[19] Serine-Type D-Ala-D-Ala Peptidases and Penicillin-Binding Proteins

By BENOÎT GRANIER, MARC JAMIN, MAGGY ADAM, MORENO GALLENI, BERNARD LAKAYE, WILLY ZORZI, JACQUELINE GRANDCHAMPS, JEAN-MARC WILKIN, CLAUDINE FRAIPONT, BERNARD JORIS, COLETTE DUEZ, MARTINE NGUYEN-DISTÈCHE, JACQUES COYETTE, MÉLINA LEYH-BOUILLE, JEAN DUSART, LÉON CHRISTIAENS, JEAN-MARIE FRÈRE, and JEAN-MARIE GHUYSEN

Substrates and Catalyzed Reactions

DD-Peptidase Activity

The catalyzed reaction is a double transfer of the *R*-L-aminoacyl(aa)-D-alanyl moiety of *R*-L-aa-D-alanyl-D-alanine carbonyl donors to the active site serine, with formation of a serine ester-linked acyl (*R*-L-aa-D-alanyl) enzyme and, from this, to an exogenous acceptor. Here E is the enzyme; D, the carbonyl donor; E · D, the Michaelis complex; E–D*, the acyl-enzyme; HY, the exogenous acceptor; P1, the leaving group of the enzyme acylation step; K, the dissociation constant, and k_{+2} and k_{+3} are the first-order rate constants. Reaction (1) occurs:

$$E + D \overset{K}{\rightleftharpoons} E \cdot D \xrightarrow[P1]{k_{+2}} E\text{–}D^* \xrightarrow[HY]{k_{+3}} E + P2 \qquad (1)$$

Here P1 is D-alanine and P2 depends on the final acceptor. When HY is D-alanine, H_2O, or a suitably structured amino compound, the carbonyl donor is regenerated, hydrolyzed (carboxypeptidase activity), or transpeptidated (transpeptidase activity), respectively; $k_{cat} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$; $K_m = Kk_{+3}/(k_{+2} + k_{+3})$; and $k_{cat}/K_m = k_{+2}/K$, i.e., the second-order rate constant for enzyme acylation.

Prototypic carbonyl donors are diacetyl (Ac_2)-L-Lys-D-Ala-D-Ala and N^{α}-acetyl-L-Lys-D-Ala-D-Ala.

Penicillin-Binding Activity

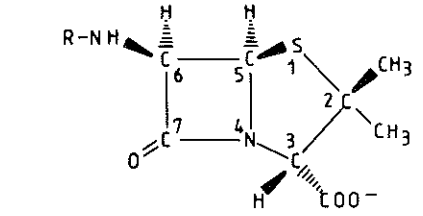
Penicillin is a suicide substrate. Because of the endocyclic nature of the scissile β-lactam amide bond, the leaving group of the enzyme acylation step remains part of the acyl enzyme. The first part only of the transfer cycle is achieved, leading to a long-lived, serine ester-linked acyl(penicilloyl)-enzyme, and the enzyme behaves as a penicillin-binding

protein (PBP). Reaction (1) becomes

$$E + D \overset{K}{\rightleftharpoons} E \cdot D \overset{k_{+2}}{\rightarrow} E\text{–}D^* \overset{k_{+3}}{\rightarrow} E + P \qquad \text{(with low } k{+}3 \text{ value)} \qquad (2)$$

The inertness of the penicilloyl enzyme is not absolute. Hydrolytic breakdown causes release of penicilloate. Rupture of the C-5–C-6 bond causes release of the thiazolidine moiety and formation of an acylic acyl-enzyme that is hydrolytically and aminolytically labile. Enzyme recovery is always a very slow process. Under conditions where acyl-enzyme breakdown is negligible (half-life = $0.69/k_{+3}$), the β-lactam concentration and the time of incubation required to immobilize 99% of the enzyme as acyl-enzyme at the steady state of the reaction are related to k_{+2}/K by $([D]\ t)_{0.99} = 4.6K/k_{+2}$.

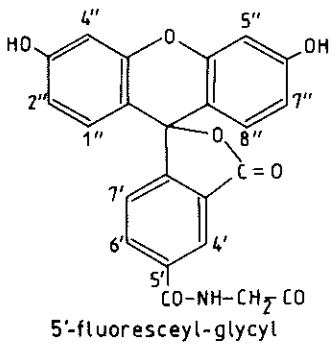
Prototypic β-lactam carbonyl donors are benzylpenicillin, [^{14}C]- and [^{3}H]benzylpenicillin, ^{125}I-labeled hydroxybenzylpenicillin, and 5′-fluoresceylglycyl-6-aminopenicillanate (5′-Flu-Gly-6APA) (Fig. 1).



6-aminopenicillanate (6 APA)

R = C_6H_5–CH_2–CO : benzylpenicillin

R = 5′-fluoresceyl-glycyl: 5′ Flu-Gly-6 APA



5′-fluoresceyl-glycyl

FIG. 1. Structure of benzylpenicillin and 5′-fluoresceyl-glycyl-6-amino-penicillanate (5′-Flu-Gly-6APA).

Esterase–Thiolesterase Activity

Reaction (1) applies. Prototypic carbonyl donors are ester S1e, C_6H_5-CONH-CH_2-COO-$CH(CH_2$-$C_6H_5)$-COO^- (racemic mixture); thiolester S2a, C_6H_5-CONH-CH_2-COS-CH_2-COO^-; and thiolester S2d, C_6H_5-CONH-$CH(CH_3)$-COS-CH_2-COO^- (D-isomer).

Low and High Molecular Weight Penicillin-Binding Proteins

All bacteria possess an assortment of low and high molecular weight membrane-bound PBPs [reaction (2)]. The PBPs belong to the penicilloyl serine transferase superfamily. Invariably, the active site serine residue belongs to the conserved tetrad SXXK.[1–3]

The low molecular weight PBPs are single catalytic entities. The bulk of the protein is on the outer face of the plasma membrane and bears a carboxy-terminal extension, the end of which serves as membrane anchor. The low molecular weight PBPs probably help control the extent of wall peptidoglycan cross-linking throughout the life cycle of the cells. The low molecular weight PBPs so far identified perform DD-peptidase, esterase, and thiolesterase activities.

