Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function

Edited by:

Paul Actor
Smith Kline & French Laboratories, Philadelphia, Pennsylvania

Lolita Daneo-Moore
Temple University, Philadelphia, Pennsylvania

Michael L. Higgins
Temple University, Philadelphia, Pennsylvania

Milton R. J. Salton
New York University School of Medicine, New York, New York

Gerald D. Shockman
Temple University, Philadelphia, Pennsylvania

1988
American Society for Microbiology
Washington, D.C. 20006

Chapter 36

Characterization of the Trypsin-Solubilized Penicillin-Binding Proteins of *Enterococcus hirae* (*Streptococcus faecium*)

Aboubaker El Kharroubi
Philippe Jacques
Graziella Piras
Jacques Coyette
Jean-Marie Ghuysen

Most penicillin-binding proteins (PBPs) behave as membrane-bound proteins which can be solubilized only by detergents or chaotropic agents, or both, and by proteolytic enzymes. When trypsin is used, several PBPs are cleaved off the membranes and lose a small terminal peptide mediating the anchorage of the proteins in the membranes. Usually, trypsin degrades the large soluble polypeptides released into smaller fragments, designated below as tPBPs, some of which are relatively stable and still active (i.e., able to hydrolyze a substrate or to bind β-lactams, or both) (2, 10).

Enterococcus hirae (*Streptococcus faecium*) strains usually possess seven PBPs ranging in size from 140 to 43 kilodaltons (kDa) (4). PBPs 2 and 3 are involved in cellular division (5). PBP 5 is a low-affinity PBP implicated in the β-lactam resistance of the *E. hirae* strains (7). PBP 6 is the only DD-carboxypeptidase activity measurable in vitro in the membranes (3).

Aboubaker El Kharroubi, Philippe Jacques, Graziella Piras, Jacques Coyette, and Jean-Marie Ghuysen • Service de Microbiologie, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman (Liège 1), Belgium

In the work presented below, we determined precise experimental conditions for solubilizing, as much as possible specifically, the different PBPs from *E. hirae* membranes, and we identified the different tPBPs released.

PBP Degradation

PBP 4

PBP 4 was shown to be spontaneously released, probably by an endogenous protease, when *E. hirae* ATCC 9790 membranes were incubated at 37°C. The soluble active polypeptide isolated, PBP 4*, was 7 kDa smaller than the membrane protein (4).

Low concentrations of trypsin (given as percentage [wt/wt]) promoted the release of tPBP 4a, which was indistinguishable from PBP 4* (identical β-lactam affinities and behavior on sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) (Fig. 1 and 2). More than 90% of the membrane PBP 4 was solubilized as tPBP 4a, which remained stable in the presence of trypsin as long as the protease concentration and the incubation time did not exceed 0.5 to 1% and 30 min, respectively. Above these limits, tPBP 4a was degraded into smaller, yet unidentified cleavage products.

PBP 5

Two batches of membranes, isolated from the resistant mutant strain R40, which contains large amounts of PBP 5 (7), were labeled differently. Under one set of conditions all the PBPs, including PBP 5, were labeled; under the other, only PBP 5 and a small fraction of PBP 6 were labeled. Comparative analysis of these differently labeled membranes allowed the identification of PBP 5 trypsin degradation products (Fig. 1).

In the presence of trypsin, PBP 5 was cleaved into four different polypeptides designated as tPBPs 5a, 5b, 5c, and 5d (Fig. 1). All were soluble and still able to bind β-lactams. Cleavage kinetics showed that tPBP 5a was the first product released. tPBP 5a was about 7 kDa smaller than PBP 5 and was then degraded into PBPs 5b and 5c. Polypeptide 5d, appearing last, was the smallest "active" product. It was stable under the experimental conditions used, but if the trypsin treatment was increased too much, it was degraded into much smaller, yet unidentified peptides.

