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Penicillin-Binding Proteins and Carboxypeptidase/Transpeptidase Activities in *Proteus vulgaris* P18 and Its Penicillin-Induced Stable L-Forms

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The originally penicillin-induced, wall-less stable L-forms of *Proteus vulgaris* P18, isolated by Tulasne in 1949 and since then cultured in the absence of penicillin, have kept the ability to synthesize the seven penicillin-binding proteins and the various DD- and LD-peptidase activities found in the parental bacteria and known to be involved in wall peptidoglycan metabolism. The stable L-forms, however, secrete during growth both the highly penicillin-sensitive, DD-carboxypeptidase-transpeptidase penicillin-binding protein PBP4 (which in normal bacteria is relatively loosely bound to the plasma membrane) and the penicillin-insensitive LD-carboxypeptidase (which in normal bacteria is located in the periplasmic region).

The rod-shaped enterobacteria can give rise by a penicillin-induced process to two types of coccal L-forms. The unstable L-forms (spheroplasts) have defects in their wall peptidoglycan and are osmotically fragile. The induced lesion is reversible, and upon removal of penicillin from the growth medium, both normal peptidoglycan synthesis and reversion to the original bacteria occur. In contrast, the stable L-forms (protoplasts) grow as permanently wall-less, ovoid organisms and can do so under conditions of normal osmotic stabilization whether penicillin is present or not (1, 8, 25). Like the other enterobacteria studied (4, 5, 20), *Proteus mirabilis* possesses seven penicillin-binding proteins (PBPs), referred to as PBP1A, 1B, 2, 3, 4, 5, and 6 (in the order of decreasing molecular weight). On the basis of the PBP pattern of the normal bacteria and that of the corresponding stable L-forms, Martin et al. (13) have suggested that the permanent inability of these L-forms to synthesize a normal peptidoglycan might be related to the hereditary and selective loss of PBP4. PBP4 is known to be a D-alanyl-D-alanine-cleaving peptidase which is able to show, on well-defined substrates, carboxypeptidase, transpeptidase, and endopeptidase activities (22). In view of the importance of the wall-less growth forms of bacteria in clinical cases, another stable L-form originating from *Proteus vulgaris* P18 has been investigated and compared with its parental strain. This stable L-form was isolated by Tulasne in 1949 as a penicillin-resistant organism (25). Since then, the cells have been subcultured twice weekly in suitable growth medium in the absence of penicillin.

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MATERIALS AND METHODS

Strains and growth conditions. *P. vulgaris* P18 was grown at 37°C with shaking in standard Merck I broth medium, and the cells were harvested at the midlog phase (optical density at 600 nm, 1). The stable L-forms were grown at 37°C either without shaking in the modified Medill-O'Kane medium (17) or with shaking in the modified Medill-Brown medium (16). Both cultures gave identical results.

Preparation of the cell envelopes. Three procedures were used to prepare the cell envelopes from the normal bacteria.

(i) Standard procedure (via spheroplasts formation). Cells (1 g, wet weight) were suspended in 30 ml of 50 mM Tris-hydrochloride buffer (pH 8.0) containing 0.75 M sucrose and 40 mg of lysozyme, and the suspensions were submitted to successive freezing and thawing. After an osmotic shock in distilled water, the cell envelopes were collected by centrifugation. For more details, see references 9 and 11.

(ii) Ribi procedure. Cells (1g, wet weight) were suspended in 5 ml of 10 mM Tris-maleate buffer (pH 7.0) containing 1 mM $MgCl_2$, and the suspension was treated with a Ribi press at 30,000 lb/in^2 and a temperature ranging between 4 and 15°C (21). The cell envelopes were collected by centrifugation.

(iii) Modified Nossal-Heppel procedure (19). Cells (collected from 1 liter of culture) were successively (i)

plasmolyzed for 15 min at 25°C in 100 ml of 10 mM Tris-hydrochloride buffer (pH 7.3) containing 30 mM NaCl, 0.5 mM EDTA, and 0.58 M sucrose (centrifugation gave rise to a supernatant S1); (ii) rapidly homogenized in 100 ml of distilled water at 4°C (centrifugation gave rise to a supernatant S2); (iii) treated for 30 min at 4°C with 5 ml of 10 mM Tris-hydrochloride buffer (pH 7.3) containing 1 M NaCl (centrifugation gave rise to a supernatant S3); and (iv) submitted to sonication with a Measuring Scientific Equipment apparatus at 25,000 Hz and at 0°C (centrifugation gave rise to a supernatant S4 and a pellet which consisted of cell envelopes). Each supernatant was dialyzed against 0.25 M Tris-hydrochloride buffer (pH 8.9) and concentrated by ultrafiltration to 4 ml.

