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The Bacterial L-Forms

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β-Lactam-Induced *Proteus* L-Forms

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I. INTRODUCTION: MYCOPLASMATALES AND L-FORMS

The mycoplasmatales, classified into the genera *Mycoplasma, Acholeplasma, Ureaplasma*, and *Spiroplasma*, form a large and heterogeneous group of wall-less prokaryotes. They occur widely in nature and have been isolated from humans, animals, plants, and insects.

The mycoplasmatales differ from the other prokaryotes not only by their complete lack of a cell wall but also by other profound cellular alterations. They are the smallest organisms capable of independent division; they have a genome of much reduced size; they lack several sequences in their 16S rRNAs and several proteins related to membrane functions; and most (but not all) species require cholesterol for growth.

The mycoplasmatales might form a separate phylogenetic class of eubacteria [1]. Thus, the extant representatives would be the surviving descendants of very primitive cells that existed before the development of a peptidoglycan-based cell wall. Alternatively, comparative enzyme immunological and other studies [2] suggest that the mycoplasmatales might have evolved from wall-containing gram-positive bacteria belonging in particular to the genera *Bacillus, Lactobacillus*, and *Streptococcus*. Mycoplasmatales are agents of (human, animal, and plant) diseases for which there are no counterpart bacterial diseases, suggesting that in their transition from wall-containing to wall-less eubacteria, their ancestors have gained new capacities for pathogenicity.

L-forms are "modern" wall-less or wall-deficient eubacteria. They can be produced from a large number of bacterial taxa through the action of wall peptidoglycan-degrading enzymes (glycosidases, peptidases and *N*-acetylmuramyl-L-alanine amidases) or antibiotics that inactivate the wall peptidoglycan-synthesizing enzyme machinery. The L-forms do not show those profound cellular alterations found in the mycoplasmatales. They resemble the normal bacteria from which they originate except for physical and chemical changes that permitted transition to life in the form of wall-less or wall-deficient bacteria (see Chap. 1).

Penicillins, whose original molecule stems back to Fleming's discovery in 1928, cephalosporins, and other β-lactams are wall peptidoglycan inhibitors that are massively used in medicine, stock farming, and the cultured fish industry. The ability of these antibiotics to induce conversion of penicillin-sensitive bacteria to penicillin-resistant wall-less or wall-deficient L-forms may have important, practical implications in chemotherapy.

The aim of this chapter is to review the various types of L-forms that proteus, a rod-shaped, gram-negative enterobacterium, produces in responses to the action of β-lactam antibiotics.

II. THE CELL ENVELOPE OF THE GRAM-NEGATIVE ENTEROBACTERIA

A. Structure

The cell envelope in all gram-negative bacteria is a multilayered structure in which the peptidoglycan is sandwiched between the inner plasma membrane and an outer membrane. There have been several reviews [3,4] and books [5,6] on these bacterial cell envelopes and the reader is referred to them for specific information. We will summarize their most relevant properties, using *Escherichia coli* as model.

1. Peptidoglycan

The peptidoglycan is a network structure (Fig. 1). Essentially, the glycan moiety consists of linear strands of alternate pyranoside residues of N-acetylglycosamine and N-acetylmuramic acid linked together by 1-4,β-bonds; the D-lactyl group of the N-acetylmuramic acid is substituted by a tetrapeptide L-Ala-γ-D-Glu(L)-meso-A_2pm-(L)-D-Ala; and tetrapeptides substituting adjacent glycan strands are covalently linked together by D-alanyl-(D)-meso-A_2pm linkages. The average glycan chain length is (depending on the authors) between 30 and 60 disaccharide

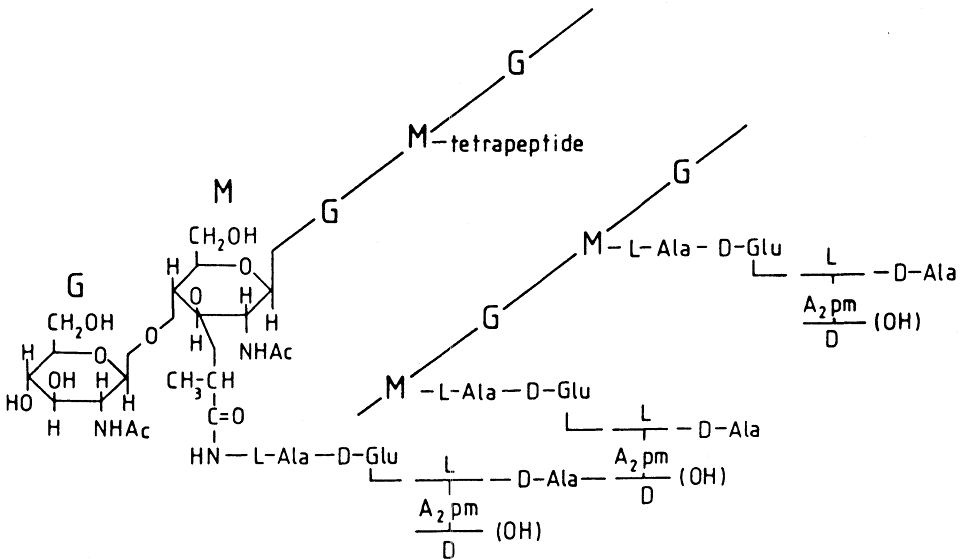


Figure 1 Structure of the peptidoglycan in gram-negative bacteria, showing sections of two glycan chains, a tetrapeptide unit, and a cross-linked peptide dimer. G = N-acetylglucosamine; M = N-acetylmuramic acid.

units and the chains are terminated by a nonreducible 1,6-anhydro-N-acetylmuramic acid residue (Fig. 2). The peptidoglycan is weakly peptide cross-linked. Approximately equal amounts of uncross-linked tetrapeptide units and the dimer of this unit make up the bulk of the peptide moiety. About 5% of the total peptide units occur as trimers and about 2-3% of the total N-acetylmuramic acid residues are substituted by truncated dipeptides L-Ala-γ-D-Glu.

2. Outer Membrane

The outer membrane is a phospholipid-lipopolysaccharide-protein structure stabilized by Mg^{2+} cations (Fig. 3). It contributes to the mechanical strength of the cell envelope and provides the bacteria with an additional permeability barrier.

The lipopolysaccharide molecules (Fig. 4) of the outer membrane are polysaccharide chains covalently linked to an unique lipid, known as lipid A. Typically, the polysaccharide moiety is composed of superficial *O*-antigen chains that cover the exterior of the cell, and a core that is characterized by the presence of two unique sugars in it, L- or D-glycero-D-mannoheptose and 2-keto-3-deoxyoctonate. The core is, in turn, linked to lipid A, the backbone of which is a disaccharide of D-glucosamine with the specific 3-D-hydroxymyristic acid in an amide linkage to the amino groups and with long-chain fatty acids in ester linkages to the hydroxyl groups.

The phospholipid molecules of the outer membrane are, in composition, qualitatively similar to those of the inner plasma membrane. Phosphatidylethanolamine and phosphatidylglycerol are the major constituents; small amounts

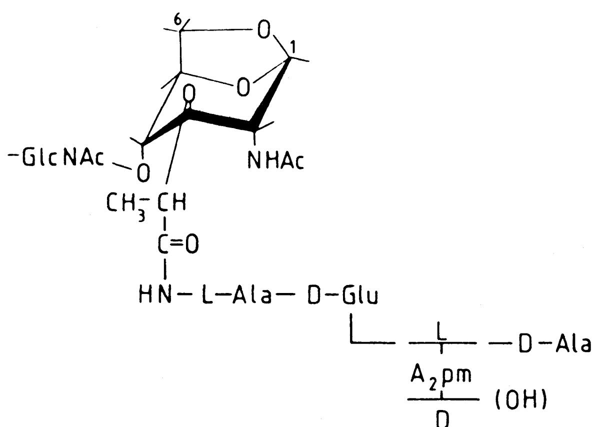


Figure 2 Tetrapeptide-substituted 1,6-anhydro-N-acetylmuramic acid occurring at the end of a glycan chain.

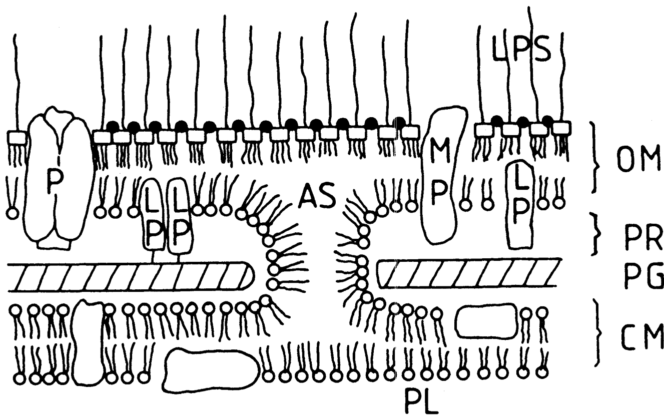


Figure 3 The cell envelope in gram-negative bacteria. OM = outer membrane; PR = periplasmic region; PG = peptidoglycan; CM = cytoplasmic membrane; P = porin (in the form of a trimer); LP = lipoprotein (either free or covalently linked to the peptidoglycan); MP = major protein; AS = adhesion site; PL = phospholipid; • = divalent cation (Mg^{2+} or Ca^{2+}); LPS = lipopolysaccharide.

of cardiolipin are also present. When compared with the plasma membrane, the outer membrane has a high ratio of protein to total phospholipid, resulting in a much decreased freedom of the membrane components for lateral diffusion.