The optimal conditions for a maximal yield of tPBP 5a depended not only on the protease concentration and the incubation duration but also on the presence of glycerol at a concentration above 1% and on the membrane concen-

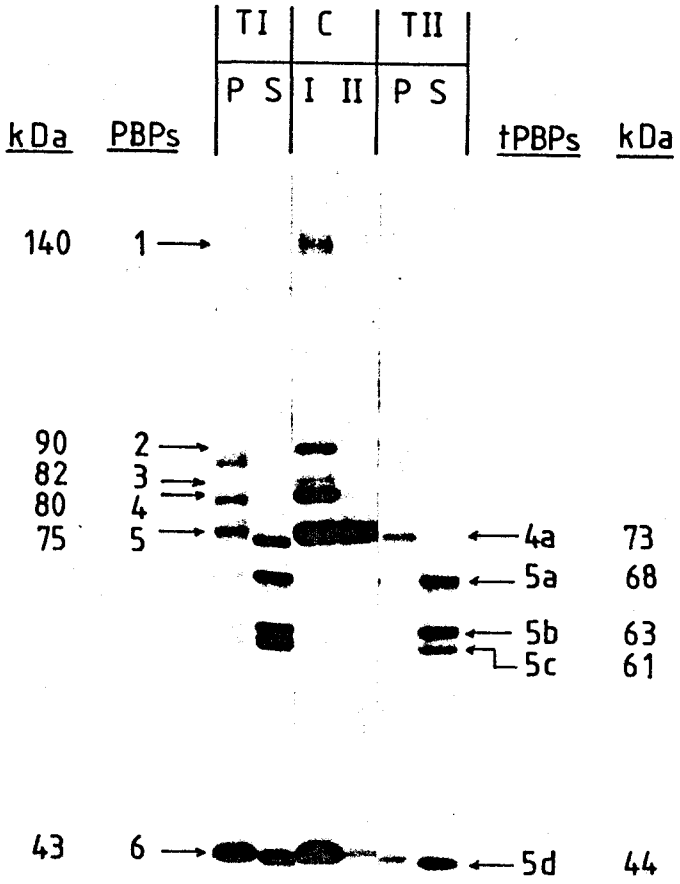

Figure 1. Identification of the trypsin degradation products of PBPs 4 and 5. Two batches of prelabeled R40 resistant mutant membranes were treated for 30 min at 37°C with 2% trypsin. Prelabeling was realized as follows: batch TI was labeled with 10^{-4} M [^{3}H]benzylpenicillin for 1 h; batch TII was preincubated with 1.5×10^{-5} M nonradioactive penicillin for 10 min and then saturated with 10^{-4} M [^{3}H]benzylpenicillin for 1 h. Control samples from each batch (CI and CII) were taken just before trypsin treatment. After proteolysis, supernatant (S) and pellet (P) fractions from a centrifugation (40,000 × *g*, 30 min) were collected and analyzed by SDS-PAGE followed by fluorography. The separating gel was 7.2% acrylamide.

tration, which should not exceed 10 mg/ml. On these bases, about 75, 3, and 4.5% of tPBPs 5a, 5b, and 5c, respectively, were released in the presence of 0.25% trypsin after 5 min of incubation at 37°C. About 18% of PBP 5 remained in the membranes. After 10 min of incubation, about 38% of total PBP 5 was already cleaved into the three smaller products.

