J.* 241:893-97
  78. Leyh-Bouille, M., Nguyen-Distèche, M., Pirlot, S., Veithen, A., Bourguignon, C., et al. 1986. *Streptomyces* K15 DD-peptidase-catalysed reaction with suicide  $\beta$ -lactam carbonyl donors. *Biochem. J.* 235:177-82
  79. Lindberg, F., Normark, S. 1986. Sequence of the *Citrobacter freundii* chromosomal *ampC*  $\beta$ -lactamase gene. *Eur. J. Biochem.* 156:441-45
  80. Marquet, A., Frère, J.-M., Ghuysen, J.-M., Loffet, A. 1979. Effects of nucleophiles on the breakdown of the benzylpenicilloyl-enzyme complex EI\* formed

- between benzylpenicillin and the exocellular D-carboxypeptidase-transpeptidase of *Streptomyces* strain R61. *Biochem. J.* 177:909-16
81. Matsuzawa, H., Asoh, S., Kunai, K., Muraiso, K., Takasuga, A., et al. 1989. Nucleotide sequence of the *rodA* gene, responsible for the rod shape of *Escherichia coli*: *rodA* and the *pppA* gene, encoding penicillin-binding protein 2, constitute the *rodA* operon. *J. Bacteriol.* 171:558-60
82. Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., Frère, J.-M. 1990.  $\beta$ -Lactamase of *Bacillus licheniformis* 749/C at 2 Å resolution. *Proteins Struct. Funct. Genet.* 7:156-71
83. Mottl, H., Terpstra, P., Keck, W. 1991. Penicillin-binding protein 4 of *Escherichia coli* shows a novel type of primary structure among penicillin-interacting proteins. *FEMS Microbiol. Lett.* 78:213-20
84. Nagasawa, H., Sakagami, Y., Suzuki, A., Suzuki, H., Hara, H., et al. 1989. Determination of the cleavage site involved in C-terminal processing of penicillin-binding protein 3 of *Escherichia coli*. *J. Bacteriol.* 171:5890-93
85. Nakagawa, J.-I., Tamaki, S., Matsuhashi, M. 1979. Purified penicillin-binding protein 1Bs from *Escherichia coli* membranes showing activities of both peptidoglycan polymerase and peptidoglycan crosslinking enzyme. *Agric. Biol. Chem.* 43:1379-80
86. Nakamura, M., Maruyama, I. N., Soma, M., Kato, J. I., Suzuki, H., et al. 1983. On the process of cellular division in *Escherichia coli*: nucleotide sequence of the gene for penicillin-binding protein 3. *Mol. Gen. Genet.* 191:1-9
87. Neugebauer, K., Sprengl, R., Shaller, H. 1981. Penicillinase from *Bacillus licheniformis*: nucleotide sequence of the gene and implications for the biosynthesis of a secretory protein in a Gram-positive bacteria. *Nucleic Acids Res.* 9:2577-88
88. Nguyen-Distèche, M., Leyh-Bouille, M., Pirlot, S., Frère, J.-M., Ghuysen, J.-M. 1986. *Streptomyces* K15 D-peptidase-catalysed reactions with ester and amide carbonyl donors. *Biochem. J.* 235:167-76
89. Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., et al. 1990. Refined crystal structure of  $\beta$ -lactamase from *Citrobacter freundii* indicates a mechanism for  $\beta$ -lactam hydrolysis. *Nature* 343:284-88
90. Ogura, T., Bouloc, P., Niki, H., D'Ari, R., Horaga, S., et al. 1989. Penicillin-binding protein 2 is essential in wild-type *Escherichia coli* but not in *lov* and *cya* mutants. *J. Bacteriol.* 171:3025-30
91. Pazhanisamy, S., Govardhan, C. P., Pratt, R. F. 1989.  $\beta$ -Lactamase-catalysed aminolysis of depsipeptides: amine specificity and steady-state kinetics. *Biochemistry* 28:6863-70
92. Pazhanisamy, S., Pratt, R. F. 1989.  $\beta$ -Lactamase-catalysed aminolysis of depsipeptides: proof of the nonexistence of a specific D-phenylalanine/enzyme complex by double-label isotope trapping. *Biochemistry* 28:6870-75