The high molecular weight PBPs involved in wall peptidoglycan assembly and cell morphogenesis are multimodule proteins. They are membrane anchored at the amino end of the polypeptide chain whereas the bulk of the protein is on the outer face of the membrane and consists of an N-terminal module, fused to a C-terminal, penicillin-binding module. Those so far identified catalyze acyl transfer reactions on esters and thiolesters, but they lack activity on D-alanyl-D-alanine-terminated peptides.

High molecular weight PBPs are also involved in the β-lactam-induced derepression of β-lactamase synthesis in *Bacillus licheniformis* and *Staphylococcus aureus* (BlaR) and in derepression of low-affinity PBP2′ synthesis in *S. aureus* (MecR). The penicillin-binding sensor is exposed on the outer face of the membrane and is fused to the carboxy end of a multitransmembrane segment signal transducer. The BlaR sensor of *B. licheniformis* lacks DD-peptidase, esterase, and thiolesterase activity and is not discussed further in this article.

[1] J. M. Ghuysen, *Annu. Rev. Microbiol.* **45,** 37 (1991).
[2] J. M. Frère, M. Nguyen-Distèche, J. Coyette, and B. Joris, *in* "The Chemistry of β-Lactams" (M. I. Page, ed.), p. 148. Chapman & Hall, Glasgow, 1992.
[3] J. M. Ghuysen and R. Hakenbeck (eds.), *New Compr. Biochem.* **27,** (1994).

Bacterial Strains, Growth Conditions, and Enzyme Production

The low molecular weight PBPs/DD-peptidases described below are peculiar in that they are obtained as soluble proteins directly from the producing strains. The high molecular weight PBPs described below are truncated, water-soluble derivatives of the corresponding, membrane-bound proteins. The encoding genes have been cloned and sequenced except for that of the membrane-bound PBP3 of *Enterococcus hirae* [American Type Culture Collection (ATCC) 9790; Rockville, MD].

DD-Peptidase/Low Molecular Weight PBP of Streptomyces K15

This enzyme (the mature protein is 262 amino acid residues)[4] is a strict transpeptidase. Hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala is negligible because the released D-alanine competes successfully with 55.5 *M* H_2O, performs aminolysis of the acyl-enzyme, and regenerates the original tripeptide. At millimolar concentrations, glycylglycine (which is structurally related to the peptidoglycan interpeptide bridge) overcomes the acceptor activities of D-alanine and water, leading to the quantitative conversion of the tripeptide into Ac_2-L-Lys-D-Ala-Gly-Gly. The *Streptomyces* K15 enzyme is membrane bound, but it lacks membrane anchors. Amplified expression in *Streptomyces lividans* TK24 harboring pDML225 results in the export of an appreciable proportion of the synthesized enzyme in the culture medium.[5]

Streptomyces lividans TK24/pDML225 is grown at 28° for 48 hr in a 20-liter Biolafitte fermentor containing 10 liters of TSB2-thiostrepton medium [trypsin soy broth (Gibco-BRL, Gaithersburg, MD), 30 g; $(NH_4)_2SO_4$, 2 g; thiostrepton, 50 mg; water, 1 liter] with an agitation speed of 250 rev/min and an airflow of 5 liters/min. The culture fluid contains the soluble DD-peptidase. Yield is 2 mg of enzyme per liter of culture.

DD-Peptidase/Low Molecular Weight PBP of Streptomyces R61

This enzyme (the mature protein is 349 amino acid residues)[6] is mainly a carboxypeptidase, but it also catalyzes transpeptidation reactions on specific donor–acceptor substrates and under proper experimental condi-

[4] P. Palomeque-Messia, S. Englebert, M. Leyh-Bouille, M. Nguyen-Distèche, C. Duez, S. Houba, O. Dideberg, J. Van Beeumen, and J. M. Ghuysen, *Biochem. J.* **279,** 223 (1991).

[5] P. Palomeque-Messia, V. Quittre, M. Leyh-Bouille, M. Nguyen-Distèche, C. J. L. Gershater, I. K. Dacey, J. Dusart, J. Van Beeumen, and J. M. Ghuysen, *Biochem. J.* **288,** 87 (1992).

[6] C. Duez, C. Piron-Fraipont, B. Joris, J. Dusart, M. S. Urdea, J. A. Martial, J. M. Frère, and J. M. Ghuysen, *Eur. J. Biochem.* **162,** 509 (1987).

tions. The wild-type strain produces the enzyme as a secretory protein. Overexpression is achieved in *S. lividans* TK24 harboring pDML115.[7]

Streptomyces lividans TK24/pDML115 is grown at 28° for 72 hr in a 1-liter Erlenmeyer flask containing 500 ml of TSB1-thiostrepton medium [trypsin soy broth (Gibco-BRL), 30 g; thiostrepton, 25 mg; water, 1 liter] with orbital agitation (250 rev/min). The culture fluid contains the soluble DD-peptidase. Yield is 30 to 40 mg of enzyme per liter of culture.

DD-Peptidase/Low Molecular Weight PBP of Actinomadura R39

This enzyme (489 amino acid residues)[8] is also a secreted carboxypeptidase/transpeptidase. Its specificity profile as a DD-peptidase differs from that of the *Streptomyces* R61 enzyme and it has a much higher penicillin affinity.

Actinomadura R39 is grown at 28° for 48 hr in a 20-liter Biolafitte fermentor containing 14 liters of TAU medium [starch (Merck), 10 g; urea (Merck), 2 g; universal peptone (Merck), 5 g; yeast extract (Difco), 10 g; K_2HPO_4, 2 g; $MgSO_4 \cdot 6H_2O$, 0.6 g; trace element solution, 1 ml; 100 m*M* Tris-HCl (pH 8.0), 1 liter] with an agitation speed of 250 rev/min and an airflow of 28 liters/min. The trace element solution contains 1 g each of $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, and $CaCl_2$ for 1 liter of water. The culture fluid contains the soluble DD-peptidase. Yield is 10 mg of enzyme per liter.

Escherichia coli PBP3p

The high molecular weight PBP3 (588 amino acid residues)[9] is involved in cell septation and is a lethal target of penicillin in *E. coli*. About 20 copies of PBP3 occur per bacterial cell. A truncated form (G57-V577) of the PBP3, i.e., PBP3p, is overexpressed in the periplasm *E. coli* HB101 or RR1 harboring pDML232 (carrying the modified *ftsI* gene) and pDML230 (carrying the chaperone SecB-encoding gene).[10] Regulation of the synthesis is at the transcriptional level and is isopropyl-β-D-thiogalactopyranoside (IPTG) dependent. Overexpression above a certain level causes conversion of a large proportion of the enzyme into inactive aggregates during the purification steps.