The outer membrane is very unusual in that the lipopolysaccharide molecules are present only in the outer leaflet whereas the glycerolphospholipid molecules are present mainly, if not exclusively, in the inner leaflet of the bilayer (Fig. 3). This extremely asymmetric distribution of the component lipids makes the basic continuum of the outer membrane very poorly permeable to both hydrophobic and hydrophilic solutes. Passage of the nutrients (and other molecules such as the antibiotics) is made possible thanks to a special class of proteins, called porins. The porins so far examined usually have molecular weights in the range of 35,000–45,000, contain large amounts of β-sheet structure, and have a characteristic property to form water-filled, transmembrane, diffusion channels both in intact cells and in reconstituted liposome systems. *E. coli* possesses two different porins called OmpF and OmpC according to the names of the structural genes, with the OmpF porin producing somewhat larger pores than the OmpC porin.

Other major components of the outer membrane are specific proteins that contain ester- and amide-linked fatty acids and for this reason, are qualified as lipoproteins. *E. coli* possesses at least two lipoproteins with apparent molecular weights of about 7200 and 21,000, respectively. The 21,000 M_r lipoprotein is

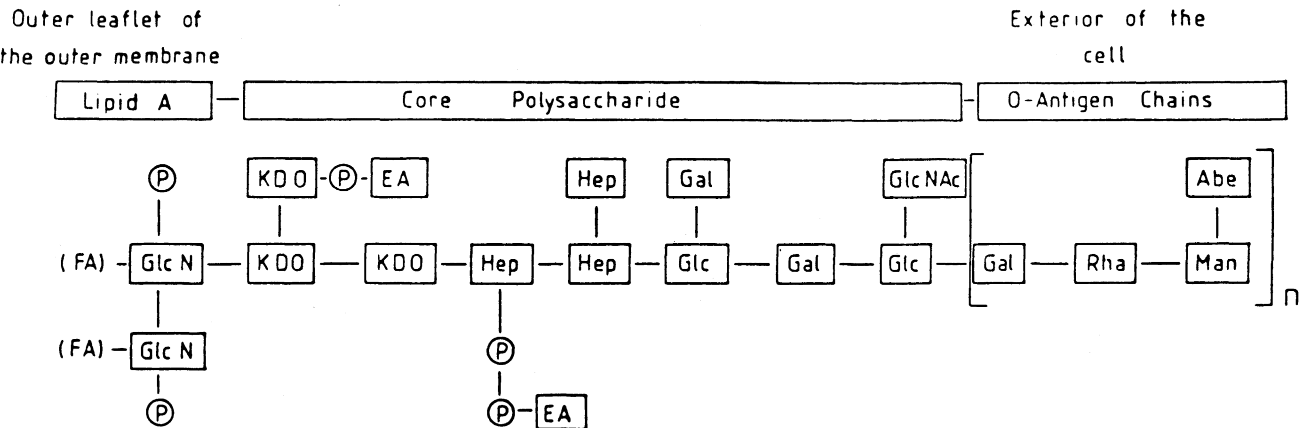


Figure 4 Structure of a complete lipopolysaccharide molecule showing the inner lipid A, the core polysaccharide, and the superficial O-antigens. FA = fatty acid; GlcN = glucosamine; KDO = 2-keto-3-deoxyoctonate; EA = ethanolamine; Hep = glycero-D-mannoheptose; Glc = glucose; Gal = galactose; GlcNAc = N-acetylglucosamine; Rha = rhamnose; Man = mannose; Abe = abequose. Incomplete lipopolysaccharides may occur with shorter polysaccharide chains.

tightly bound, but not covalently linked, to the peptidoglycan. The 7200 M_r lipoprotein occurs both in a free form and in covalent linkage with the underlying peptidoglycan (Fig. 5). The attachment site is at the carboxyl terminal of the protein where the ε-amino group of a lysine residue is bound to the carboxyl group at the L-center of meso-A_2pm of peptide units of the peptidoglycan (i.e., where a D-alanine residue occurs in a conventional tetrapeptide unit). The attachment site of the lipid moiety is at the amino terminal of the protein. One fatty acid is bound as an amide of the α-amino group of a cysteine and two other fatty acids occur as esters of the hydroxyl groups of S-glycerylcysteine.

Much has been written on the importance that, altogether, the peptidoglycan, the lipopolysaccharide, and the major proteins and lipoproteins play in the integrity of the cell envelope of the gram-negative bacteria. A mechanism has been proposed in which outer membrane vesicles, containing none of the covalently bound 7200 M_r lipoprotein and little free lipoprotein, are released from *E. coli* cells during normal growth when the outer membrane expands faster than the underlying peptidoglycan layer [7]. Outer membrane blebs are produced by *E. coli* mutants lacking the 7200 M_r lipoprotein (lpp^-) [8]. Single *lpp* or *ompA* proteim mutants are rod-shaped (*ompA* is a major protein of the outer membrane playing a role in stabilization of mating aggregates in F-pilus-mediated conjugation), but double mutants of *lpp* and *ompA* grow as spheres.

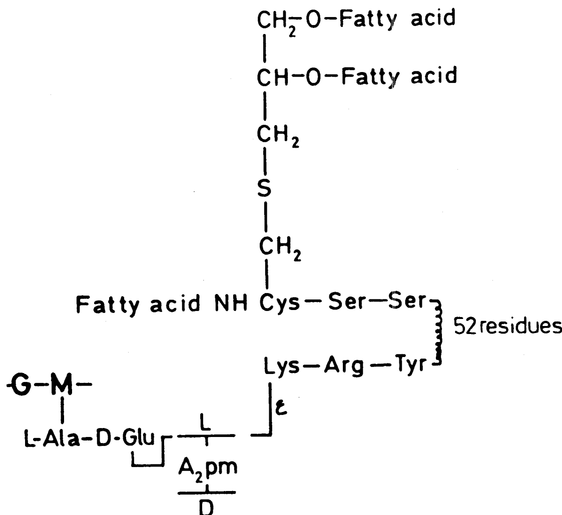


Figure 5 Peptidoglycan-linked lipoprotein. In *E. coli*, the 7200-M_r lipoprotein contains 58 amino acid residues. G = N-acetylglucosamine; M = *N*-acetylmuramic acid.

Finally, there are many distinct areas in the cell envelope at which both outer and plasma membranes are physically attached to each other. About 200 to 400 such adhesion sites (Fig. 3) are seen in a growing cell of *E. coli*. They are considered as physical channels through which the outer membrane components, such as the lipopolysaccharide molecules, are translocated across the peptidoglycan layer once they have been synthesized at the plasma membrane.

B. DD-Peptidases

A discussion of the enzyme machinery responsible for the synthesis and assembly of the various components of the cell envelope of the gram-negative enterobacteria is beyond the scope of this article. But the DD-peptidases—which are the specific targets of the β-lactam antibiotics—deserve attention. For specific information, the reader is referred to Ref. 9-11.

Following the synthesis of the nucleotide precursors in the cytoplasm and the lipid-facilitated transfer of the disaccharide-pentapeptide units across the plasma membrane, peptidoglycan assembly requires the action of several membrane-bound enzymes (Fig. 6). Essentially, transglycosylases catalyze extension of the glycan strands and DD-transpeptidases catalyze insolubilization of the expanding network by peptide cross-linking. In combination, these two types of enzyme activities permit initial incorporation of newly synthesized disaccharide peptide units. Recent data [12] support the view that this material is incorporated directly in the preexisting peptidoglycan sacculus without first existing as a "soluble, nascent" intermediate. In turn, maturation of the peptidoglycan, wall remodelling throughout the bacterial life cycle and control of the extent of peptide cross-linking require additional DD-transpeptidase/carboxypeptidase/endopeptidase activities. Remarkably, β-lactam antibiotics are susceptible to inactivate (but with widely varying efficiency) all the DD-peptidases (but not the transglycosylases) involved in these various steps of peptidoglycan metabolism. Finally, modeling of the peptidoglycan within the bacterial cell envelope also involves covalent attachment of some appendages, such as lipoprotein molecules. It has been proposed, but never proved nor disproved, that lipoprotein attachment might be catalyzed by a cell envelope-associated, β-lactam-insensitive, LD-transpeptidase (Fig. 7). Whatever its nature, this enzyme does not discriminate between peptide monomers, dimers, and trimers [12].