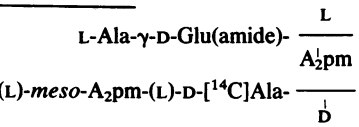
The cell envelopes of the stable L-forms were prepared according to the standard procedure described above, but without supplemental lysozyme. In all cases, the cell envelopes were suspended in 0.25 M Tris-hydrochloride buffer (pH 8.9) at a concentration of about 50 mg of protein per ml. They were stored in the frozen state at −20°C.

Protein determination. The proteins were estimated by the technique of Lowry et al. (10) with bovine serum albumin as a standard and in the case of the cell envelope, in the presence of sodium dodecyl sulfate as described previously (23).

PBP analysis. Samples (45 μl) of the cell envelopes (150 μg of protein) or the solubilized enzyme preparations (at a proper concentration) were incubated with [^{14}C]benzylpenicillin (from the Radiochemical Centre, Amersham; final specific radioactivity, 50 mCi/mmol) at the indicated concentration for 10 min at 37°C, and the reaction was terminated by the addition of 8 mM (final concentration) nonradioactive benzylpenicillin and 1% (wt/vol, final concentration) Sarkosyl. After centrifugation (in the case of the cell envelopes), the samples were boiled in the presence of 1% sodium dodecyl sulfate and 11.6% mercaptoethanol, and the PBPs were separated by polyacrylamide (10%) slab gel electrophoresis at pH 8.3 in the presence of sodium dodecyl sulfate and visualized by fluorography. The time of exposure at −70°C to prefogged X-ray film was 8 weeks. For more details, see reference 24. Relative band intensities on the fluorograms were estimated with a microdensitometer (Beckman DU-8; Beckman Instruments, Inc., Fullerton, Calif.) with peak integration. Saturation of the PBPs was carried out by using increasing concentrations of [^{14}C]benzylpenicillin (up to 5×10^{-4} M). The affinities of various nonradioactive β-lactam compounds for the PBPs were determined by competition with [^{14}C]benzylpenicillin and expressed as the concentrations necessary to inhibit by 50% further binding of [^{14}C]benzylpenicillin. For this purpose, the cell envelopes were exposed to serial dilutions of the various antibiotics for 10 min at 37°C, and then [^{14}C]benzylpenicillin (0.2 mM, final concentration) was added. After a further 10 min of incubation, the reaction was terminated as above by the addition of nonradioactive benzylpenicillin and Sarkosyl. The half-lives of the adducts formed between the PBPs and [^{14}C]benzylpenicillin were estimated by the technique of Spratt (24). The thermolability of the native PBPs (before reaction with radioactive benzylpenicillin) was determined as described by Ohya et al. (20). The apparent molecular weights of the PBPs were determined by comparing their mobilities on the gels with those of myoglobin (M_r, 17,800), chymotrypsinogen A (M_r, 25,000), ovalbumin (M_r, 43,000), and bovine serum albumin (M_r, 68,000).

Analytical polyacrylamide gel electrophoresis at pH 8.3 under nondenaturing conditions. Electrophoresis was carried out on cylindrical gels (0.9 by 12 cm; containing 7% acrylamide and 0.2% *NN'*-methylene bisacrylamide) in 25 mM Tris-glycine buffer (pH 8.3). After preelectrophoresis of the gels for 1 h at 1 mA/gel, the enzyme samples were submitted to electrophoresis for about 3 h at 4°C and 2 mA/gel. Bromophenol blue was used as a marker. After electrophoresis, the gels were sliced into 2-mm-thick disks, the disks were eluted with 300 μl of 0.1 M Tris-hydrochloride buffer (pH 8.9), and the eluates were assayed for enzyme activity.

DD-Carboxypeptidase, DD-transpeptidase, and LD-carboxypeptidase assays. All reactions were carried out at 37°C in 30 μl (final volume) of 0.25 M Tris-hydrochloride (pH 8.9) containing 2×10^{-4} M dithiotreitol. For details, see reference 18. D-Alanyl-D-alanine-cleaving carboxypeptidase (in short, DD-carboxypeptidase) activity was estimated by measuring the amount of D-[^{14}C]Ala released from the nucleotide-pentapeptide UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-[^{14}C]Ala-D-[^{14}C]Ala (1.33 mM; specific radioactivity, 22 mCi/mmol). D-Alanyl-D-alanine-cleaving transpeptidase (in short, DD-transpeptidase) activity was estimated by measuring the amount of radioactive monoamidated peptide dimer