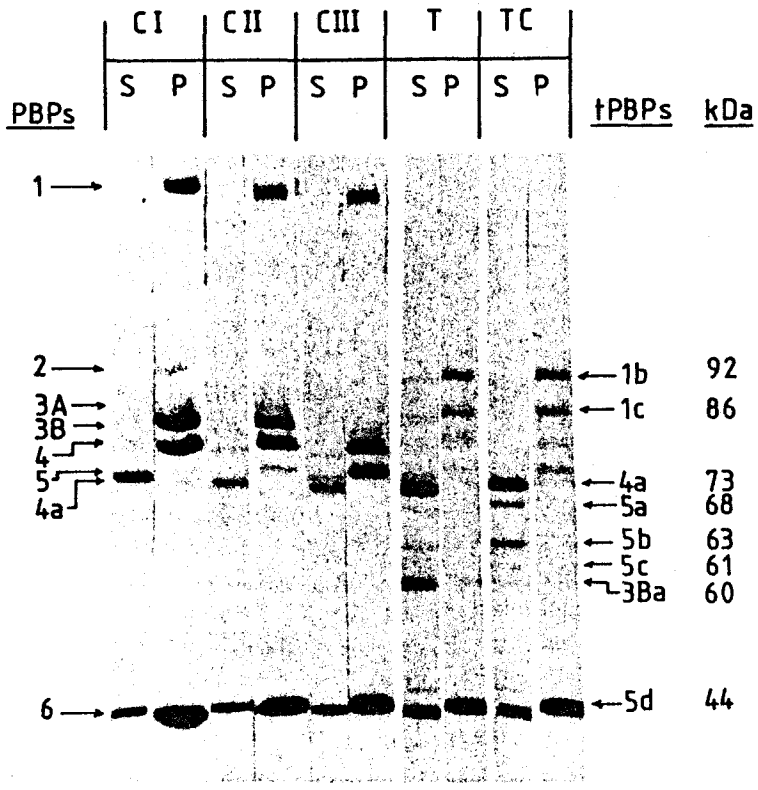

Figure 2. Identification of the trypsin degradation products of PBP 3B from the NT1/20 mutant membranes. Two membrane samples (150 μg/15 μl) were incubated with 2×10^{-5} M benzyl[^{35}S]penicillin for 20 min. One was kept as a control (CII). The other (T) was treated with 1% trypsin for 10 min. Proteolysis was stopped by the addition of a 10× excess of trypsin soybean inhibitor. Two other membrane samples were incubated with 5×10^{-6} M cefotaxime for 10 min and then with 10^{-4} M benzyl[^{35}S]penicillin for 30 min. One was kept as a control (CIII). The other (TC) was treated with trypsin as described for sample T. Membranes from *E. hirae* ATCC 9790 were used as a control (CI) and labeled as samples CII and T. After incubation in the presence or absence of trypsin, all the samples were centrifuged and separated into supernatant (S) and pellet (P) fractions which were submitted to SDS-PAGE and fluorography. The separating gel was 7.2% acrylamide.

PBP 3B

The degradation of PBP 3B was studied on membranes isolated from the thermosensitive division mutant NT1/20 of *E. hirae* ATCC 9790, which is constitutively devoid of PBP 2 (1). Labeling of NT1/20 membranes with ben-

zyl[^{35}S]penicillin, with or without previous incubation in the presence of a low cefotaxime concentration, allowed us to find the PBP 3B derivatives among the different PBP degradation products released after trypsin treatment (Fig. 2).

The main cleavage product, tPBP 3Ba, still able to bind β-lactams, had a size of 60 kDa. No intermediate-size degradation product was identified. Kinetics of release showed that the optimal yields reached 95% when the proteolysis was done under the following conditions: 1% trypsin for 10 min at 37°C in 1 mM $MgCl_2$–5% glycerol–40 mM phosphate buffer (pH 7). After 15 min of incubation, degradation of PBP 3Ba began. The half-life of the polypeptide under these conditions was about 40 min. Thus tPBP 3Ba was relatively less stable in the presence of trypsin than tPBPs 4a and 5d.

PBP 6 (DD-Carboxypeptidase)

PBP 6 solubilization by trypsin was already described in 1980 (4). The experimental conditions for solubilization were reexamined and improved as described here. On the basis of the results presented above and to avoid the loss of different soluble tPBPs, membranes were treated with 1% trypsin for 10 min at 37°C prior to the trypsin treatment required for DD-carboxypeptidase solubilization. PBP 3B, 4, and 5 derivatives were found in the supernatant of a centrifugation done after the trypsin pretreatment (Fig. 3). They represented, respectively, about 60, 90, and 85% of the untreated membrane proteins. Usually, a small, apparently soluble fraction of intact PBP 6 was also present in this supernatant. Experimental results (not shown here) indicated that this fraction corresponded to micellar or microparticular PBP 6.

High trypsin concentrations induced the formation of two PBP 6 cleavage derivatives. Depending on the experimental conditions used, the largest polypeptide, tPBP 6a (40 kDa), was found after centrifugation either in supernatants or in pellets, in this latter case thus being still associated in some way with the treated membranes. It appeared to be an intermediate in the DD-carboxypeptidase degradation as it never exceeded 10% of the total PBP 6. The second derivative, tPBP 6b (formerly designated as PBP 6*) accumulated during the solubilization as a 30-kDa soluble polypeptide still possessing the enzymatic properties of the membrane PBP 6.

Optimal yields of solubilization of this 30-kDa DD-carboxypeptidase were obtained by using a two-step trypsin treatment (Fig. 3). DD-Carboxypeptidase activity or β-lactam binding capacity was measured in the two supernatants and the residual membrane suspension obtained.

Maximal solubilization reached 90 to 97% of the activity present in the pretreated membranes in analytical as well as preparative experiments.