93. Pazhanisamy, S., Pratt, R. F. 1989.  $\beta$ -Lactamase-catalysed aminolysis of depsipeptides: peptide inhibition and a new kinetic mechanism. *Biochemistry* 28:6875-82
94. Perkins, H. R., Nieto, M., Frère, J.-M., Leyh-Bouille, M., Ghuysen, J.-M. 1973. *Streptomyces* D-carboxypeptidases as transpeptidases. The specificity for amino compounds acting as carboxyl acceptors. *Biochem. J.* 131:707-18
95. Pigott, J. J., Coote, J. G. 1976. Genetic aspects of bacterial endospore formation. *Bacteriol. Rev.* 40:908-62
96. Piras, G., El Kharroubi, A., Van Beumen, J., Coeme, E., Coyette, J., et al. 1990. Occurrence and characterization of a penicillin-binding protein 3 of low penicillin affinity in *Enterococcus hirae*. *J. Bacteriol.* 172:6856-62
97. Pratt, J. M., Jackson, M. E., Holland, I. B. 1986. The C-terminus of penicillin-binding protein 5 is essential for localisation to the *E. coli* inner membrane. *EMBO J.* 5:2399-2405
98. Robinson, A. C., Begg, K. J., Sweeney, J., Condie, A., Donachie, W. D. 1988. Mapping and characterization of mutants of the *Escherichia coli* cell division gene, *ftsA*. *Mol. Microbiol.* 2:581-88
99. Samuni, A., Citri, N. 1979. How specific is the effect of penicillins on the conformation of penicillinase? An experimental model. *Mol. Pharmacol.* 16:250-55
- 99a. Schuster, C., Dobrinski, B., Hakenbeck, R. 1990. Unusual septum formation in *Streptococcus pneumoniae* mutants with an alteration in the D, D-carboxypeptidase penicillin-binding protein 3. *J. Bacteriol.* 172:6499-6505
100. Song, M. D., Wachi, M., Doi, M., Ishi-

- no, F., Matsuhashi, M. 1987. Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. *FEBS Lett.* 221:167-71
101. Sougakoff, W., Petit, A., Goussard, S., Sirot, D., Bure, A., et al. 1989. Characterization of the plasmid genes *blaT-4* and *blaT-5* which encode the broad-spectrum  $\beta$ -lactamases TEM-4 and TEM-5 in Enterobacteriaceae. *Gene* 78:339-48
  102. Spratt, B. G. 1988. Hybrid penicillin-binding proteins in penicillin-resistant strains of *Neisseria gonorrhoeae*. *Nature* 332:173-76
  103. Spratt, B. G., Cromie, K. D. 1988. Penicillin-binding proteins of Gram-negative bacteria. *Rev. Infect. Dis.* 10: 699-711
  104. Spratt, B. G., Pardee, A. B. 1975. Penicillin-binding proteins and cell shape in *E. coli*. *Nature* 254:516-17
  105. Spratt, B. G., Zhang, Q.-Y., Jones, D. M., Hutchison, A., Brannigan, J. A., et al. 1989. Recruitment of a penicillin-binding protein gene from *Neisseria flavescens* during the emergence of penicillin-resistance in *Neisseria meningitidis*. *Proc. Natl. Acad. Sci. USA* 86: 8988-92
  106. Stoker, N. G., Broome-Smith, J. K., Edelman, A., Spratt, B. G. 1983. Organization and subcloning of the *dacA-rodA-ppbA* cluster of cell shape genes in *Escherichia coli*. *J. Bacteriol.* 155:847-59
  107. Suzuki, S., van Heijenoort, Y., Tamura, T., Mizoguchi, J., Hirota, Y., et al. 1980. *In vitro* peptidoglycan polymerization catalysed by penicillin-binding protein 1b of *Escherichia coli* K12. *FEBS Lett.* 110:245-49
  108. Tamaki, S., Nakajima, S., Matsuhashi, M. 1978. Thermosensitive mutation in *Escherichia coli* simultaneously causing defects in penicillin-binding protein-1Bs and in enzyme activity for peptidoglycan synthesis *in vitro*. *Proc. Natl. Acad. Sci. USA* 74:5472-76
  109. Taschner, P. E. M., Huls, P. G., Pas, E., Woldringh, C. L. 1988. Division behavior and shape changes in isogenic *ftsZ*, *ftsQ*, *ftsA*, *ppbB* and *ftsE* cell division mutants of *Escherichia coli* during temperature shift experiments. *J. Bacteriol.* 170:1533-40