[7] A. M. Hadonou, M. Jamin, M. Adam, B. Joris, J. Dusart, J. M. Ghuysen, and J. M. Frère, *Biochem. J.* **282,** 495 (1992).

[8] B. Granier, C. Duez, S. Lepage, S. Englebert, J. Dusart, O. Dideberg, J. Van Beeumen, J. M. Frère, and J. M. Ghuysen, *Biochem. J.* **282,** 781 (1992).

[9] M. Nakamura, I. N. Maruyama, M. Soma, J. I. Kato, H. Suzuki, and Y. Hirota, *Mol. Gen. Genet.* **191,** 1 (1983).

[10] C. Fraipont, M. Adam, M. Nguyen-Distèche, W. Keck, J. Van Beeumen, J. A. Ayala, B. Granier, H. Hara, and J. M. Ghuysen, *Biochem. J.* **298,** 189 (1994).

Escherichia coli HB101 (or RR1)/pDML232/pDML230 is grown at 37° in a 20-liter Biolafitte fermentor containing 15 liters of LB/kanamycin/tetracycline medium [Bacto-tryptone (Difco), 12 g; Bacto-yeast extract, 5 g; NaCl, 10 g; kanamycin, 25 mg; tetracycline, 12.5 mg; water, 1 liter] with an agitation speed of 250 rev/min and an airflow of 5 liters/min. When the culture reaches an absorbance of 0.8 at 550 nm, 2 m*M* IPTG is added to induce PBP3p synthesis and the culture continues for 3 hr. The cells are collected by centrifugation and spheroplasted at 20° in 1150 ml of 30 m*M* Tris-HCl (pH 8.0)/27% sucrose/5 m*M* EDTA/600 mg of lysozyme. The spheroplast suspension is supplemented with 15 m*M* $CaCl_2$ and 0.5 *M* NaCl and centrifuged at 30,000 *g* for 15 min. The supernatant, i.e., the periplasmic fraction, contains the truncated PBP3p. Yield is about 2 mg of enzyme per liter of culture.

*Streptococcus pneumoniae PBP2x**

The high molecular weight PBP2x (750 amino acid residues)[11] is a major target of the β-lactam antibiotics in *Streptococcus pneumoniae*. PBP2x is the first PBP to be altered in its amino acid sequence in cefotaxime-resistant laboratory mutants and transformation with DNA segments encoding low-affinity variants of PBP2x from clinical isolates gives rise to transformants with increased penicillin resistance. A truncated form of PBP2x lacking the 30 hydrophobic amino acid residues at positions 19–48, i.e., PBP2x*, is overexpressed in *E. coli* DH5α harboring pCG31.[12]

Escherichia coli DH5α/pCG31 is grown at 37° in a 20-liter Biolafitte fermentor containing 15 liters of LB-chloramphenicol medium [Bacto-tryptone (Difco), 10 g; Bacto-yeast extract, 5 g; NaCl, 10 g; chloramphenicol, 0.5 g; water, 1 liter] with an agitation speed of 200 rev/min and an airflow of 15 liters/min. When the culture reaches an absorbance of 1.2 at 550 nm, the cells are collected by centrifugation, suspended in 25 ml of 10 m*M* Tris-HCl (pH 8.0), and disrupted with a French press (1000 psi). After centrifugation at 20,000 *g* for 60 min, the supernatant, which contains PBP2x*, is dialyzed at 4° twice for 2 hr against 10 m*M* Tris-HCl (pH 8.0). Yield is 6–7 mg of enzyme per liter of culture.

Enterococcus hirae ATCC 9790 t-PBP3

The 78-kDa PBP3 of *E. hirae* ATCC 9790 is involved in cell septation and is the specific target of cefotaxime in this organism.[13] It can be con-

[11] G. Laible, R. Hakenbeck, M. A. Sicard, B. Joris, and J. M. Ghuysen, *Mol. Microbiol.* **3,** 1337 (1989).
[12] G. Laible, W. Keck, R. Luiz, H. Mottl, J. M. Frère, M. Jamin, and R. Hakenbeck, *Eur. J. Biochem.* **207,** 943 (1992).
[13] G. Piras, A. El Kharroubi, J. Van Beeumen, E. Coeme, J. Coyette, and J. M. Ghuysen, *J. Bacteriol.* **172,** 6856 (1990).

verted into a water-soluble 58-kDa t-PBP3 by trypsin digestion of the isolated membranes.

Enterococcus hirae ATCC 9790 is grown at 37° in a 100-liter fermentor containing 95 liters of SB medium [yeast extract, 10 g; peptone, 10 g; $NaH_2PO_4 \cdot H_2O$, 16.45 g; $Na_2HPO_4 \cdot 2H_2O$, 31.85 g; KH_2PO_4, 0.42 g; K_2HPO_4, 0.305 g; glucose, 20 g (sterilized separately as a 20% aqueous solution); H_2O, 1 liter, pH 6.5 at 20°] with an agitation speed of 150 rev/min and an airflow of 100 liters/min. When the culture reaches an absorbance of 5.0 at 550 nm (late exponential phase), the cells are collected and suspended in 2800 ml of 5 m*M* sodium phosphate (pH 7.0)/1 m*M* $MgCl_2$. The suspension is divided into seven samples and each sample is supplemented with 40 mg of lysozyme, 800 μg of DNase, 400 μg of RNase, and 4 mg of *Streptomyces globisporus* muramidase 1 (Sigma, St. Louis, MO), resulting in cell lysis. The membranes are collected by centrifugation at 40,000 *g* for 30 min and washed several times in 5 m*M* sodium phosphate (pH 7.0). Yield is 0.16 mg of membrane-bound PBP3 per liter of culture.

Enzyme Purification

Unless otherwise specified, the purification steps are routinely carried out at 4°.