formed from a mixture of radioactive pentapeptide L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-[^{14}C]Ala-D-[^{14}C]Ala (1.33 mM; specific radioactivity, 22 mCi/mmol) and amidated tetrapeptide L-Ala-γ-D-Glu(amide)-(L)-*meso*-A_2pm-(L)-D-Ala (13.3 mM). *meso*-Diaminopimelyl-(L)-D-alanine-cleaving carboxypeptidase (in short, LD-carboxypeptidase) activity was estimated by measuring the amount of D-Ala released from the nucleotide-tetrapeptide UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala (1.33 mM). Free D-Ala was estimated as described previously (2). One unit of these enzymes hydrolyzed or catalyzed the synthesis of 1 μeq of appropriate linkages per min.

ID_{50} values. The DD-peptidases (but not the LD-carboxypeptidase) were sensitive to β-lactam antibiotics. The ID_{50} values were the antibiotic concentrations which inhibited the enzyme activities by 50%.

Isolation of the highly penicillin-sensitive DD-peptidase PBP4, the moderately penicillin-sensitive DD-peptidase PBP5, and the penicillin-insensitive LD-carboxypeptidase. Normal bacteria (90 g, wet weight) suspended in 450 ml of 10 mM Tris-maleate buffer (pH 7) were disrupted with a Ribi press (see above), and the resulting preparation was submitted to centrifuga-

tion for 6 h at 200,000 × g. The supernatant fraction and the membrane pellet were treated as follows (all the operations were carried out at 4°C).

The supernatant (465 ml) was submitted to $(NH_4)_2SO_4$ fractionation. The precipitate collected at 30 to 60% saturation, which contained 75% of the highly penicillin-sensitive DD-peptidase and 95% of the insensitive LD-peptidase, was dissolved in 60 ml of 0.25 M Tris-hydrochloride buffer (pH 8.9) supplemented with 0.2 mM dithiothreitol. After dialysis against the same buffer, the enzyme solution was filtered through a column (2.6 by 31 cm) of DEAE-Sephadex. The column was washed and then treated with two successive NaCl gradients (made in the same buffer), first from 0 to 0.15 M NaCl, under which conditions the DD-peptidase was eluted (at about 0.07 M NaCl), and then from 0.15 to 0.4 M NaCl, under which conditions the LD-carboxypeptidase was eluted (at about 0.25 M NaCl). The relevant fractions (containing the separated peptidases) were pooled, and the solutions were dialyzed against the Tris-hydrochloride (plus dithiotreitol) buffer (pH 8.9) and concentrated by ultrafiltration.

The above procedure permitted a 180-fold enrichment of the highly penicillin-sensitive DD-peptidase (from 5 to 970 mU/mg protein; carboxypeptidase assay) with a total yield of 24% and a 300-fold enrichment of the LD-carboxypeptidase (from 2.4 to 760 mU/mg of protein) with a total yield of 46%. The DD-peptidase could be detected and identified as PBP4 after the DEAE-Sephadex treatment. Further purification of the DD-peptidase to a final specific activity of 4 U/mg of protein (carboxypeptidase assay) could be achieved by filtration on a column of Ultrogel AcA4-4 in 0.25 M Tris-hydrochloride buffer (pH 8.9) containing 0.2 mM dithiotreitol, followed by polyacrylamide gel electrophoresis at pH 8.3 under nondenaturing conditions. Due to the instability of the purified enzyme, the final yield was poor (1%), but a constant ratio between DD-carboxypeptidase activity, DD-transpeptidase activity, and [^{14}C]benzylpenicillin-binding capacity was found throughout the protein peak eluted from the Ultrogel column. Similarily, gel electrophoresis showed that these three activities were attributable to a same protein which, under the conditions used, migrated 3 cm toward the anode.

The membrane pellet originating from the normal bacteria was treated (at 25°C for 30 min) with 10 mM Tris-maleate buffer (pH 7.0) containing 1 mM $MgCl_2$ and 0.5% Genapol X-100 (a gift from Farbwerke, Hoechst, Belgium). Under these conditions, the moderately penicillin-sensitive DD-peptidase was solubilized. The extract was dialyzed against 0.25 M Tris-hydrochloride buffer (pH 8.9) containing 0.2 mM dithiotreitol and 0.5% Genapol X-100 and filtered through an ampicillin-linked Sepharose column (equilibrated against the same buffer). The column was then treated with the same buffer as above, but containing 1 M NaCl, under which conditions the DD-peptidase was eluted (12). Each step of the purification gave rise to a parallel enrichment in both DD-peptidase activity and PBP5. The final enzyme preparation had a specific carboxypeptidase activity of 60 mU/mg of protein. No PBP other than PBP5 could be detected.