Basically, the DD-peptidase active sites catalyze transfer of the electrophilic group L-Ala-γ-Glu-(L)-meso-A_2pm-(L)-D-Ala from pentapeptides L-Ala-γ-D-Glu-(L)-meso-A_2pm-(L)-D-Ala-D-Ala to a nucleophilic acceptor. They are specifically designed to attack peptide bonds that are located in α-position to a free carboxylate and extend between two carbon atoms having the D-configuration. The unique optical specificity of these enzymes justifies their qualification as DD-peptidases.

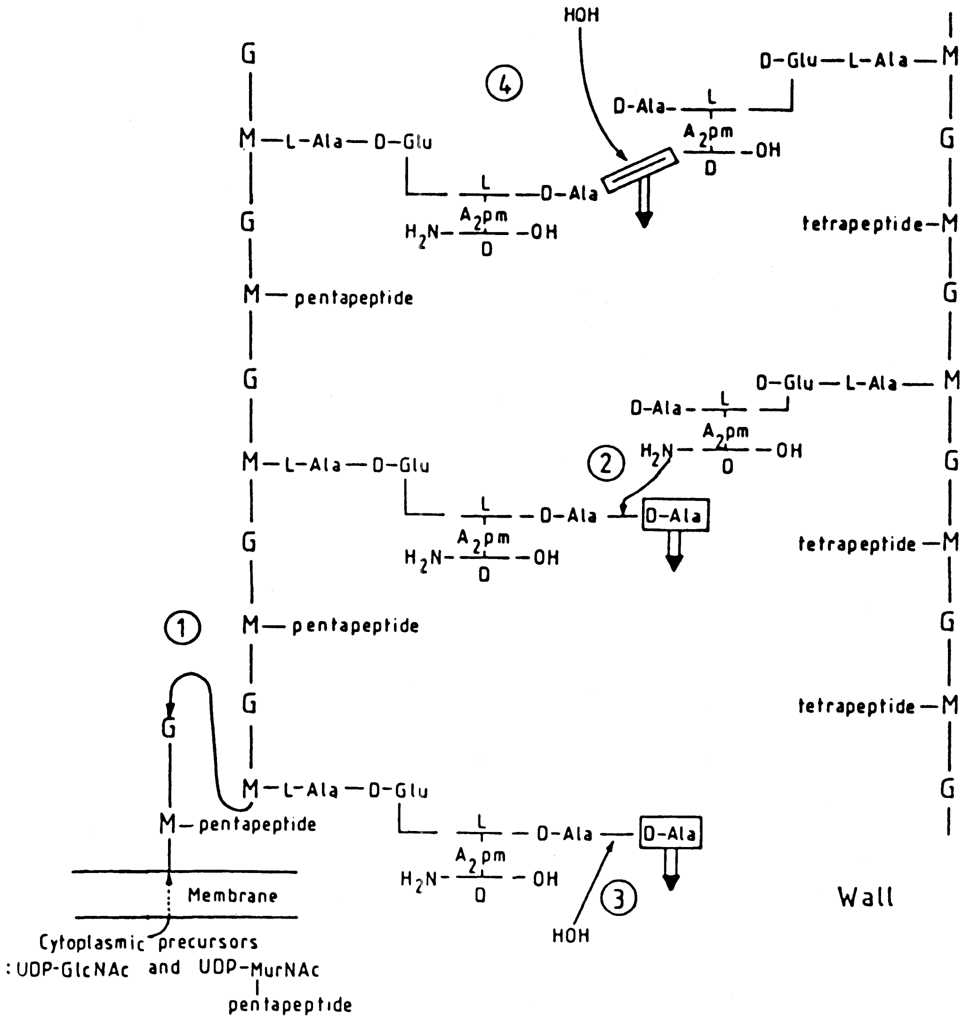


Figure 6 Enzyme activities involved in the last steps of peptidoglycan metabolism in gram-negative bacteria. 1 = transglycosylase activity; 2 = DD-transpeptidase activity; 3 = DD-carboxypeptidase activity; 4 = DD-endopeptidase activity.

The exact reaction catalyzed by the DD-peptidases depends on the nature of the acceptor which is effectively utilized in the transfer reaction (Fig. 6). If the acceptor is the amino group located on the D-center of meso-A_2pm of another peptide unit, transpeptidation occurs. If the acceptor is water, carboxypeptidation, i.e., hydrolysis, occurs. DD-carboxypeptidases also exist which can

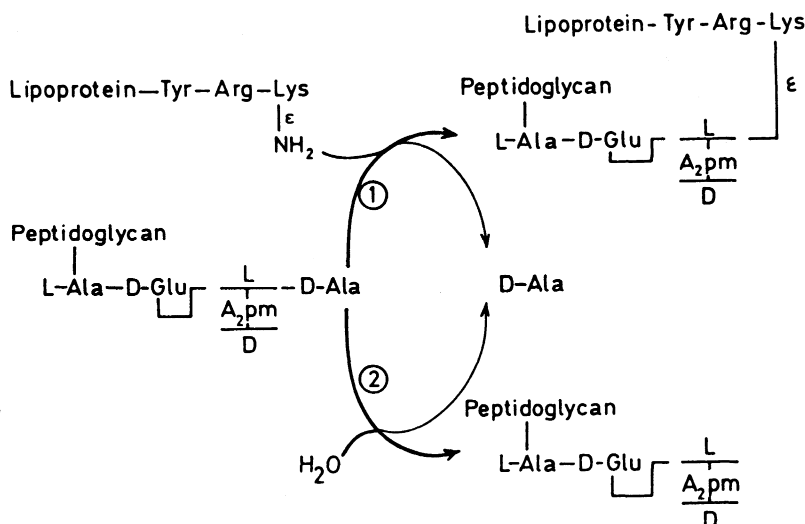


Figure 7 Possible mechanism of enzyme-catalyzed attachment of lipoprotein to peptidoglycan by LD-transpeptidase activity (1) and hydrolysis of tetrapeptide into tripeptide by LD-carboxypeptidase activity (2).

accommodate D-centers with very bulky side-chains at the carboxy-terminal position of the carbonyl donor peptide. Such enzymes can hydrolyze into tetrapeptide units, cross-linked peptide dimers or oligomers previously made by transpeptidation, thus performing peptidoglycan hydrolase activity (or "endopeptidase" activity).

Several serine DD-peptidases, isolated from gram-positive bacilli and actinomycetes, have been studied in detail. They operate by covalent catalysis through the transitory formation of a serine-ester-linked acyl enzyme intermediate. When in operation on D-Ala-D-Ala-terminated carbonyl donor substrates, the process is highly effective and the reaction flux is to reaction products (Fig. 8). When in operation on β-lactams (which are suicide substrates of the DD-peptidases), the reaction flux stops at the level of the acyl (penicilloyl, cephalosporoyl, etc.) enzyme intermediate (Fig. 9), thus conferring on the serine DD-peptidases the property to behave as "penicillin binding proteins" (PBPs). These adducts (i.e., acyl enzyme intermediates) are sufficiently stable to be submitted to gel electrophoresis in the presence of sodium dodecylsulfate. If [^{14}C] or [^{3}H]-benzylpenicillin (or another radioactive β-lactam) is used, fluorography of the gels permits visualization and quantitation of the DD-peptidases in the form of PBPs.

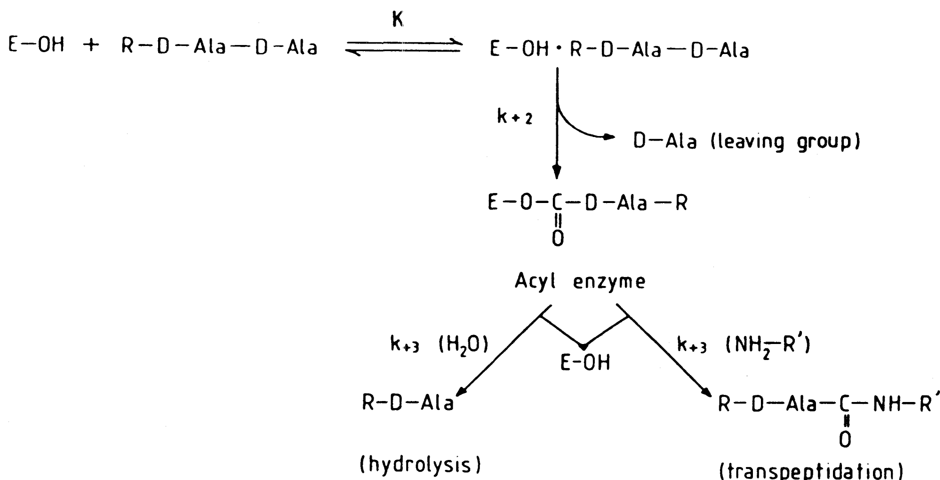


Figure 8 Mechanism of action of the serine DD-peptidases (E-OH) on D-Ala-D-Ala-terminated carbonyl donor peptides.