Streptomyces K15 Enzyme

Step 1. The culture supernatant (10 liters, 11.7 g of total proteins, 20 mg of enzyme) is clarified by filtration through a 0.2-μm-pore size poly(vinylidene difluoride) membrane (Millipore, Bedford, MA), concentrated to 1 liter with a Flowgen Ultrasette Tangential Flow ultrafiltration cell and supplemented with an equal volume of cold (−20°) acetone. The mixture is maintained at −20° for 30 min, resulting in the precipitation of the enzyme. The pellet, collected by centrifugation at 7500 *g* for 30 min, is dissovled in 100 ml of 25 m*M* Tris-HCl (pH 8.0)/0.2 m*M* dithiothreitol/0.5 *M* NaCl.

Step 2. The solution is dialyzed against the Tris–dithiothreitol buffer without salt, resulting in the precipitation of the enzyme. The pellet collected by centrifugation is dissolved in 10 ml of 25 m*M* Tris-HCl (pH 7.2)/0.2 m*M* dithiothreitol/0.4 *M* NaCl. The solution is divided into five aliquots, each of which is applied to a 35-ml Q-Sepharose column equilibrated against the same buffer. The enzyme is eluted in the void volume while the contaminating nucleic acids are absorbed onto the ion exchanger.

Final yield is 75% and purity is noted below. The preparation is stored at −20° in the presence of a 0.4 *M* NaCl and at a protein concentration of 1 mg per ml to avoid precipitation.

Filtration of the enzyme preparation on Sephadex G-75 in 25 m*M* Tris-HCl (pH 8.2)/0.2 m*M* dithiothreitol/0.5 *M* NaCl yields a single protein fraction, the elution volume of which is compatible with a M_r value of 27,000 for the protein. SDS–PAGE, however, reveals the presence of a 52,000 M_r PBP in addition to the expected 27,000 M_r PBP. The two proteins each bind penicillin in a 1 : 1 molar ratio, react with the anti-*Streptomyces* K15 enzyme antiserum, and exhibit the same amino-terminal sequence. The 52-kDa PBP completely disappears when, prior to the SDS–PAGE, the preparation is treated with 7 *M* guanidinium chloride in 0.1 *M* Tris-HCl, pH 8.5, at 100° for 3 min, and is dialyzed successively against 8 *M* urea and 0.1% SDS, suggesting that the 52-kDa PBP might be due to incomplete denaturation of the protein.

Streptomyces R61 Enzyme

Step 1. The culture supernatant (1 liter; 300 mg of total proteins; ≈30–40 mg of enzyme) is stirred at 20° with 100 g of Amberlite CG-50 (Serva, Heidelberg, Germany) and the suspension is adjusted to pH 4.0 with glacial acetic acid. The exchanger is collected by filtration, suspended in 50 m*M* Tris-HCl (pH 8.0; 2 ml of buffer per gram of wet exchanger), and the pH is adjusted to 8.0 with concentrated NH_3. After filtration, the exchanger is treated as above with a second batch of buffer. The combined eluates are dialyzed against 10 m*M* Tris-HCl (pH 8.0)/50 μM EDTA and concentrated fivefold with an Amicon (Danvers, MA) ultrafiltration unit.

Step 2. The solution is loaded on a 2.5 × 20-cm Q-Sepharose fast flow column equilibrated against 10 m*M* Tris-HCl (pH 8.0)/50 μM EDTA. After washing, the enzyme is eluted with a linear NaCl gradient from 0 to 0.3 *M* over 800 ml at a flow rate of 8 ml/min. The active fractions (about 0.12 *M* NaCl) are concentrated to 20 ml.

Step 3. The solution is filtered through a 4 × 85-cm Sephadex G-75 column equilibrated against 10 m*M* Tris-HCl (pH 8.0)/50 μM EDTA. The active fractions are pooled, dialyzed against 10 m*M* Tris-HCl (pH 7.0)/50 μM EDTA, and concentrated to 30 ml.

Step 4. The solution is injected onto a prepacked Hiload 26/10 Q-Sepharose Fast Flow column (Pharmacia, Piscataway, NJ) conditioned with 10 m*M* Tris-HCl (pH 7.0)/50 μM EDTA. Treatment with a linear NaCl gradient from 0 to 0.2 *M* over 1100 ml at a flow rate of 5 ml/min yields two active fractions. One of them is eluted at about 73 m*M* NaCl and the other at about 85 m*M* NaCl. Each fraction is dialyzed against 10 m*M* Tris-HCl (pH 8.0)/50 μM EDTA and concentrated to 5 mg of enzyme per milliliter.

Final yield is ≈50% (taking the two fractions into account) and purity is noted below. The preparation is stored at −20°.

The two enzyme isoforms each behave as homogeneous proteins by nondenaturing PAGE, SDS–PAGE, and gel electrofocusing. They each bind penicillin in a 1 : 1 molar ratio with comparable constant values. They might result from different processings at the N or C terminus of the protein. Such a microheterogeneity is not observed when the enzyme is produced by the wild-type *Streptomyces* R61.

Actinomadura R39 Enzyme

Step 1. The culture supernatant (12 liters, 25 g of total proteins, 120 mg of enzyme) is stirred for 15 min at 20° with 240 g of DEAE-cellulose previously equilibrated against 50 m*M* Tris-HCl (pH 8.0). The ion exchanger is collected by filtration, packed in a 4 × 10-cm column, and washed with 2.5 liters of 100 m*M* Tris-HCl (pH 8.0)/0.15 *M* NaCl. The adsorbed enzyme is eluted with a curvilinear NaCl gradient in 100 m*M* Tris-HCl (pH 8.0; mixing chamber at constant volume, 250 ml of 0.15 *M* NaCl; added solution, 0.5 *M* NaCl; flow rate, 2 ml/min). The active fractions (0.35 *M* NaCl) are concentrated to 25 ml with an Amicon ultrafiltration unit.

Step 2. The solution is filtered through a 4 × 85-cm Sephadex G-100 column in 100 m*M* Tris-HCl (pH 8.0). The active fractions are pooled (140 ml) and loaded on a 2.6 × 10-cm Q-Sepharose Fast Flow column in the Tris buffer. After washing with 1 liter of 0.35 *M* NaCl in buffer, the enzyme is eluted with a linear NaCl gradient from 0.35 to 0.45 *M* over 700 ml at a flow rate of 5 ml/min. The active fractions are pooled, dialyzed against the Tris buffer, and concentrated to 2 mg of enzyme per milliliter.

Final yield is 74% and purity is higher than 95%. The preparation is stored at −20°.