The same procedures as those described above were applied to the isolation of the corresponding peptidases from growing stable L-forms. The DD-peptidase PBP5 was isolated from the plasma membrane, but the DD-peptidase PBP4 (final specific activity, 90 mU/mg of protein in the carboxypeptidase assay) and the LD-carboxypeptidase (final specific activity, 43 mU/mg of protein) were isolated from culture fluids.

RESULTS

P. vulgaris P18 had the rod-shaped morphology and the multilayered cell envelope structure typical of the enterobacteria. In contrast, the stable L-forms were ovoid organisms of various sizes (from 0.2 to 10 μm in diameter). As shown by electron microscopy after metal shadowing, they exhibited a smooth surface and possessed multiple flagella. Examination of thin sections revealed that the cell envelope consisted of only two layers of irregular thickness (plasma membrane). Both the peptidoglycan layer and the outer membrane were absent. It has been shown (14) that unstable L-forms (i.e., osmotically fragile spheroplasts) can be obtained from *P. mirabilis* with either benzylpenicillin or cefoxitin alone, but that the addition of cefoxitin to growing benzylpenicillin-induced, unstable L-forms caused an immediate inhibition of cellular growth, showing a clear cooperative effect of these two β-lactam compounds. Such effects were not seen with the stable (protoplast-like) L-form of *P. vulgaris* P18. Benzylpenicillin, cephalothin, or cefoxitin alone as well as the combinations benzylpenicillin-cephalothin and benzylpenicillin-cefoxitin (800 and 80 μg/ml, respectively, in each case) did not inhibit cellular growth.

PBP patterns of *P. vulgaris* P18 and its stable L-form. The cell envelopes isolated from the normal bacteria via spheroplast formation (standard procedure) possessed seven PBPs (Fig. 1, track 1). After reaction with a saturating concentration of [^{14}C]benzylpenicillin (0.2 mM), PBP1A (M_r, 84,000), PBP1B (M_r, 77,000), PBP2 (M_r, 68,000), PBP3 (M_r, 63,000), PBP4 (M_r, 46,000) and PBP5/6 (M_r, 43,000) occurred with a relative abundance of 14, 15, 4, 3, 5, and 59%, respectively (Table 1). Half-saturation occurred at ≃0.2 μM benzylpenicillin for PBP4, ≃2 μM for PBP1A, 10 to 15 μM for PBP2, 3, and 5/6, and finally 80 μM for PBP1B (Table 2). Cephalothin action was preferentially directed against PBP1A, and PBP2 was a very specific target for mecillinam. PBP1B was the only protein whose ability to bind [^{14}C]benzylpenicillin was not affected by heating the cell envelopes for 10 min at 55°C. The adducts formed between the PBPs and [^{14}C]benzylpenicillin showed varied stabilities with half-lives ranging from 10 min (PBP5/6) to 300 min (PBP1A) (Table 3). All these data showed that *P. vulgaris* P18 had a PBP pattern typical of the enterobacteria (4) and that its PBPs had the properties that one expected for a

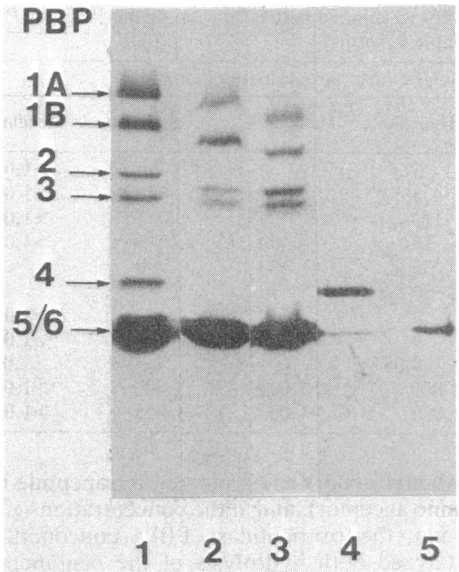


FIG. 1. PBP patterns of *P. vulgaris* P18 (tracks 1 and 2) and its stable L-forms (track 3). Analyses of the purified PBP4 (track 4) and PBP5 (track 5) isolated from normal cells. The cell envelopes of the normal bacteria were prepared via spheroplast formation (track 1) or by using the Ribi press (track 2). The isolated PBP4 was water soluble. The isolated PBP5 was in Genapol. For experimental conditions, see the text.

member of this taxonomic group (20). PBP4 was especially loosely bound to the isolated cell envelopes from which it was selectively released by treatment with 1 M NaCl. Moreover, PBP4-free cell envelopes were obtained by submitting the bacteria to the modified Nossal-Heppel procedure, in which case PBP4 was recovered in both fractions S3 and S4, or by disrupting the bacteria with the Ribi press (Fig. 1, track 2), in which case PBP4 was recovered in the supernatant fraction.