The reason for the abortive reaction between β-lactams and serine DD-peptidases is that the scissile amide bond of the β-lactam ring is endocyclic (Fig. 9). What should be regarded as the leaving group of the enzyme acylation step cannot leave the enzyme active site, which, therefore, remains occupied. Enzyme regeneration, however, may occur so that either the bound acyl moiety is released by transfer to water (in which case the DD-peptidase functions as a classical β-lactamase of very weak efficiency) or that the bound acyl moiety is first fragmented so that the "leaving group" can diffuse away and the active site can undergo deacylation. Whatever the case, enzyme regeneration is a slow or very slow process.

The above model of β-lactam action on the serine DD-peptidases can be represented schematically by the reaction

$$E + I \overset{K}{\rightleftharpoons} E\cdot I \xrightarrow{k_{+2}} E\text{-}I^* \xrightarrow{k_{+3}} E + \text{reaction products} \qquad [1]$$

where E = DD-peptidase; I = β-lactam; E-I* = acyl enzyme; K = dissociation constant; k_{+2} and k_{+3} = first-order reaction rates. Thus it follows that the higher the bimolecular rate constant of enzyme acylation (k_{+2}/K) and the smaller the rate constant of enzyme deacylation k_{+3} (whatever the underlying mechanism), the more potent a β-lactam as a DD-peptidase inactivator, or the higher the capacity of a β-lactam of immobilizing a DD-peptidase in the form of a PBP. Depending on both the DD-peptidase and the structure of the β-lactam, the

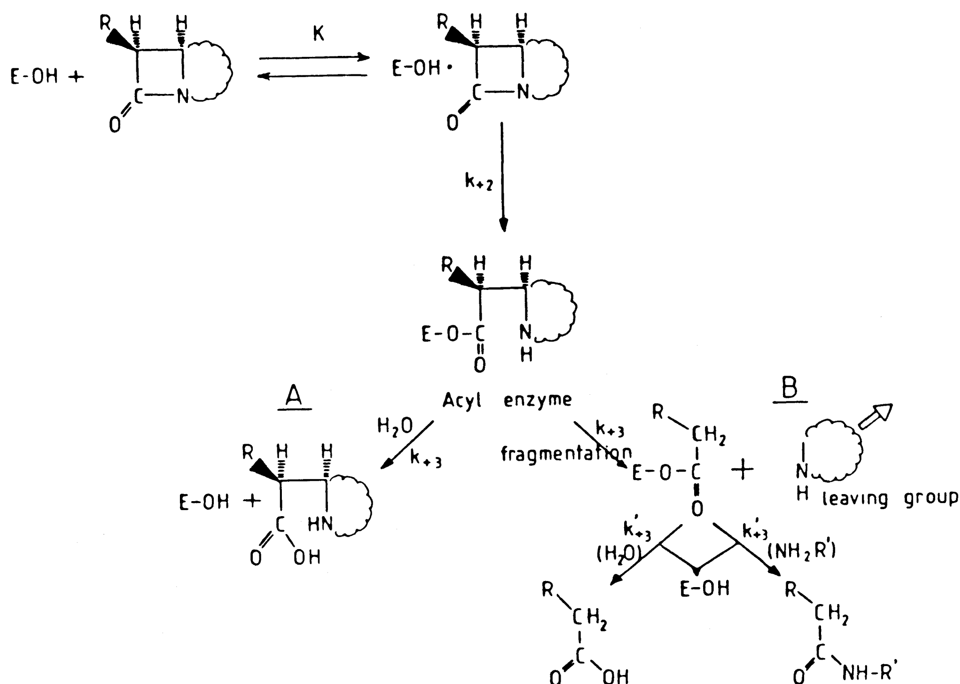


Figure 9 Mechanism of action of the serine DD-peptidases (E-OH) on β-lactams. (A) Acyl enzyme breakdown occurs by direct release of the acyl (penicilloyl, cephalosporoyl, etc.) moiety. (B) Acyl enzyme breakdown involves prior fragmentation of the acyl (penicilloyl, cephalosporoyl, etc.) moiety. In this pathway, fragmentation (k+3) is rate-limiting.

values of these kinetic parameters vary widely. These observations suggest variations in the geometry and properties of the DD-peptidase active sites and multiple modes of binding of the β-lactams, leading to enzyme-ligand associations of highly varying complementarity and productiveness.

C. Penicillin Binding Proteins (PBPs)

Fluorography of the gels obtained by SDS electrophoresis of bacterial plasma membranes previously exposed to a radioactive β-lactam shows that all bacteria possess a set of membrane-bound proteins that bind penicillin and behave as PBPs [11,13]. Very few of these proteins have been characterized as serine DD-peptidases but, as an extension of these studies, it is assumed that, in all cases, penicillin binding is by acylation of a serine residue, implying that all PBPs are serine DD-peptidases (and function as shown in Figs. 8 and 9).

The interactions between PBPs and β-lactams have been studied at least superficially. Most often, the "affinity" of a radioactive β-lactam for a PBP is roughly expressed as the antibiotic concentration, which under given conditions of temperature, pH, and duration of the incubation, leads to 50% of maximal binding. If the β-lactam is not available in a radioactive form, the "affinity" relates to the concentration which is necessary to inhibit by 50% the binding of, most often, radioactive benzylpenicillin. With those PBPs that have been characterized as DD-peptidases, "sensitivity" to a β-lactam is expressed as the β-lactam concentration that causes 50% inhibition of the enzyme activity.

Using these techniques, the PBP patterns (number, apparent molecular weight, relative abundance, thermostability and protease sensitivity, "affinity" for β-lactams, stability of the adducts formed, sensitivity of enzymatic activity) have been determined for many bacterial species. However imprecise they may be, these studies have led to valuable conclusions. One of them is that the PBP pattern is highly species specific, and great variations are observed between taxonomically unrelated bacteria.

All the enterobacteria possess at least seven PBPs that are usually qualified as high-molecular-weight PBPs (PBPs 1A, 1Bs, 2, and 3) and low-molecular-weight PBPs (PBPs 4, 5, and 6) [14]. Because of the close similarity in the PBP patterns, it is thought that the individual corresponding PBPs in all enterobacteria catalyze similar reactions and perform similar physiological functions. In *E. coli*, detailed biochemical and genetic studies [13] have shown that the high-molecular-weight PBPs play specific functions in wall elongation (PBPs 1A and 1Bs), rod shape maintenance (PBP2), and initiation of cell septation (PBP3), respectively. PBPs 1A, 1Bs, and possibly PBPs 2 and 3 have been identified as bifunctional transglycosylase-DD-transpeptidase enzymes [15]. In contrast, PBPs 4, 5, and 6 are believed to be involved in processes related to maturation and remodelling of the wall peptidoglycan (see above). They have been identified as monofunctional DD-peptidases performing, to various extents, transpeptidase/carboxypeptidase/endopeptidase activities.

Characteristic features of the PBP pattern in the enterobacteria are the relatively high abundance of the low-molecular-weight PBPs 5 and 6, the high stability of PBP 1Bs to heat and detergent, and the highly specific targeted action of some β-lactams [14]. Thus, mecillinam binds exclusively to PBP2 over a wide range of concentrations (causing generation of osmotically stable round forms) while, in contrast, cefoxitin has an especially low affinity for this PBP2. Azthreonam and to a lesser extent, cefuroxim bind preferentially to PBP3 (and are effective in inhibiting cell division and causing cell filamentation). Cephaloridin has highest "affinity" for PBPs 1A/1B (and is effective in causing spheroplast formation and rapid cellular lysis). PBPs 4 and 5 show relatively high and low "sensitivity," respectively, to several penams such as benzylpenicillin, ampicillin, carbenicillin, and methicillin.

The first direct demonstration that penicillin inhibits peptide cross-linking during wall peptidoglycan metabolism stems from the work of Wise and Park [53] and Tipper and Strominger [16]. In particular [16], it was shown that *Staphylococcus aureus* grown in the presence of sublethal doses of benzylpenicillin accumulates the nucleotide precursor UDP-*N*-acetylmuramyl-pentapeptide in the cytoplasm and has a higher proportion of D-Ala-D-Ala-terminated peptide units and a lower extent of interpeptide cross-linking in the wall peptidoglycan than the control cells grown in the absence of the antibiotic. Since then, inhibition of transpeptidation has been regarded as the foundation stone of the mechanism of penicillin action.