Step 1. The periplasmic fraction (1.15 liter; 1.5 g of total proteins, 30 mg of enzyme) is dialyzed against 10 m*M* Tris-HCl (pH 8.0)/10% (v/v) glycerol, resulting in the precipitation of the enzyme. The pellet is collected by centrifugation and dissolved in 30 ml of Tris-glycerol/0.5 *M* NaCl. The solution is dialyzed against Tris–glycerol/0.2 *M* NaCl, under which conditions 70–80% of PBP3p remains in solution.

Step 2. The solution is applied to a 100-ml column of Procion blue MX4GD (mix 1591)-Fractogel (Merck) equilibrated against Tris–glycerol/0.2 *M* NaCl. The adsorbed PBP3 is eluted by a linear NaCl gradient from 0.15 to 1 *M* over 375 ml at a flow rate of 4 ml/min. The active fractions (0.5 *M* NaCl) are pooled and concentrated.

Final yield is 30% and purity is 80%. The preparation is stored at 20° in the presence of 0.5 *M* NaCl and at a protein concentration of 0.5 mg per ml to avoid precipitation.

*Streptococcus pneumoniae PBP2x**

Step 1. The dialyzed cell extract (3.9 g of total proteins; 98 mg of PBP2x*; in 25 ml 10 m*M* Tris-HCl, pH 8.0) is loaded on a 2.6 × 14-cm Q-Sepharose Fast Flow column equilibrated against the Tris buffer. The adsorbed PBP2x* is eluted with a linear NaCl gradient from 0 to 1 *M* over 320 ml at a flow rate of 4 ml/min. The active fractions (0.5 *M* NaCl) are pooled and dialyzed for 2 hr against 10 m*M* sodium acetate adjusted to pH 5.0 with 12 *N* HCl.

Step 2. The solution is divided into five 20-ml samples, each of which is adsorbed on a Mono S HR5/5 Pharmacia column equilibrated against the sodium acetate buffer. The adsorbed PBP2x* is eluted by a linear NaCl gradient from 0 to 1 *M* over 20 ml at a flow rate of 1 ml/min. Three active fractions are collected at 0.25, 0.32, and 0.61 *M* NaCl, respectively, and are dialyzed against 10 m*M* sodium phosphate (pH 7.0). The three enzyme isoforms each are homogeneous by SDS–PAGE and bind penicillin in a 1 : 1 molar ratio with similar constant values.

Final yield is 68% (taking the three fractions into account) and purity is at least 95%. The preparations (2.5 mg of enzyme per ml) are stored at −20° in the presence of 10% glycerol.

Enterococcus hirae t-PBP3

Step 1. A membrane suspension [1 g of total proteins; 2.5 mg of membrane-bound PBP3 in 65 ml of 50 m*M* Tris-HCl (pH 7.5)/0.5% Triton X-100] is incubated in the presence of 10 mg of trypsin type XI for 30 min at 37°. After centrifugation at 40,000 *g* for 60 min, the supernatant (containing the 58-kDa t-PBP3) is loaded on a 2.6 × 40-cm Q-Sepharose Fast Flow column equilibrated against 20 m*M* Bis–Tris (pH 6.3)/0.015% (v/v) Triton X-100. The adsorbed t-PBP3 is eluted with a linear NaCl gradient from 0 to 1 *M* over 1500 ml, at a flow rate of 5 ml/min. The active fractions (0.2 *M* NaCl) are pooled and concentrated to 10 ml with an Amicon ultrafiltration unit, and the solution is dialyzed against 10 m*M* sodium phosphate (pH 7.0).

Step 2. Samples (5 ml each) are filtered through a 1-ml Mono Q HR5/5 column equilibrated against the phosphate buffer. The adsorbed t-PBP3 is eluted with a linear NaCl gradient from 0 to 1 *M* over 31 ml at a flow rate of 0.6 ml/min. The active fractions (0.25 *M* NaCl) are dialyzed against the phosphate buffer.

Yield is 35% and purity is 7.5%. The preparation contains no PBP other than the 58-kDa t-PBP3. It is stored at −20° at a final concentration of 1.9 mg total proteins per milliliter.

Enzyme Assays and Kinetic Parameters

Assays are performed in 10 m*M* sodium phosphate (pH 7.0) for the *Streptomyces* R61 enzyme, *S. pneumoniae* PBP2x*, and *E. hirae* t-PBP3, in 10 m*M* phosphate (pH 7.0) or 50 m*M* Tris-HCl (pH 7.5)/4 m*M* $MgCl_2$ for the *Actinomadura* R39, in 10 m*M* phosphate (pH 7.0)/0.25 *M* NaCl for the *E. coli* PBP3p, and in 25 m*M* Tris-HCl (pH 7.2)/0.2 m*M* dithiothreitol/0.4 *M* NaCl for the *Streptomyces* K15 enzyme.

Table I gives the K_m, k_{cat}, and k_{cat}/K_m (i.e., k_{+2}/K) values for the peptide, ester, and thiolester carbonyl donors [reaction (1)]. Table II gives the k_{+2}/K and k_{+3} values for benzylpenicillin and 5′-Flu-Gly-6APA [reaction (2)].

Peptides

The enzymatic estimation of D-alanine has been modified, resulting in a twofold increased sensitivity.[14]

i. *o*-Dianisidine is replaced by *o*-dianisidine dihydrochloride (10 mg per ml of water); the pyrophosphate buffer is replaced by 100 m*M* Tris-HCl, pH 8.0; and the methanol–water mixture (which is added just before the spectrophotometric measurements) is replaced by a mixture of sulfuric acid/methanol/water (60 : 50 : 40; v/v/v). The readings are recorded at 535 nm.

ii. Alternatively, *o*-dianisidine is replaced by 2,2′-azinodi(3-ethylbenzthiazoline sulfonate) (ABTS) and the experimental conditions are modified as follows.

Reagents

Citrate–phosphate, pH 5.3: citric acid, 8.5 g; Na_2HPO_4, 20.5 g; water, 1 liter.

FAD (monosodium; Boehringer Mannheim, Germany): 0.1 mg per ml of 100 m*M* Tris-HCl, pH 8.0.

D-Amino-acid oxidase (Boehringer): a suspension of 5 mg per ml of ammonium sulfate solution, as supplied.

ABTS (Boehringer): 5 mg per ml of water.