The stable L-forms were disrupted under those conditions which had permitted isolation from the normal bacteria of cell envelopes with a full assortment of PBPs. Analysis of the stable L-form cell envelopes (Fig. 1, track 3; Tables 1, 2, and 3) showed that PBP1A, 1B, 2, 3, and 5/6 were present and had the same properties (relative abundance, affinity for β-lactam antibiotics, thermostability, and stability of the adducts formed with [^{14}C]benzylpenicillin) as those of the normal bacteria. In some experiments (Fig. 1) the PBP1A and 1B of the stable L-forms had, somehow, reduced apparent molecular weights, but this was not always the case. PBP4, however, was always absent. Further analysis showed that the stable L-forms had not lost their ability to synthesize PBP4, but had lost their ability to anchor it in their cell envelope. During growth, PBP4 was therefore secreted in the culture medium from which it could be isolated.

DD- and LD-peptidase activities of *P. vulgaris* P18 and its stable L-forms. The DD- and LD-peptidase activities of *P. vulgaris* P18 and its stable L-forms are summarized in Table 4. Cell envelopes possessing a full assortment of PBPs (i.e., prepared from normal bacterial via spheroplast formation) catalyzed both DD-carboxypeptidase and DD-transpeptidase activities. The specific activities given in Table 4 were determined on reaction mixtures containing Triton X-100 at a 1% (vol/vol) final concentration, a condition that increased enzyme activity 10-fold. The effects of increasing concentrations of benzylpenicillin showed that a major part (≃80%) of the total DD-carboxypeptidase activity was highly sensitive to the antibiotic ($\pm ID_{50} \simeq 0.02$ to 0.06 μM), and that a minor part (≃20%) was attributable to an enzyme of moderate penicillin sensitivity ($\pm ID_{50} \simeq 2$ to 6 μM).

Cell envelopes lacking PBP4 (i.e., prepared from normal bacteria after disruption with the Ribi press or from the stable L-forms) were not competent in catalyzing transpeptidation reactions and had only a low level of DD-carboxypeptidase activity of moderate benzylpenicillin sensitivity. This DD-peptidase activity was not enhanced by the presence of Triton X-100. It was isolated (in the presence of Genapol X-100) and identified as PBP5 as described above (Fig. 1, track 5).

Both the supernatant fraction obtained after disruption of the normal bacteria with the Ribi press and the culture filtrate of the growing L-forms contained the highly penicillin-sensitive DD-carboxypeptidase-transpeptidase. This DD-peptidase was isolated and identified as PBP4 as described above (Fig. 1, track 4). An antiserum prepared against the DD-peptidase PBP4 isolated

TABLE 1. Relative abundance of the PBPs present in the cell envelopes of *P. vulgaris* P18 and its stable L-forms as revealed by reaction with a saturating concentration (0.2 mM) of [^{14}C]benzylpenicillin

PBP	Relative abundance (%) of PBPs from:		
	Normal bacteria[a]		L-forms
	Spheroplast prepn	Ribi press prepn	
1A	14	11	12
1B	15	13	12
2	4	4	12
3	3	5	8
4	5	0	0
5/6	59	67	56

[a] The cell envelopes of the normal bacteria were prepared via spheroplast formation or by disruption with a Ribi press.

TABLE 2. Concentration of β-lactam antibiotics necessary to achieve half-saturation of the PBPs of *P. vulgaris* P18 and its stable L-forms

PBP	Organisms	Concn (μM) of antibiotic to achieve half-saturation of PBP					
		Benzyl-penicillin	Ampicillin	Carbenicillin	Methicillin	Cephalothin	Mecillinam
1A	Normal bacteria	1.8	1	5	6	0.04	>1,000
	L-forms	0.5	1	3	18	0.01	>1,000
1B	Normal bacteria	80	70	100	90	1	>1,000
	L-forms	35	40	30	500	1	>1,000
2	Normal bacteria	9	4	30	400	90	<10
	L-forms	4	3	20	70	40	<10
3	Normal bacteria	15	3	9	20	10	>1,000
	L-forms	3	3	9	10	2	>1,000
4	Normal bacteria	0.2	0.02	0.08	1	3	1,000
5/6	Normal bacteria	10	250	1,000	>1,000	1,000	>1,000
	L-forms	6	>1,000	>1,000	>1,000	>1,000	>1,000

from *P. vulgaris* P18 (specific carboxypeptidase activity, 4 U/mg of protein) maximally inhibited the activities of the enzyme by 80%. It had exactly the same effect on the corresponding DD-peptidase-PBP4 of the stable L-forms.