In fact, the picture is much more complex. It is now well known (but not well understood) that PBP inactivation may have multiple effects on each of the bacterial cell envelope integuments, may affect both the peptide and the glycan moieties of the peptidoglycan, and does not necessarily nor always result in an overall decrease of peptide cross-linking. Thus, at those concentrations (30 μg/ml) that in *E. coli* inhibit cell division and induce cell filamentation by impairing septum initiation, benzylpenicillin both enhances the attachment of newly synthesized peptidoglycan units to the wall sacculus by transpeptidation, resulting in an increased degree of peptide cross-linking, and, subsequently, causes a sudden increase of incorporation of lipopolysaccharide in the outer membrane [17]. The same effects are seen with mecillinam at those concentrations (2 μg/ml) that induce conversion of *E. coli* into round cells. But (as estimated by measuring the amounts of anhydromuramic acid present), mecillinam, under these conditions, also reduces the average glycan chain length of the peptidoglycan to about half its normal value [17,18]. Alterations in the phospholipid composition of the plasma membrane in response to changes in the cell wall induced by β-lactams and other wall inhibitors [19-23] show the close interdependency that exists between the peptidoglycan and both the outer and inner membranes. Finally, complications in the interpretation of the observed phenomena also arise from the fact that, under partial PBP inactivation, PBPs left intact may compensate for the loss of other PBPs [13].

III. THE CELL ENVELOPE OF THE ROD-SHAPED *PROTEUS*

Basically, the above description of the cell envelope of the gram-negative enterobacteria applies to *Proteus* spp. But there are peculiarities.

A. Peptidoglycan

The peptidoglycan of proteus possesses a remarkable marker (Fig. 10) which is that more than half of the N-acetylmuramic acid residues are substituted by an ester-linked O-acetyl group on C6 [24]. Non-O-acetylated peptidoglycan

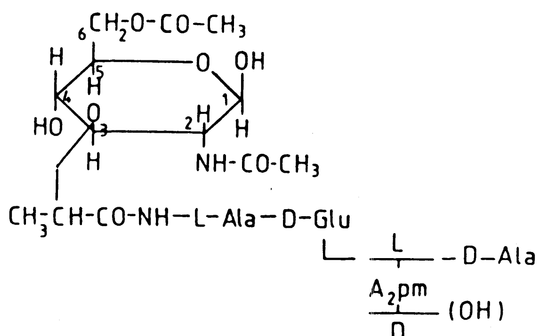


Figure 10 Tetrapeptide-substituted di-O-acetylmuramic acid.

disaccharide peptide units are incorporated in the growing sacculus and it is at a later stage that part of them become O-acetylated. O-acetylation is accompanied by an increase in peptide cross-linking and there is suggestive evidence that a peptidoglycan strand (or strand section) has to be properly cross-linked before acetylation can occur. The presumed O-acetylase (which, so far, has not been characterized) seems not to discriminate between N-acetylmuramic acid residues on the basis of their peptide substituents. Enzymatic degradation of the glycan strands into disaccharide units yields all possible O-acetylated and non-O-acetylated disaccharide-L-Ala-D-Glu, disaccharide-tetrapeptide, bis-disaccharide peptide dimers and tris-disaccharide peptide trimers. O-acetylation, increased peptide cross-linking and generation of a limited amount of L-Ala-D-Glu dipeptides [25] are considered as "maturation" processes of the wall peptidoglycan.

B. Outer Membrane

The outer membrane of proteus also possesses one interesting marker, which is the occurrence, side by side, of two types of lipopolysaccharides. One of them has much shorter O-specific polysaccharide side-chains attached to the core-lipid A structure and thus is more hydrophobic than the other [26].

Another feature of the outer membrane in proteus is the complete absence in exponential-phase cultures of lipoprotein covalently linked to the peptidoglycan [27]. Stationary cells, however, contain a covalently linked lipoprotein of low molecular weight ($M_r \cong 7200$) in amounts similar to those found in *E. coli* where, irrespective of the growth phase, statistically one lipoprotein is covalently linked to about every tenth to twelfth peptidoglycan unit. The proteus 7200 M_r lipoprotein closely resembles that of *E. coli*. However, it lacks methionine and contains more acidic and fewer basic residues and has a somewhat different distribution of amide- and ester-linked fatty acids [28].

Proteus manufactures at least three other major proteins for export in the outer membrane. On the basis of their ability to form hydrophilic pores in reconstituted membranes, the 39,000 M_r protein and the peptidoglycan-associated 36,000 M_r protein appear to be porins [29]. (The term "peptidoglycan-associated protein" is meant to emphasize that separation of the protein from the peptidoglycan requires drastic treatments such as extraction with SDS at 100°C.) In turn, the peptidoglycan-associated, 15,000 M_r protein mediates little permeation of low-molecular-weight solutes and has been characterized as a second lipoprotein [30]. This latter lipoprotein might play an important role in outer membrane assembly. This suggestion seems to be especially pertinent since proteus, which lacks the covalently linked lipoprotein of low molecular weight in exponentially growing cultures, contains, when compared with *E. coli*, much larger quantities of the peptidoglycan-associated lipoprotein of high molecular weight. It has also been hypothesized that *O*-acetylation might confer increased hydrophobicity to the peptidoglycan, thus facilitating its association with the inner leaflet of the outer membrane [27].

C. PBPs and DD-Peptidases

The PBP pattern in *Proteus* spp. is very similar to that of *E. coli* [31] and the corresponding PBPs in these two organisms are thought to play similar functions. Using saturating concentrations of [^{14}C]benzylpenicillin, the relative abundance of the PBPs in *Proteus vulgaris* (Fig. 11) is about 14-15% for PBP1A and PBP1B, 3-5% for PBPs 2, 3, and 4, and 59% for PBPs 5/6 [32].

PBPs 4 and 5 have been isolated from both *P. mirabilis* [33] and *P. vulgaris* [32]. In vitro and on peptide substrate analogues, PBP4 ($M_r \cong 48{,}000$) functions as a carboxypeptidase and, to a lower extent, as a transpeptidase. It also performs endopeptidase activity. PBP4 shows high sensitivity to several penams. The half-inhibitory concentration of benzylpenicillin is about 0.01-0.05 μM and the penicilloyl enzyme "intermediate" has a long half-life of about 200-300 min (which corresponds to a k_{+3} value of Equation [1] of about $0.5 \times 10^{-4} s^{-1}$). Enzyme regeneration is by fragmentation of the bound penicilloyl moiety with formation of phenylacetylglycine (Fig. 9B).

Under similar conditions, PBP5 ($M_r \cong 43{,}000$) shows no endopeptidase activity, may perform low transpeptidase activity (at least the *P. mirabilis* enzyme) and functions essentially as a carboxypeptidase. It exhibits relatively low sensitivity to benzylpenicillin (half-inhibitory concentrations, 1-4 μM) and forms a short-lived benzylpenicilloyl enzyme "intermediate" (half-life, 7-10 min). Enzyme regneration proceeds through the direct release of penicilloate so that PBP5 behaves as a β-lactamase of very low efficacy (Fig. 9A).

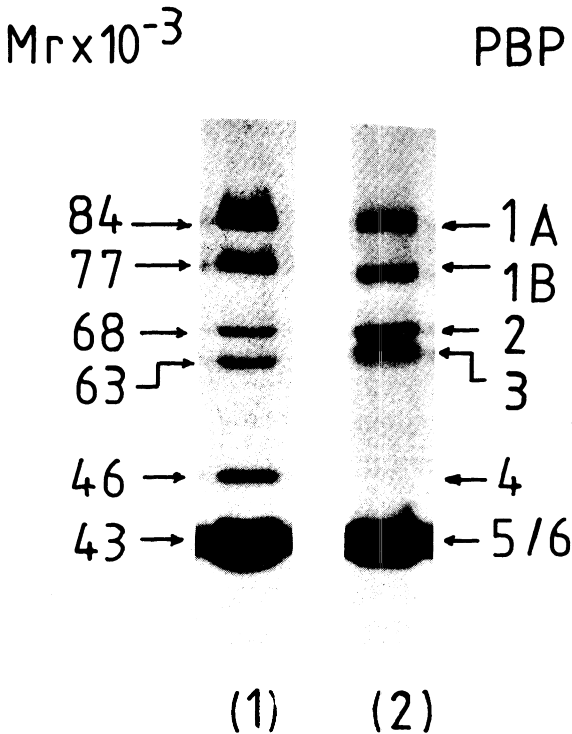


Figure 11 PBP patterns of *Proteus vulgaris* P18 (1) and the corresponding stable protoplast L-form (2).

IV. THE CELL ENVELOPE OF β-LACTAM-INDUCED L-FORMS OF *PROTEUS*

A. Conversion of *Proteus* to L-Form Growth

Benzylpenicillin converts the rod-shaped proteus cells into filaments at low antibiotic concentration (1 μM) and into osmotically fragile spheroplasts at higher antibiotic concentration (15 μM) (Fig. 12a–f). Prolonged treatment results in cell lysis. In spite of this, proteus is notable for its ability to escape, under certain conditions, the lethal consequences of benzylpenicillin action by making a transition to peculiar growth types called L-forms.