[14] J.-M. Frère, M. Leyh-Bouille, J. M. Ghuysen, M. Nieto, and H. R. Perkins, this series, Vol. 45, p. 610.

TABLE I
KINETIC PROPERTIES OF ACYCLIC CARBONYL DONORS

Enzyme	Ac_2-L-Lys-D-Ala-D-Ala			α-Ac-L-Lys-D-Ala-D-Ala			Ester S1e			Thiol ester S2a			Thiol ester S2d		
	k_{cat} (sec^{-1})	K_m (mM)	k_{+2}/K (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	k_{+2}/K (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	k_{+2}/K (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	k_{+2}/K (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	k_{+2}/K (M^{-1} sec^{-1})
Streptomyces K15	0.31	6.2	50 (a)	0.45	9	48 (b)	ND	ND	<0.2	ND	ND	8	0.11	1.5	75
													0.34	4.5	75 (c)
Streptomyces R61	55	14	4000	0.25	15	17	4.6	0.9	5200	5	0.05	100,000	70	0.1	700,000
Actinomadura R39	18	0.8	22,500	32	0.2	160,000	0.33	0.05	6600	0.9	0.08	11,500	6	0.015	400,000
E. coli PBP3p		No activity			No activity			No activity			No activity		0.25	3	80
S. pneumoniae PBP2x*		No activity			No activity		0.33	2.8	100	0.47	0.8	600	30	5.6	5000
E. hirae t-PBP3		No activity			No activity			No activity		—	—	<20	6	1.8	3200

[a] From reaction (1). The reactions are carried out at 37° in buffer or in buffer containing 2 mM Gly-Gly (a), 10 mM Gly-L-Ala (b), and 1.5 mM Gly-Gly (c). ND, Not determined.

TABLE II
KINETIC PROPERTIES OF β-LACTAM CARBONYL DONORS[a]

Enzyme	Benzylpenicillin		5′-Flu-Gly-6APA	
	k_{+2}/K (M^{-1} sec^{-1})	k_{+3} (sec^{-1})	k_{+2}/K (M^{-1} sec^{-1})	k_{+3} (sec^{-1})
Streptomyces K15	135	1.1×10^{-4}	25	ND
Streptomyces R61	18,000	1.1×10^{-4}	6300	5×10^{-5}
Actinomadura R39	300,000	3×10^{-6}	70,000	8×10^{-6}
E. coli PBP3p	4000	4×10^{-5}	25	ND
S. pneumoniae PBP2x*	58,000 (±5000)	$5.5\ (\pm 0.5) \times 10^{-5}$	1600	4×10^{-5}
E. hirae t-PBP3	>100,000	ND	600	ND

[a] From reaction (2). The reactions are carried out in buffer at 37°. ND, Not determined.

Peroxidase (Boehringer): 50 μg per ml of water.
Enzyme–coenzyme mixture (freshly prepared). FAD: D-amino-acid oxidase, 30 : 1 (v/v).
H_2O_2 assay mixture (freshly prepared). Citrate–phosphate, pH 5.3 : ABTS : peroxidase, 100 : 1 : 1 (v/v/v).

Assay. Samples containing D-alanine (30 μl) and the enzyme–coenzyme mixture (60 μl) are incubated together for 5 min at 37°. The H_2O_2 assay mixture (400 μl) is added and the absorbance is read at 415 nm. Note that the D-amino-acid oxidase must be completely catalase-free.

Sensitivity. D-Alanine (10 nmol in a final mixture of 490 μl) gives an absorbance of 0.6–0.7.

Esters

The values of the kinetic parameters derive from complete time courses obtained by monitoring the absorbance increase at 254 nm ($\Delta\varepsilon = 500\ M^{-1}\ cm^{-1}$).[15,16]

Thiol esters

The time-dependent decrease of absorbance is monitored at 250 nm ($\Delta\varepsilon = -2200\ M^{-1}\ cm^{-1}$).[15,16] Low K_m values (<500 μ*M*) are derived from complete time courses. In the other cases, the initial rates are determined

[15] M. Adam, C. Damblon, B. Plaitin, L. Christiaens, and J. M. Frère, *Biochem. J.* **270,** 525 (1990).
[16] M. Adam, C. Damblon, M. Jamin, W. Zorzi, V. Dusart, M. Galleni, A. El Kharroubi, G. Piras, B. G. Spratt, W. Keck, J. Coyette, J. M. Ghuysen, M. Nguyen-Distèche, and J. M. Frère, *Biochem. J.* **271,** 601 (1991).

and the k_{cat} and K_m values are obtained by fitting the results on a hyperbola with a nonlinear regression (Enzfitter, Elsevier Biosoft, Cambridge, UK) program or with the help of the Hanes linearization of the Henri–Michaelis equation.

Radioactive and Nonradioactive β-Lactams

Determination of the k_{+3} value of acyl-enzyme breakdown rests on measurements of the recovery of the enzyme activity as a function of time and/or the release of the acyl-enzyme from preformed radioactively labeled acyl-enzyme.[17,18] Knowing the k_{+3} value, determination of the k_{+2}/K value rests on measurements of the pseudo-first-order rate constants of acyl-enzyme formation at varying concentrations of the β-lactam. Direct measurement of acyl-enzyme formation requires the use of a radioactive β-lactam. If the β-lactam is not available in a radioactively labeled form, an indirect procedure can be used in which the enzyme left free after a first incubation with varying concentrations of the nonradioactive β-lactam is fully acylated in a second step, by a radioactive β-lactam. It is important that precise experimental conditions are observed to avoid misleading results.[17,18]

The procedure described below[2] allows the k_{+2}/K value for a nonradioactive β-lactam 1 to be determined in a one-step competition with a radioactive β-lactam 2 for which the k_{+2}/K value is known. With $[D_1]$ and $[D_2] \gg E_0$, the nonradioactive β-lactam 1 decreases the maximal extent of acylation of the protein by the radioactive β-lactam 2, and the k_{+2}/K value for the β-lactam 1 can be computed by measuring the amounts of radioactive acyl enzyme formed with the β-lactam 2 in the absence ($[E - D_2^*]_0$) and in the presence ($[E - D_2^*]$) of the β-lactam 1. With $[E - D_1^*] = [E - D_2^*]_0 - [E - D_2^*]$, the use of the following equation is justified if the acylation reactions are completed within a time much shorter than the half-lives of the acyl-enzymes, so that the k_{+3} steps can be neglected.

$$\left(\frac{k_{+2}}{k}\right)_1 = \left(\frac{k_{+2}}{K}\right)_2 \frac{[E - D_1^*]}{[E - D_2^*]} \frac{[D_2]}{[D_1]}$$

The reactions can be made extremely fast by using high concentrations of the β-lactams 1 and 2, because $[E - D_1^*]/[E - D_2^*]$ depends only on $[D_1]/[D_2]$. Utilization of an internal standard is necessary to avoid errors due to losses on deposition of the samples on the electrophoresis gels.