P. vulgaris P18 and its stable L-forms also synthesized a penicillin-resistant (and therefore not detectable as a PBP) *meso*-diaminopimelyl-(L)-D-alanine-cleaving peptidase (equivalent to the carboxypeptidase 2 of *Escherichia coli* [7]). This LD-peptidase was found to be periplasmic in the normal bacteria and was selectively recovered in fractions S1 and S2 when the Nossal-Heppel technique was employed. The same LD-peptidase was secreted in the culture medium by the stable L-forms during growth (Table 4). The LD-peptidase was partially purified as described above.

Properties of the isolated DD- and LD-peptidases of *P. vulgaris* P18 and its stable L-forms. (i) Highly penicillin-sensitive DD-carboxypeptidase-transpeptidase PBP4. The water-soluble DD-peptidase PBP4 had an apparent molecular weight of about 46,000 (on the basis of its migration by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) or 49,000 (on the basis of its K_d value by Ultrogel filtration). It was anionic at pH 8.3.

With the cosubstrates, free pentapeptide (the carbonyl donor) and amidated tetrapeptide (the amino acceptor), and at the concentrations given above, the DD-peptidase PBP4 concomitantly catalyzed both hydrolysis of the pentapeptide (Hy) and transpeptidation (T), with a Hy/T ratio of about 10 (under conditions where less than 25% of the radioactive pentapeptide was utilized). Optimal pH for enzyme activity was 8.8 in 0.25 M (or less) Tris-hydrochloride buffer. The addition of 0.2 M NaCl to the same buffer inhibited the enzyme activity by 70%. UDP-*N*-acetylmuramyl-pentapeptide was not used as carbonyl donor for the transpeptidation reaction although it was a substrate (K_m = 0.6 mM) for the carboxypeptidase activity.

The DD-peptidase PBP4 exhibited wide variations in its sensitivity to β-lactam antibiotics (Table 5). The ID_{50} values thus obtained related well with the corresponding values of Table 2, except for benzylpenicillin and mecillinam. Somehow, these two antibiotics had much higher affinities for the water-soluble PBP4 than for the membrane-bound PBP4. On the basis of the rates of enzyme recovery (3), the adducts formed between the DD-peptidase and benzylpenicillin and ampicillin had half-lives of 240 and 170 min, respectively. The inhibition of the DD-peptidase by carbenicillin was of the competitive type. The K_m value for the substrate (UDP-*N*-acetylmuramyl-pentapeptide) varied depending on the carbenicillin concentrations used (Fig. 2). This observation was at variance with that made previously with the corresponding DD-carboxypeptidase-transpeptidase PBP4 of *P. mirabilis*, in which case the inhibition appeared to be clearly noncompetitive (11).

TABLE 3. Stability of the complexes formed between [^{14}C]benzylpenicillin and the PBPs of *P. vulgaris* P18 and its stable L-forms

PBP	Half-life (min) of the complexes	
	Normal bacteria	L-forms
1A	300	320
1B	240	220
2	120	110
3	180	155
4	180	Absent
5/6	10	12

(ii) Moderately penicillin-sensitive DD-carboxypeptidase PBP5. DD-Carboxypeptidase PBP5 did not catalyze transpeptidation reaction with the above system of cosubstrates. It functioned solely as a carboxypeptidase on either UDP-*N*-acetylmuramyl-pentapeptide or the free pentapeptide. It was half inhibited by a 1 μM benzyl-

TABLE 4. Specific activities of DD- and LD-peptidases of *P. vulgaris* and its stable L-forms

Organisms	Prepn	Sp act (mU/mg) of peptidase[a]		
		DD-Carboxy-peptidase	DD-Trans-peptidase	LD-Carboxy-peptidase
Normal bacteria	Cell envelopes prepared via spheroplast formation[b]	10 (H and M)[c]	1 (H)	ND[d]
	Cell envelopes disrupted in Ribi press	0.5 (M)	ND	ND
	Supernatant after disruption with Ribi press	5 (H)	0.5 (H)	2.4 (R)
L-forms	Cell envelopes	0.7 (M)	ND	ND
	Culture filtrate	5.7 (H)	0.4 (H)	2.5 (R)

[a] The letters within parentheses indicate sensitivity to benzylpenicillin as follows: H, highly sensitive (ID_{50}, ≈0.01 to 0.06 μM); M, moderately sensitive (ID_{50}, ≈2 to 6 μM); R, resistant.