As defined by H. H. Martin [34], L-forms qualify as "all artificially induced or spontaneously arising aberrant growth states of previously normal bacteria, where multiplication occurs in the form of fragile, osmotically sensitive cells, mostly of spherical shape, but often also pleomorphic, resulting from the

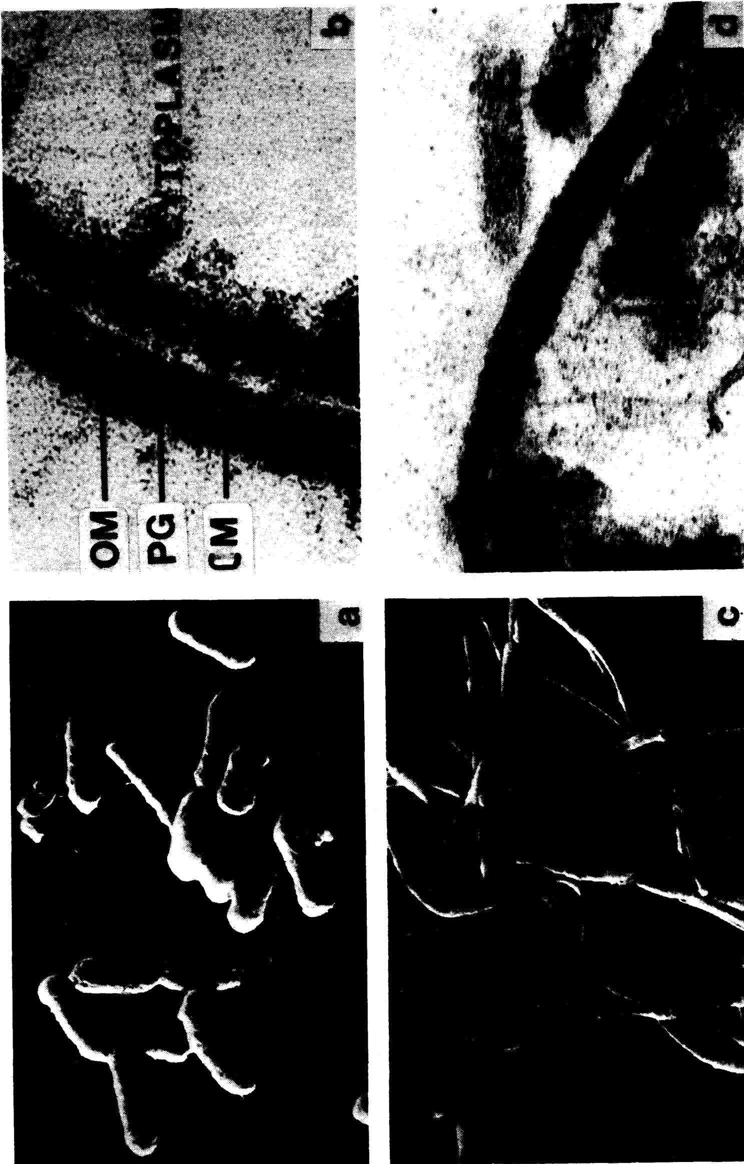
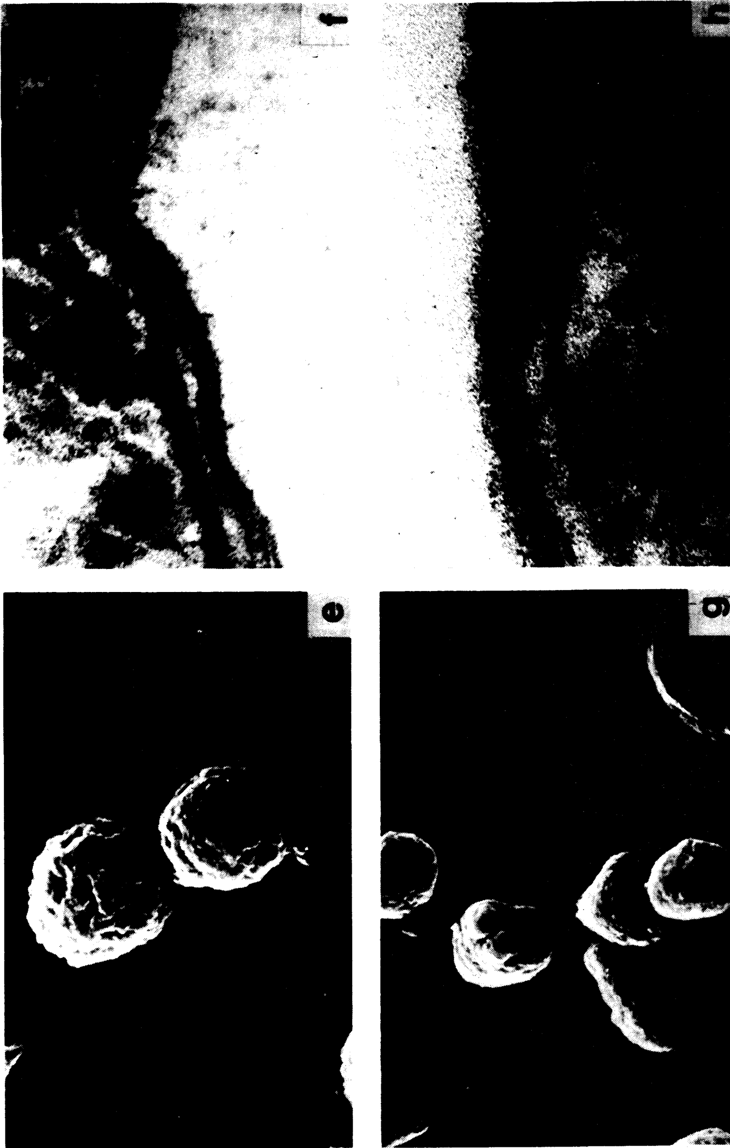


Figure 12 Morphology of *Proteus vulgaris* P18 and effects caused by growing the cells in the presence of benzylpenicillin and mecillinam. Scanning (*a,c,e,g*) and thin-layer (*b,d,f,h*) electron microscopy of: normal bacteria (*a* and *b*); filaments induced by ≅ 1 μM benzylpenicillin (*c* and *d*); spheroplasts induced by ≅ 15 μM



benzylpenicillin (*e* and *f*); ovoid cells induced by ≅ 15 μM mecillinam (*g* and *h*). Magnifications: × 1250 in *c*, × 10,000 in *a*,*e*, and *g*; × 181,200 in *f*; × 239,400 in *d*; × 350,000 in *h*; and × 400,000 in *b*. OM = outer membrane; PG = peptidoglycan; CM = cytoplasmic membrane.

response of a pliable cell surface to external forces." Osmotic fragility relates to high (although varying) sensitivity to mechanical stress (fragmentation can be achieved by simple freezing and thawing), osmotic shock (dilution of an L-form shake culture into distilled water causes rapid lysis), and to the dissolving action of anionic detergents such as SDS. Normal proteus cells are unimpaired by these treatments.

Proteus may give rise to two main types of L-form survivors to β-lactam action. The unstable spheroplast L-forms, on the one hand, retain portions of a defective cell wall and can revert to the normal, rod-shaped bacteria upon removal of the β-lactam from the culture medium. The stable protoplast L-forms, on the other, have the plasma membrane as sole cell integument and do not revert to normal bacteria under any conditions.

Experiments leading to the simultaneous appearance of these two types of L-forms involve, classically, exposure of proteus to a β-lactam in a rich complex agar medium supplemented with an osmotic stabilizer (0.5 *M* sucrose; 20% v/v NaCl or 4% w/v polyvinylpyrollidone) and 5-10 vol% of defibrinated horse serum. Colonies of protoplast L-forms are small (diameter, 0.2-0.3 mm), compact, and exhibit a granular appearance. Colonies of spheroplast L-forms are 5-10 times larger and have a typical "fried-egg" appearance. Subcultures on solid media and, eventually, liquid shake cultures, in the absence of serum and with only little osmotic stabilization, can then be achieved.

In principle, the above procedure applies to many gram-negative bacteria. But, the difficulty and percentage of failures greatly vary depending on the cases. There is no general rapid and simple way of obtaining β-lactam-induced spheroplasts and protoplast L-forms from these bacteria. *P. mirabilis*, however, is a remarkable exception at least when the goal pursued is only the exclusive preparation of homogeneous populations of unstable spheroplast L-forms. In this case, the presence of an osmotic stabilizer and of serum supplement in the primary cultures can be omitted. Furthermore, with some β-lactams including benzylpenicillin (but not with all of them), spheroplast L-form growth can be initiated by direct inoculation of *P. mirabilis* into liquid medium supplemented with the selected β-lactam. *P. vulgaris*, however, is much more restricted in this respect (for more information, see Ref. 34).