  110. Taschner, P. E. M., Ypenburg, N., Spratt, B. G., Woldringh, C. L. 1988. An amino acid substitution in penicillin-binding protein 3 creates pointed polar caps in *Escherichia coli*. *J. Bacteriol.* 170:4828-37
  111. Tesch, W., Ryffel, C., Strässle, A., Kayser, F. H., Berger-Bächi, B. 1990. Evidence of a novel staphylococcal *mecR* encoded element (*mecR*) controlling expression of penicillin-binding protein 2'. *Antimicrob. Agents Chemother.* 34: 1703-6
  112. Tipper, D. J., Strominger, J. L. 1965. Mechanism of action of penicillins: a proposal based on the structural similarity to acyl-D-alanyl-D-alanine. *Proc. Natl. Acad. Sci. USA* 54:1131-41
  113. Todd, J. A., Bone, E. J., Ellar, D. J. 1985. The sporulation-specific penicillin-binding protein 5a from *Bacillus subtilis* is a D $\alpha$ -carboxypeptidase *in vitro*. *Biochem. J.* 230:825-28
  114. Todd, J. A., Bone, E. J., Piggot, P. J., Ellar, D. J. 1983. Differential expression of penicillin-binding protein structural genes during *Bacillus subtilis* sporulation. *FEMS Microbiol. Lett.* 18:197-202
  115. Todd, J. A., Roberts, A. N., Johnston, K., Piggot, P. J., Winter, G., et al. 1986. Reduced heat resistance of mutant scores after cloning and mutagenesis of the *Bacillus subtilis* gene encoding penicillin-binding protein 5. *J. Bacteriol.* 167:257-64
  116. Tormo, A., Vicente, M. 1984. The *ftsA* gene product participates in formation of the *Escherichia coli* septum structure. *J. Bacteriol.* 157:779-84
  117. Tsukamoto, K., Kihura, R., Ohno, R., Sawai, T. 1990. Substitution of aspartic acid-217 of *Citrobacter freundii* cephalosporinase and properties of the mutant enzymes. *FEBS Lett.* 264:211-14
  118. Tsukamoto, K., Ohno, R., Saway, T. 1990. Extension of the substrate spectrum by an amino acid substitution at residue 219 in the *Citrobacter freundii* cephalosporinase. *J. Bacteriol.* 172: 4348-51
  119. Ubukata, K., Nonoguchi, R., Matsuhashi, M., Song, M. D., Konno, M. 1989. Restriction maps of the regions coding for methicillin and tobramycin resistances on chromosomal DNA in methicillin-resistant staphylococci. *Antimicrob. Agents Chemother.* 33:1624-26
  120. van Heijenoort, Y., Leduc, M., Singer, H., van Heijenoort, J. 1987. Effect of moemycin on *Escherichia coli*. *J. Gen. Microbiol.* 133:667-74
  121. Varetto, L., Bellefroid-Bourguignon, C., Frère, J.-M., Nguyen-Distèche, M., Ghuyssen, J.-M., et al. 1987. The pH-dependence of the active-site serine D $\alpha$ -

- peptidase of *Streptomyces* R61. *Eur. J. Biochem.* 162:525–31
122. Ward, J. E., Lutkenhaus, J. F. 1985. Overproduction of FtsZ induces minicells in *Escherichia coli*. *Cell* 42:941–49
123. Waxman, D. J., Strominger, J. L. 1981. Primary structure of the COOH-terminal membranous segment of a penicillin-sensitive enzyme purified from two Bacilli. *J. Biol. Chem.* 256:2067–77
124. Yi, Q. M., Lutkenhaus, J. F. 1985. The nucleotide sequence of the essential cell division gene *ftsZ*. *Gene* 36:241–47
125. Zhu, Y. F., Curran, I. J., Joris, B., Ghuysen, J.-M., Lampen, J. O. 1990. Identification of BlaR, signal transducer for  $\beta$ -lactamase production in *Bacillus licheniformis* as a penicillin-binding protein with strong homology to the Oxa-2  $\beta$ -lactamase (class D) of *Salmonella typhimurium*. *J. Bacteriol.* 172:1137–41