[b] As determined in reaction mixtures containing 1% Triton X-100. The presence of the detergent caused an approximately 10-fold increase in enzyme activity as compared with reaction mixtures containing the same cell envelopes without Triton X-100. The addition of Triton X-100 had little or no effect in all other cases.

[c] The inhibitory effect exerted by increasing concentrations of benzylpenicillin was biphasic, showing that, under the assay conditions used, the moderately penicillin-sensitive peptidase activity represented 10 to 20% of the total activity.

[d] ND, Not detected.

penicillin concentration. The removal of Genapol X-100 caused irreversible enzyme denaturation.

(iii) Penicillin-insensitive LD-carboxypeptidase. Filtration of water-soluble LD-carboxypeptidase on Ultrogel AcA4-4 indicated a molecular weight of 32,000. The enzyme activity increased as the pH of the reaction mixture increased from pH 7.5 (Tris-hydrochloride buffer) to 11 (Glycine-NaOH buffer). At pH 8.9, variations of the molarity of the Tris-hydrochloride buffer from 0.02 to 0.22 were without any effect, and the K_m value for the hydrolysis of UDP-*N*-acetylmuramyl-tetrapeptide was 0.4 mM. The free tetrapeptide and the amidated tetrapeptide had equivalent substrate activities.

DISCUSSION

The present investigation confirms the results previously obtained by Martin et al. (13) on the L-form strains of *P. mirabilis* LVI and LD52 (8) and expands them by showing that the stable L-form derived from *P. vulgaris* P18 has kept the ability of the parental strain to synthesize all of the PBPs and the DD- and LD-peptidases known to be involved in peptidoglycan cross-linking and its further maturation during the bacterial life cycle. However, both the DD-carboxypeptidase-transpeptidase PBP4, which in the parental strain is loosely bound to the outer surface of the plasma membrane, and the LD-carboxypeptidase, which is located in the periplasmic region, are secreted in the culture medium during growth by the protoplast-like, stable L-forms. Mutants of *E. coli* lacking PBP4 (15) grow normally under a wide range of laboratory condi-

TABLE 5. Antibiotic concentrations required to inhibit by 50% the purified DD-carboxypeptidase-transpeptidase PBP4[a]

Antibiotic	ID_{50} value (μM)
Benzylpenicillin	0.01
Ampicillin	0.006
Carbenicillin	0.08
Methicillin	1
Cephalothin	1
Mecillinam	2

[a] The enzyme used had a specific activity of 4 U/mg of protein (carboxypeptidase assay).

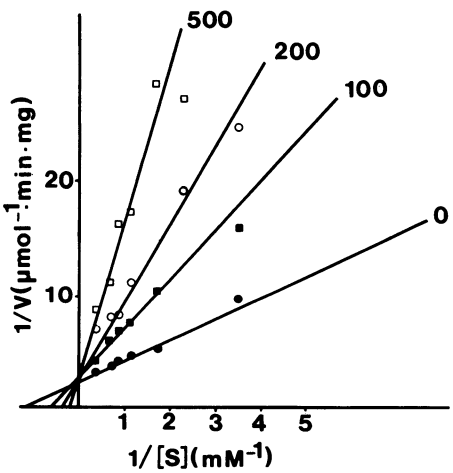


FIG. 2. Inhibition of the DD-carboxypeptidase activity of the isolated PBP4 by increasing concentrations of carbenicillin (0, 100, 200, and 500 nM). Lineweaver-Burk plot.

tions, strongly suggesting that this PBP is dispensable. On this basis, a lack of integration of PBP4 within the plasma membrane is probably not at least the main defect for the hereditary and permanent inability of the stable L-forms to synthesize wall peptidoglycan. This inability may be due to defects in the early stages of peptidoglycan synthesis or, more likely, to defects in the subsequent lipid cycle possibly caused by the physical and chemical changes in the plasma membrane which permitted transition of *Proteus mirabilis* to life in the form of an envelopeless, osmotically stable L-form (6).