B. Unstable Spheroplast L-Forms of *P. mirabilis*

Established liquid shake cultures of unstable spheroplast L-forms of *P. mirabilis* can be obtained with almost every available β-lactam (although, in some cases, conversion to L-form growth is possible only on agar medium), with final titers, at the stationary phase, of 2×10^8 to 3×10^8 colony-forming units. The average doubling time during exponential growth varies depending on the β-lactam. Unstable L-forms grow with a generation time of 45 min with cefoxitin, 60 min

with benzylpenicillin, and 90 min with amoxycillin and ampicillin (instead of 30 min for the original bacterial cells in the absence of β-lactam [34].

1. Benzylpenicillin-Induced Spheroplast L-Forms

One way to readily obtain such L-forms is, for example, to inoculate agar slants of suitable medium containing 120 μg of benzylpenicillin/ml with 2×10^8 stationary-phase cells of *P. mirabilis*. Heavy growth can be obtained after incubation for 2-3 days at 37°C; the harvested cells can then be transferred to liquid media containing 120 μg of benzylpenicillin/ml and final adaptation to growth in the presence of high antibiotic concentrations and with a generation time of about 60 min is usually obtained after a few serial transfers.

Morphologically, the benzylpenicillin-induced spheroplast L-forms appear as flagellated spherical cells covered with fimbriae and loosely attached superficial wall materials [35]. Their cell envelope contains both an outer membrane and a peptidoglycan layer; the latter can be isolated after extraction with phenol and further purified with trypsin (as it is done with the normal proteus cells). Hence, a full assortment of wall integuments is present but with defect(s) causing loss of both rod shape and mechanical strength. The induced lesion(s) is (are) unstable; upon removal of benzylpenicillin from the growth medium, reversion to the original rod-shaped bacteria occurs.

When compared with the normal bacteria, the benzylpenicillin-induced spheroplast L-forms have more than one alteration and both the outer membrane and the peptidoglycan layer are affected. The outer layer has reduced amounts of lipopolysaccharide [36], enterobacterial common antigen [37], and total proteins (but still possesses the 40,000 M_r and 36,000 M_r porins) [38]. In turn, the peptidoglycan layer has a slight decrease in the overall extent of peptide cross-linking [39], shows alterations in the tetrapeptide to tripeptide (L-Ala-γ-D-Glu-(L)-meso-A_2pm) pattern [39], and exhibits a large decrease in the degree of O-acetylation of the N-acetylmuramic acid residues [39].

While all the tripeptide units present in the peptidoglycan of stationary phase cells of proteus serve to anchor the lipoprotein molecules, an appreciable amount of free tripeptides occurs in the unstable spheroplast L-forms. If, as suggested above, covalent attachment of lipoprotein to peptidoglycan is catalyzed by a penicillin-insensitive LD-transpeptidase, then one indirect consequence of the benzylpenicillin-induced conversion of proteus to unstable spheroplast L-forms might be the conversion of this LD-transpeptidase into an LD-carboxypeptidase. As shown in Figure 7, utilization of water as acceptor of the transfer reaction causes hydrolysis of the tetrapeptides into tripeptides, preventing lipoprotein attachment. Note that a penicillin-insensitive LD-peptidase that effectively performs hydrolysis of the tetrapeptides can be readily isolated from the bacterial cells where, to all appearances, it is part of the periplasmic compartment [32].

The O-acetylation pattern of the peptidoglycan can be expressed by the ratio of non-O-acetylated to O-acetylated N-acetylmuramic acid residues. The ratio value is about 0.5 in the normal bacterial cells. It is much higher, about 1.3, in the peptidoglycan of the unstable spheroplast L-forms. On the basis of these observations, it has been suggested that in proteus, the peptidoglycan might be built up from different areas of the polymer differing in the O-acetyl content and that, in the unstable spheroplast L-forms grown in the presence of benzylpenicillin, the synthesis of the O-acetyl-rich peptidoglycan is selectively depressed whereas the synthesis of the O-acetyl-low peptidoglycan remains virtually unaffected [39].

2. Effects of Pairs of β-Lactams

The continued synthesis of a peptide cross-linked (but defective) peptidoglycan by the unstable spheroplast L-forms in the presence of benzylpenicillin can be attributed either to some unknown penicillin-insensitive DD-peptidase enzyme system or to the functioning of one or several PBPs that escape complete inactivation by benzylpenicillin. In this latter case, the unstable spheroplast L-forms should possess a reduce assortment of PBPs left in a free form (and thus capable of subsequently binding [^{14}C]benzylpenicillin) and, moreover, complete L-form growth inhibition should be made possible by the addition of a second β-lactam able to complement benzylpenicillin action. As shown below, experimental evidence supports this latter view.

In the continuous presence of a β-lactam, as it occurs during β-lactam-induced spheroplast L-form growth, the percentage of a given PBP which remains constantly present in a free active form is given by

$$\mathrm{PBP_{free}} = \mathrm{PBP_{total}} - \mathrm{PBP_{bound}}$$

with

$$\mathrm{PBP_{bound}} = \mathrm{PBP_{total}} \frac{1}{1 + \frac{k_{+3}}{k_{+2}}\left(1 + \frac{K}{[\mathrm{I}]}\right)} \qquad [2]$$

where K, k_{+2}, and k_{+3} are the constants of Reaction [1] and [I] is the β-lactam concentration which is actually accessible to the PBP. This latter value depends on both the ease with which the β-lactam permeates through the outer membrane and the competition between the various PBPs present for the amount of β-lactam available. Assuming [I] = 100 μM; K = 10 mM; $k_{+2} = 1\ \mathrm{s^{-1}}$ and $k_{+3} = 3 \times 10^{-3}\ \mathrm{s^{-1}}$ (which corresponds to a half-life of the bound PBP of about 5 min), then 22% of the PBP under consideration remains in a free form all the time. Under identical conditions but with $k_{+3} = 3 \times 10^{-4}\ \mathrm{s^{-1}}$ (half-life = 38 min), only 3% of the PBP remains free.

Many β-lactams other than benzylpenicillin induce good spheroplast L-form growth but, unfortunately, the exact resulting biochemical lesions are not known in most cases and the available data do not permit quantitation of the PBPs left in a free form on the basis of Equation [2]. Nevertheless, with, for example, the pair benzylpenicillin-cefoxitin, it is known [40] that PBP4 forms an adduct (i.e., PBP_{bound}) that is very stable in the case of benzylpenicillin (half-life, 300 min) but short-lived in the case of cefoxitin (half-life, 14 min). Conversely, PBPs 5/6 form adducts that are very stable in the case of cefoxitin (half-life, 900 min) but short-lived in the case of benzylpenicillin (half-life, 7 min). It is therefore not surprising (as the experience shows) that benzylpenicillin-induced L-forms contain no free PBP4 but appreciable amounts of free PBPs 5/6, together with free PBP2. Similarly, the cefoxitin-induced L-forms (which also contain a normally cross-linked peptidoglycan) grow and divide with appreciable amounts of free PBPs 4 and 2 but vastly reduced quantities of free PBPs 5/6 and some high-molecular-weight PBPs.

Benzylpenicillin and cefoxitin complement each other at the level of the PBPs. Most likely, they also complement each other at the level of the lesions that each of them induces in the cells. Following this expectation, benzylpenicillin and cefoxitin, in combination, effectively inhibit spheroplast L-form growth, and, remarkably, cause a 50% decrease of peptide cross-linking in the peptidoglycan [40].

Following the above approach, many β-lactams were tested to find other suitable pairs of compounds that would complement each other to achieve complete and permanent inactivation of all essential targets in *P. mirabilis*. Most of the β-lactams did not show complementation. However, the pair ampicillin-cefoxitin was found to be very effective (H. H. Martin, personal communication), and the pair benzylpenicillin-mecillinam deserves special attention.

The amidinopenicillin, mecillinam, binds exclusively to PBP2 over a wide range of concentrations. When exposed to mecillinam, *Proteus* is massively converted into large spherical cells [39]. These cells remain resistant to osmotic shock (and thus cannot be regarded as L-forms), exhibit a multilayered cell envelope (Fig. 12.g–h), and possess a peptidoglycan that has virtually the same extent of peptide cross-linking and the same degree of O-acetylation as those found in the normal bacteria [39]. By analogy with similar studies carried out on *E. coli* [17], one may assume that the peptidoglycan has glycan strands of reduced length. No growth inhibition or cell death occurs at mecillinam concentrations up to 100 μg/ml, and shake cultures of spherical cells grown in the presence of 15 μg/ml divide with a generation time of 54 min. The mecillinam-induced spherical cells are unstable. Upon removal of mecillinam from the medium, reversion to rod-shaped normal bacteria occurs.