[17] B. Joris and J. M. Frère, *CRC Crit. Rev. Microbiol.* **11,** 299 (1985).
[18] J. M. Ghuysen, J. M. Frère, M. Leyh-Bouille, M. Nguyen-Distèche, and J. Coyette, *Biochem. J.* **235,** 159 (1986).

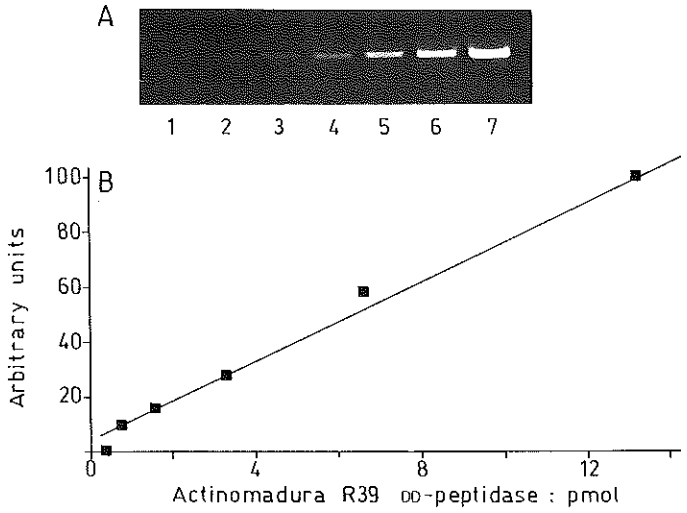


FIG. 2. Detection of the *Actinomadura* R39 DD-peptidase labelled with 5′-Flu-Gly-6APA, by SDS–PAGE and densitometry measurements. (A) Photograph of the gel illuminated with a UV lamp (λ_{max}, 312 nm). Enzyme amounts: 0.22, 0.44, 0.88, 1.65, 3.3, 6.6 and 13.2 pmol in lanes 1–6, respectively. (B) Calibration curve obtained after densitometry of (A). Reprinted with permission from Ref. 19.

Fluorescent β-Lactams

Fluorescein-coupled penicillins replace the radioactive β-lactams.[19] 5′-Flu-Gly-6APA (Fig. 1) serves to illustrate the technique.

i. Quantification by densitometry. The *Actinomadura* R39 enzyme (3 μ*M*) is incubated with 5′-Flu-Gly-6APA (7 μ*M*) in 50 m*M* Tris-HCl, pH 7.5, for 30 min at 37°, under which conditions the enzyme is fully acylated. Solutions containing varying concentrations of the acyl-enzyme, from 10 to 600 n*M*, in Tris buffer/2% (v/v) glycerol/0.2% (w/v) SDS/0.2% (v/v) 2-mercaptoethanol/0.02% (w/v) bromophenol blue are heated at 100° for 1 min. Samples (20 μl) are submitted to 12% SDS–PAGE using a 9.0 × 7.0 × 0.075-cm slab gel, for 45 min at 200 V (15 mA). Detection is made under UV light (λ_{max} = 312 nm) and quantification is made with the help of a two-dimensional densitometer (Cybertech CS-1, Dalton, Waalwijk, The Netherlands). Amounts as low as 0.44 pmol of enzyme can be detected with the naked eye and there is a good linear correlation between the fluorescence intensities and the amounts of enzyme (Fig. 2).

[19] M. Galleni, B. Lakaye, S. Lepage, M. Jamin, I. Thamm, B. Joris, and J. M. Frère, *Biochem. J.* **291,** 19 (1993).

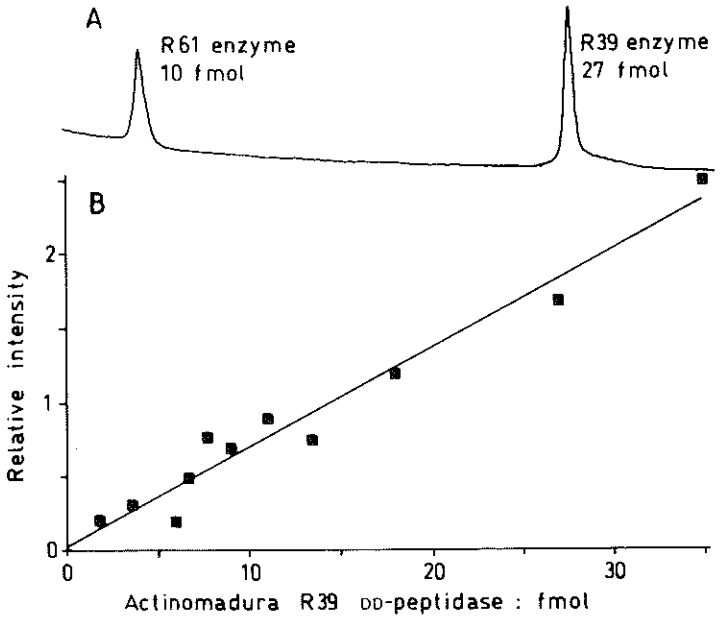


FIG. 3. Identification of *Actinomadura* R39 DD-peptidase and *Streptomyces* R61 DD-peptidase labeled with 5′-Flu-Gly-6APA, by SDS–PAGE using the ALF DNA sequencer. (A) Recording of the fluorescent tracing given by 27 fmol of the R39 enzyme and 10 fmol of the R61 enzyme. (B) Calibration curve showing the ratios of the areas under the peaks corresponding to the R39 and R61 enzymes (relative intensity) versus the amounts of the R39 enzyme. Reprinted with permission from Ref. 19.