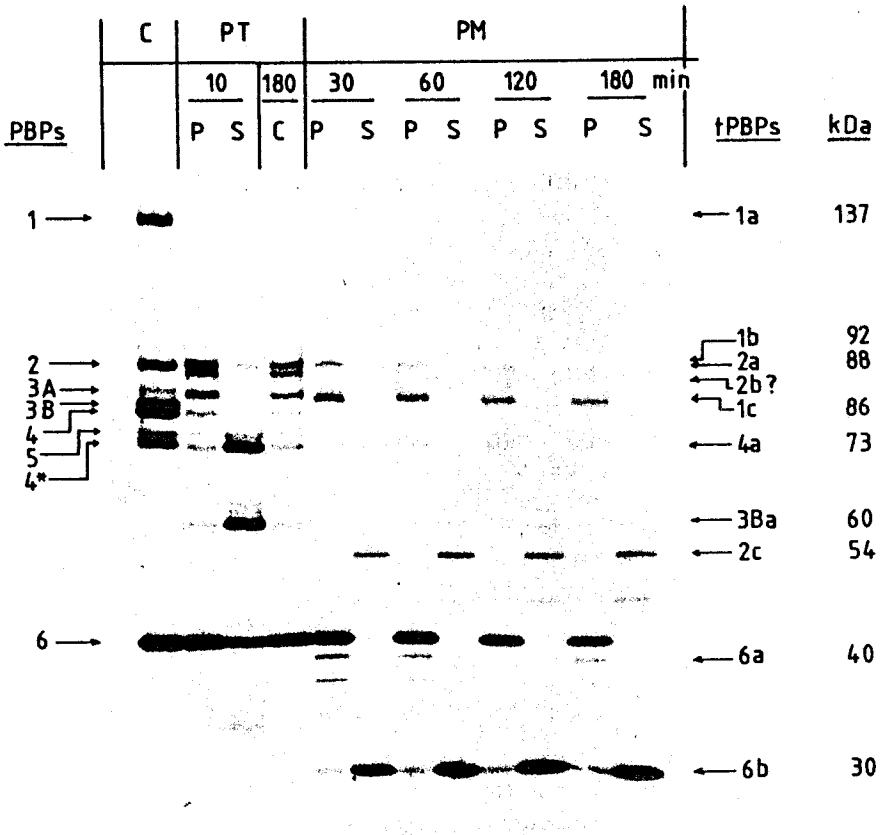

Figure 3. Conversion by trypsin of membrane-bound PBP 6 into water-soluble tPBP 6b. Samples of membranes (130 μg/15 μl), prelabeled with 5×10^{-5} M benzyl[^{35}S]penicillin for 30 min at 37°C, were incubated for 10 min at 37°C with 1% trypsin, centrifuged (40,000 × *g*, 30 min), and separated in pellets (P) and supernatants (S). The pellets, suspended in 100 mM NH_4HCO_3–0.1 mM $CaCl_2$ solution (pH 7.8), were treated at 37°C in the presence of 10% trypsin for increasing times. After the second incubation, the soluble products were collected in supernatants (S) after centrifugation, and the residual membranes (P) were resuspended in the original volume (27 μl). The different fractions were submitted to SDS-PAGE (7.2% acrylamide) and fluorography. C, Control membranes; PT, pretreated membranes; PM, pretreated membranes submitted to the second trypsin treatment; 180C, control pretreated membranes incubated for 180 min in the absence of trypsin.

PBP 2

Present in trypsic hydrolysates of strain ATCC 9790 membranes and absent in similar hydrolysates of strain NT1/20 membranes, two PBP 2 derivatives were identified with certainty. Other minor cleavage products probably

derived from PBP 2, but their identifications were not pursued (Fig. 3). One derivative of 88 kDa (2 kDa smaller than the native PBP) remained associated with the membranes and seemed to be an intermediate in the hydrolysis of PBP 2 (0.85 to 1% trypsin, 15 to 30 min). The second tPBP 2, 54 kDa, was solubilized with 5% trypsin after 2 h of incubation (Fig. 3). Optimal conditions for release were not determined, nor was its binding capacity further examined.