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LITERATURE CITED

1. **Fleck, J.** 1963. Etude de la croissance en milieu liquide d'une souche de *Proteus* et de sa forme L. C. R. Soc. Biol. **157**:183–185.
2. **Frère, J. M., M. Leyh-Bouille, J. M. Ghuysen, M. Nieto, and H. R. Perkins.** 1976. Exocellular DD-carboxypeptidases-transpeptidases from *Streptomyces*. Methods Enzymol. **45**:610–636.
3. **Frère, J. M., M. Leyh-Bouille, J. M. Ghuysen, and H. R. Perkins.** 1974. Interaction between β-lactam antibiotics and exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61. Eur. J. Biochem. **50**:203–214.
4. **Georgopapadakou, N. H., and F. M. Liu.** 1980. Penicillin-binding proteins in bacteria. Antimicrob. Agents Chemother. **18**:148–157.
5. **Ghuysen, J. M.** 1980. Antibiotics and peptidoglycan metabolism, p. 9–117. *In* P. G. Sammes (ed.), Topics in antibiotic chemistry, vol. 5. Ellis Horwood, Chichester.
6. **Gmeiner, J., and H. H. Martin.** 1976. Phospholipid and lipopolysaccharide in *Proteus mirabilis* and its stable protoplast L-form. Difference in content and fatty acid composition. Eur. J. Biochem. **67**:487–494.
7. **Izaki, K., and J. L. Strominger.** 1968. Biosynthesis of peptidoglycan of bacterial cell walls. XIV. Purification and properties of two D-alanine carboxypeptidases from *Escherichia coli*. J. Biol. Chem. **243**:3193–3201.
8. **Kandler, O., and G. Kandler.** 1956. Trennung and Charakterisierung verschiedener L-Phasen-Typen von *Proteus mirabilis*. Z. Naturforsch. **11**:252–259.
9. **Kohn, A.** 1960. Lysis of frozen and thawed cells of *Escherichia coli* by lysozyme and their conversion into spheroplasts. J. Bacteriol. **79**:697–706.
10. **Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
11. **Martin, H. H., C. Maskos, and R. Burger.** 1975. D-alanyl-D-alanine carboxypeptidase in the bacterial form and L-forms of *Proteus mirabilis*. Eur. J. Biochem. **55**:465–473.
12. **Martin, H. H., W. Schilf, and C. Maskos.** 1976. Purification of the membrane-bound DD-carboxypeptidase of the unstable spheroplast L-form of *Proteus mirabilis* by affinity chromatography. Eur. J. Biochem. **71**:585–593.
13. **Martin, H. H., W. Schilf, and H. G. Schieffer.** 1980. Differentiation of mycoplasmatales from bacterial protoplast L-forms by assay for penicillin-binding proteins. Arch. Microbiol. **27**:297–299.
14. **Martin, H. H., M. Tonn-Ehlers, and W. Schilf.** 1980. Cooperation of benzylpenicillin and cefoxitin in bacterial growth inhibition. Phil. Trans. R. Soc. London B **289**:365–367.
15. **Matsuhashi, M., Y. Takagaki, I. N. Maruyama, S. Tamaki, Y. Nishimura, H. Suzuki, U. Ogino, and Y. Hirota.** 1977. Mutants of *Escherichia coli* lacking in highly penicillin-sensitive D-alanine carboxypeptidase activity. Proc. Natl. Acad. Sci. U.S.A. **74**:2976–2979.
16. **Medill-Brown, M., W. G. Hutchinson, and E. Cocklin.** 1960. The L-forms of *Proteus mirabilis*. Ann. N.Y. Acad. Sci. **79**:372–379.
17. **Minck, R., A. Kirn, and M. Galleron.** 1957. Recherches sur la transformation L en milieux synthétiques d'une souche de *Proteus*. Ann. Inst. Pasteur **92**:138–141.
18. **Nguyen-Distèche, M., J. M. Ghuysen, J. J. Pollock, P. E. Reynolds, H. R. Perkins, J. Coyette, and M. R. J. Salton.** 1974. Enzymes involved in wall peptide crosslinking in *Escherichia coli* K12 strain 44. Eur. J. Biochem. **41**:447–455.
19. **Nossal, N. G., and L. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241**:3055–3062.
20. **Ohya, S., M. Yamazaki, S. Sugawara, and M. Matsuhashi.** 1979. Penicillin-binding proteins in *Proteus* species. J. Bacteriol. **137**:474–479.
21. **Ribi, E., T. Perrine, R. List, W. Brown, and G. Goode.** 1959. Use of pressure cell to prepare cell walls from Mycobacteria. Proc. Soc. Exp. Biol. Med. **100**:647–649.
22. **Schilf, W., and H. H. Martin.** 1980. Purification of two DD-carboxypeptidases-transpeptidases with different penicillin sensitivities from *Proteus mirabilis*. Eur. J. Biochem. **105**:361–370.
23. **Shepherd, S. T., H. A. Chase, and P. E. Reynolds.** 1977. The separation and properties of two penicillin-binding proteins from *Salmonella typhimurium*. Eur. J. Biochem. **78**:521–523.
24. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. **72**:341–352.
25. **Tulasne, R.** 1949. Existence of L-forms in common bacteria and their possible importance. Nature (London) **164**:876–877.