ii. Quantification using the ALF automatic DNA sequencer. After reaction with 5′-Flu-Gly-6APA, solutions are made as above, containing varying concentrations, from 0.2 to 3.5 n*M*, of the acylated *Actinomadura* R39 enzyme and a fixed concentration, 1 n*M*, of the acylated *Streptomyces* R61 enzyme. The solutions are heated at 100° for 1 min. Samples (10 μl) are submitted to 12% SDS–PAGE using a 18 × 30 × 0.05-cm slab gel and an ALF DNA sequencer.[20] Detection of the fluorescent proteins is performed with a laser beam (λ_{ex}, 494 nm; λ_{em}, 512 nm) and the areas under the fluorescent bands are determined by triangulation. Amounts as low as 2 fmol of enzyme can be detected and satisfactory quantification is obtained down to 7 fmol (Fig. 3).

iii. Comparison with radioactive penicillin. Table II gives the k_{+2}/K and k_{+3} values for the interactions between benzylpenicillin and 5′-Flu-

[20] W. Ansorge, B. Sproat, J. Stegemann, C. Schawger, and M. Zenke, *Nucleic Acids Res.* **15,** 4593 (1987).

TABLE III
DETECTION OF *Streptomyces* R61 DD-PEPTIDASE/PBP WITH RADIOACTIVE AND FLUORESCENT PENICILLINS[a]

Enzyme concentration (pmol)	[^{14}C]Benzylpenicillin (55 mCi/mmol)	[^{3}H]Benzylpenicillin (5000 mCi/mmol)	^{125}I-Labeled hydroxybenzylpenicillin (2000 mCi/mmol)	5′-Flu-Gly-6APA
13.2	15 hr	15 hr	<6 hr	2 hr (A)
3.3	2 days	1 day	<6 hr	2 hr (A)
0.82	6 days	6 days	12 hr	6 hr (B)
0.22	10 days	7 days	1 day	6 hr (B)
0.05	>10 days	10 days	>1 day	6 hr (B)
0.01	>10 days	>10 days	>1 day	6 hr (B)

[a] Detection with 5′-Flu-Gly-6APA is made by densitometry (A) or with the ALF DNA sequencer (B).

Gly-6APA, respectively, and the enzymes and PBPs studied. Table III compares the total experience times required to detect varying amounts of the *Streptomyces* R61 DD-peptidase/PBP using [^{14}C]benzylpenicillin, [^{3}H]benzylpenicillin, ^{125}I-labeled hydroxybenzylpenicillin, and 5′-Flu-Gly-6APA as labeling agents.

iv. Detection of the membrane-bound PBPs of freezed-thawed cells of *E. coli* HB101. Cells (from a 2-ml sample of a culture at an absorbance of 1.0 at 550 nm) are suspended in 50 μl of 50 m*M* sodium phosphate, pH 7.0, submitted to three cycles of freezing and thawing, and treated with 5′-Flu-Gly-6APA at a 10 μ*M* final concentration, for 30 min at 30°. After centrifugation, the pellet is dissolved in 50 μl of denaturing buffer/2-mer-

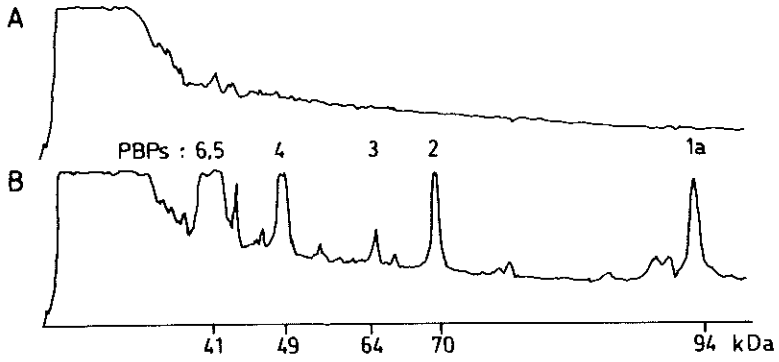


FIG. 4. Detection of the PBPs in freezed and thawed cells of *Escherichia coli* labeled with 5′-Flu-Gly-6APA, by SDS–PAGE using the ALF DNA sequencer. (A) Samples are pretreated with 200 μM benzylpenicillin before labelling with 10 μM 5′-Flu-Gly-6APA. (B) Samples are treated directly with 5′-Flu-Gly-6APA. Reprinted with permission from Ref. 19.

captoethanol and the suspension is heated at 100° for 1 min. Samples (10 μl) are analyzed as above using the ALF DNA sequencer. The same experiment is repeated on freezed-thawed cells preincubated with 200 μM nonradioactive benzylpenicillin, under which conditions all the PBPs are saturated. Figure 4 shows that multiple proteins in *E. coli* interact competitively with 5′-Flu-Gly-6APA and benzylpenicillin. The proposed protein numbering is based on the known molecular masses of the *E. coli* PBPs.

v. Specificity profile. Depending on the structure of the fluorescent β-lactam used, the rate of acylation of a given PBP may vary greatly. Thus, for example, 5′-Flu-Gly-6APA, 5′-fluoresceylampicillin, and 6′-fluoresceylampicillin acylate the *E. coli* PBP3p with k_{+2}/K values of 25 M^{-1} sec^{-1} (Table II), 13,000 M^{-1} sec^{-1}, and 2000 M^{-1} sec^{-1}, respectively.

Acknowledgments

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Note added in proof: Biotinylated β-lactams can also be used for the analysis of Penicillin Binding Proteins (M. Dargis and F. Malouin, *Antimicrob. Ag. Chemother.* **38,** 973 (1994).

[20] Cleavage of LexA Repressor

By JOHN W. LITTLE, BAEK KIM, KENNETH L. ROLAND, MARGARET H. SMITH, LIH-LING LIN, and STEVE N. SLILATY

Introduction

The SOS regulatory system of *Escherichia coli* controls the response of the cell to treatments that damage DNA or inhibit DNA replication.[1,2] In normally growing cells, about 20 SOS genes are turned off by the LexA repressor. On inducing treatments, LexA undergoes specific proteolytic cleavage; cleavage inactivates LexA and leads to derepression of the SOS genes. This specific cleavage reaction is therefore of biological interest, because it controls the state of the SOS regulatory system.

[1] J. W. Little and D. W. Mount, *Cell* (*Cambridge, Mass.*) **29,** 11 (1982).
[2] G. C. Walker, *Microbiol. Rev.* **48,** 60 (1984).