PBP 1

No soluble PBP 1 derivatives have been identified yet, but three membrane tPBPs 1 were found. One, tPBP 1a (sometimes seen in untreated membranes; Fig. 3, C), appeared in the presence of low trypsin concentrations (0.1% for 5 to 10 min). It was very unstable and degraded into tPBPs 1b and 1c (92 and 86 kDa, respectively). These membrane tPBPs 1b and 1c were quite stable, as they were still present after 30 min of treatment with 10% trypsin (Fig. 3, PM, pellets).

Both PBPs 1b and 1c were easily identified in NT1/20 trypsin-treated membranes (Fig. 2, T and TC). Optimal conditions for degradation or solubilization, as well as binding capacity, were not further examined.

PBP 3R

Membranes of *E. hirae* S185, isolated from pig intestine and showing about the same β-lactam resistance as the R40 mutant, contained large amounts of a PBP with low β-lactam affinity which had the same apparent size as PBP 3.

Identification of PBP 3R trypsin cleavage products was done as described above for those isolated from PBP 5. Two main soluble polypeptides originated from PBP 3R. Both were different from PBP 3 and PBP 5 derivatives (Fig. 4). tPBPs 3Ra and 3Rb (66.5 and 42 kDa, respectively) were still able to bind benzylpenicillin.

Up to now, no conditions were found under which tPBP 3Ra was released alone. Under the best conditions, tPBPs 3Ra and 3Rb were found in a ratio of 1:1 after 95% degradation of PBP 3R. The membranes were treated with 4% trypsin for 30 min in 1 mM $MgCl_2$–5% glycerol–40 mM phosphate buffer (pH 7). A slight change in the buffer allowed the isolation of tPBP 3Rb alone. About 80% of PBP 3R was degraded when membranes suspended in 50 mM phosphate buffer, pH 7.8, were subjected to 4% trypsin treatment for 30 min. No tPBP 3Ra and almost no PBP 3R were observed. The influence of glycerol on PBP 3R cleavage is under current investigation.

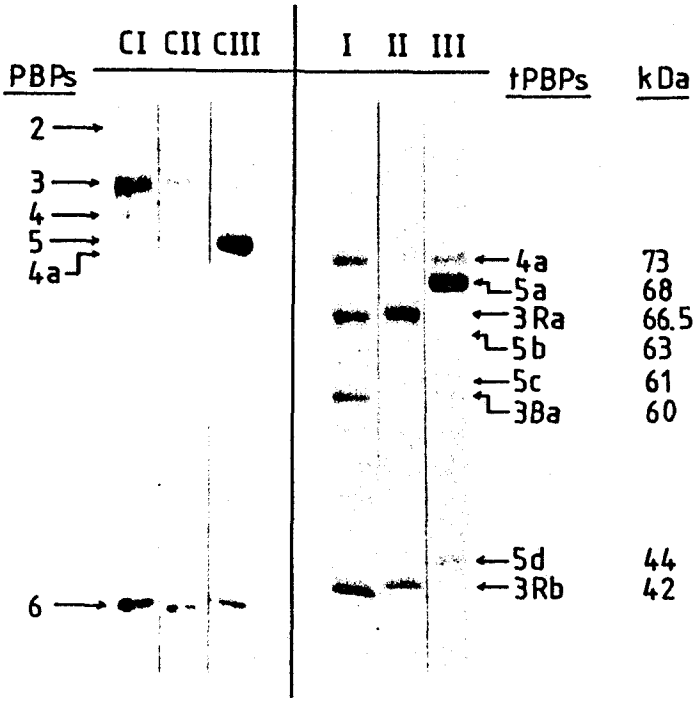

Figure 4. Identification of the trypsin degradation products of PBP 3R from the S185 β-lactam-resistant mutant. Membranes (1 mg/90 μl) were labeled with 5×10^{-4} M benzyl[^{14}C]penicillin for 1 h (sample I) or preincubated with 10^{-6} M benzylpenicillin for 30 min and then labeled with 3×10^{-4} M benzyl[^{14}C]penicillin for 1 h (sample II). Samples were collected after labeling as controls (CI and CII, respectively). The remaining membranes were treated with 4% trypsin for 30 min and then centrifuged (40,000 × *g*, 30 min). The supernatants were collected and submitted, with the control samples, to SDS-PAGE and fluorography. Membranes from the R40 mutant, fully labeled (CIII) as for sample I, were treated with 0.2% trypsin for 5 min. Soluble tPBPs collected after centrifugation (III) were treated like the other samples. The separating gel was 7.2% acrylamide.

Conclusions

To avoid the difficulties of purification of detergent-extracted PBPs from *E. hirae*, we decided to explore the potentialities of membrane proteolysis for the isolation of hydrosoluble, active PBP derivatives. The results presented here clearly show that trypsin proteolysis can be used to isolate such soluble PBP derivatives in good yields. This is certainly the case for PBPs 3B, 4, 5, and 6 from either strain ATCC 9790 or its resistant mutant R40, as well as for PBP 3R from the resistant strain S185. Under the conditions used, the majority of tPBPs were still able to bind β-lactams, some of them even after a 45 to

50% reduction of size. These results are in accordance with those published by others concerning PBPs from different species (6, 8, 9).

Our investigations show in addition that some tPBPs were obtained under such specific conditions that the trypsin lysates contained only the derivatives of one or two PBPs. These lysates are good candidates for a purification of PBP derivatives.

Finally, the PBPs present striking differences in their mode of association with the membranes of *E. hirae*. If PBPs 4 and 5 appear to be anchored via a small terminal peptide, PBPs 2, 3, and probably 6 are inserted into the membranes through a large hydrophobic domain. PBP 1 is the most hydrophobic of them. Indeed, tPBP 1c, the smallest PBP 1 derivative observed, was still associated with trypsin-treated membranes even though it had lost a 50-kDa terminal portion.

ACKNOWLEDGMENTS. This work was supported by the Fonds de Recherche de la Faculté de Médecine, the Fonds de la Recherche Scientifique Médicale, Brussels (contracts 3.4522.86 and 3.4507.83), the Gouvernement belge (action concertée 86/91-90), and the Région wallonne (C2/C16/conv. 246/20428).

LITERATURE CITED

1. **Canepari, P., M. Del Mar Lleo, G. Satta, R. Fontana, G. D. Shockman, and L. Daneo-Moore.** 1983. Division blocks in temperature-sensitive mutants of *Streptococcus faecium* (*S. faecalis* ATCC 9790). *J. Bacteriol.* **156**:1046–1051.
2. **Clément, J. M.** 1983. On the insertion of proteins into membranes. *Biochimie* **65**:325–338.
3. **Coyette, J., J. M. Ghuysen, and R. Fontana.** 1978. Solubilization and isolation of the membrane-bound DD-carboxypeptidase of *Streptococcus faecalis* ATCC9790. *Eur. J. Biochem.* **88**:297–305.
4. **Coyette, J., J. M. Ghuysen, and R. Fontana.** 1980. The penicillin-binding proteins in *Streptococcus faecalis* ATCC9790. *Eur. J. Biochem.* **110**:445–456.
5. **Coyette, J., A. Somzé, J. J. Briquet, and J. M. Ghuysen.** 1983. Function of the penicillin-binding protein 3 in *Streptococcus faecium*, p. 523–530. *In* R. Hakenbeck, J. V. Höltje, and H. Labischinski (ed.), *The Target of Penicillin*. Walter De Gruyter, Berlin.
6. **Ellerbrok, H., and R. Hakenbeck.** 1984. Penicillin-binding proteins of *Streptococcus pneumoniae*: characterization of tryptic-peptides containing the β-lactam-binding site. *Eur. J. Biochem.* **144**:637–641.
7. **Fontana, R., R. Cerini, P. Longoni, A. Grossato, and P. Canepari.** 1983. Identification of a streptococcal penicillin-binding protein that reacts very slowly with penicillin. *J. Bacteriol.* **155**:1343–1350.
8. **Waxman, D. J., and J. L. Strominger.** 1979. Cleavage of a COOH-terminal hydrophobic region from D-alanine carboxypeptidase, a penicillin-sensitive bacterial membrane enzyme. *J. Biol. Chem.* **254**:4863–4875.
9. **Waxman, D. J., and J. L. Strominger.** 1981. Limited proteolysis of the penicillin-sensitive

D-alanine carboxypeptidase purified from *Bacillus subtilis* membranes. Active, water-soluble fragments generated by cleavage of a COOH-terminal membrane anchor. *J. Biol. Chem.* **256:**2059–2066.

10. **Waxman, D. J., and J. L. Strominger.** 1983. Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. *Annu. Rev. Biochem.* **52:**825–869.