Mecillinam action results in profound morphological alterations of the cells but does not produce any apparent striking biochemical lesions. In particular, it

does not cause the extensive shift in the O-acetylation pattern observed with benzylpenicillin. Nevertheless, mecillinam stops growth of benzylpenicillin-induced spheroplast L-forms (presumably by inactivating the PBP2 left in free form in these organisms) in a rather spectacular manner. Upon addition of 15 μg of mecillinam/ml to a shake culture of spheroplast L-forms growing logarithmically in the presence of 120 μg benzylpenicillin/ml, one observes a further two- to fourfold increase in cell density, conversion of the L-forms into nongrowing, gigantic spherical cells with a diameter of 20 μm, and (as observed with the pair benzylpenicillin-cefoxitin) a drastic decrease of peptide cross-linking in the peptidoglycan [39].

Study of the effects of suitable pairs of β-lactams shows that the O-acetyl groups in proteus are important markers of peptidoglycan synthesis. O-acetylation is also a crucial event in other gram-negative and gram-positive bacteria. Thus, similar to the situation found in proteus, *Neisseria gonorrhoeae* (which has only three major PBPs and where acquisition of intrinsic resistance to β-lactams is accompanied by stepwise decreases in the penicillin "affinity" of PBPs 1 and 2) possesses O-acetyl groups in its peptidoglycan [41]. Examination of isogenic sensitive and resistant strains shows that the primary effects of benzylpenicillin at the corresponding growth inhibitory concentrations differ strikingly depending on the strains. The effect is only a slight change in peptide cross-linking but a sharp decline in the degree of O-acetylation in the penicillin-sensitive gonococcus. In marked contrast, it is a very moderate change in O-acetylation but a substantial decline in cross-linking in the penicillin-resistant gonococcus. On the basis of the extent of saturation of the individual PBPs found under these conditions, it has been suggested that (1) PBP2 has a role in controlling (directly or indirectly) the degree of *O*-acetylation; (2) PBP1 is probably implicated in the transpeptidation reaction of the peptidoglycan; and (3) PBP3 probably fulfils a "maturation" function (analogous to the *E. coli* low-molecular-weight PBPs). Mention should also be made of more recent investigations revealing the presence of a secondary transpeptidase in gonococci that appears to be insensitive to penicillin action [41]. The possible function of this enzyme system during prolonged exposure to penicillin has not yet been reported.

C. Stable Spheroplast L-Forms of *P. mirabilis*

Prolonged maintenance of the benzylpenicillin-induced, unstable spheroplast L-forms in a growing state is probably not feasible. Indeed, the longer the subcultivation period in the presence of benzylpenicillin, the higher the proportion of spheroplast L-forms that lose their ability to revert to normal bacteria and, concomitantly, acquire resistance to the combined action of benzylpenicillin and mecillinam. On the basis of this double resistance to benzylpenicillin

and mecillinam, stable spheroplast L-forms can be detected in freshly prepared cultures of unstable spheroplast L-forms already after three or four transfers [39].

When compared with the unstable spheroplast L-forms, the stable spheroplast L-forms exhibit a much more sharply defined surface which is sparsely covered with slender fimbriae (but without any loosely attached material) [35]. They also show a classical multilayered cell envelope from which a normally peptide cross-linked peptidoglycan can be isolated as a defined entity but they contain higher amounts of both lipopolysaccharide and proteins (although in lesser amounts than in the normal bacteria). These properties suggest a tighter organization of the outer membrane. In spite of this, the stable spheroplast L-forms remain mechanically and osmotically sensitive.

How the originally reversible lesions present in the unstable spheroplast L-forms are perpetuated as hereditary defects, with concomitant acquisition of mecillinam resistance, in the stable spheroplast L-forms is a question that remains to be explored. The process is best explained as the spontaneous appearance in the benzylpenicillin-induced spheroplast L-forms population of mutants that rapidly accumulate by a still unknown selective mechanism [39]. Chemically induced and frequently appearing spontaneous mutants of *E. coli* have been described showing mecillinam resistance and, depending on the cases, spherical or rod cell shape.

D. Stable Protoplast L-Forms of *P. mirabilis* and *P. vulgaris*

Conversion to stable protoplast L-form growth is the ultimate response of proteus to penicillin action. The stable protoplast L-forms are wall-less, ovoid organisms (Fig. 13). They grow in the absence of extensive osmotic stabilization or other protective measures, do not reverse to normal bacteria under any conditions, and show complete resistance to β-lactams, including mecillinam, alone or in combination. Their cell envelope consists of one single membrane that, in thin sections, appears as two layers of irregular thickness. They exhibit a smooth surface and possess multiple flagella. The protoplast L-forms do not divide at regular intervals (perhaps because of the missing wall) so that the resulting unbalanced growth yields cells of different sizes (0.1–10 μm in diameter), including the so-called small bodies (0.1-0.4 μm). Vigorous growth in liquid shake culture can be obtained with generation times of about 2 hr but propagation of individual protoplasts is erratic and often abortive.

The first stable protoplast L-form was isolated by Tulasne in 1949 as a benzylpenicillin-resistant organism originated from *P. vulgaris* [42]. Since then, these organisms have been subcultured twice a week in suitable growth medium in the absence of penicillin. Soon after Tulasne, Kandler and Kandler [43] isolated similar stable protoplast L-forms from strains of *P. mirabilis*. Propagation

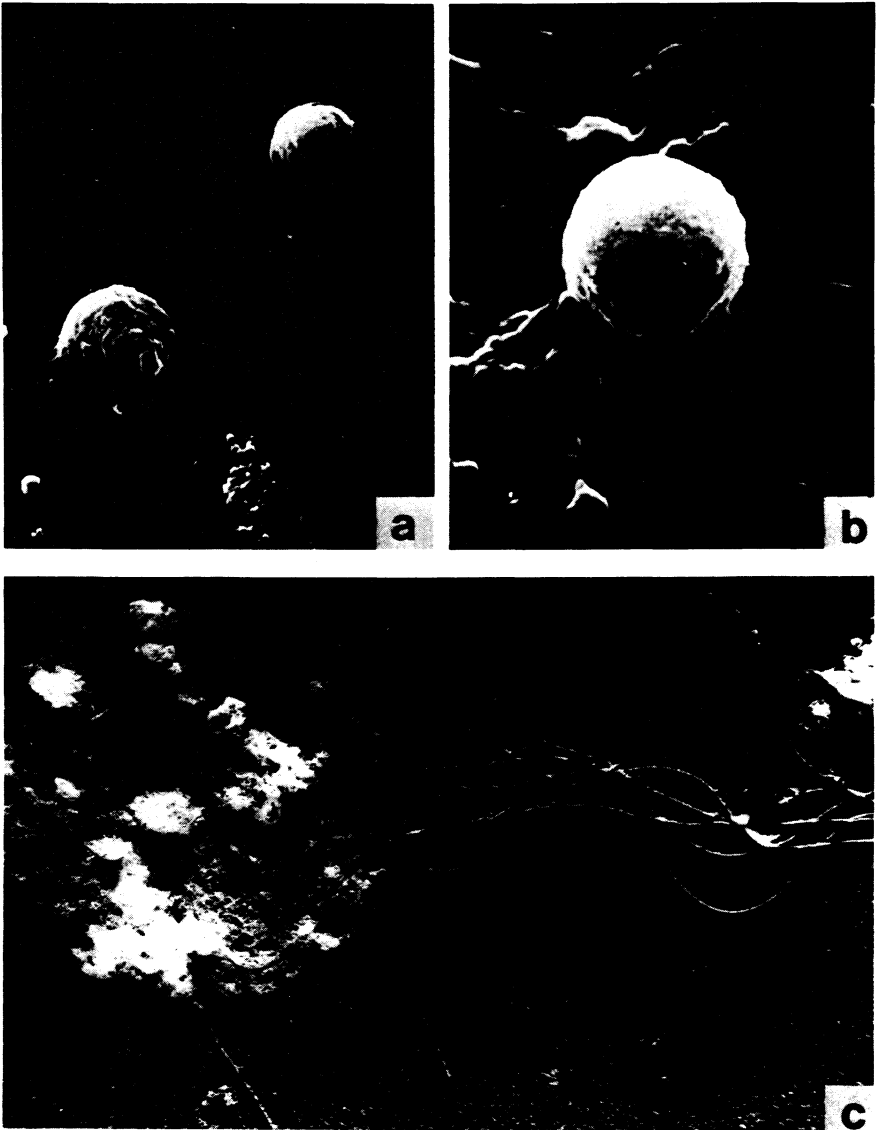


Figure 13 Morphology of the stable protoplast L-forms derived from *Proteus vulgaris* P18. Scanning (*a,b*), metal shadowing (*c*), and thin-layer (*d,e*) electron microscopy. Magnifications: × 10,000 in *a*; × 20,000 in *b*; × 24,000 in *c*; × 60,000 in *e*; × 400,000 in *d*.

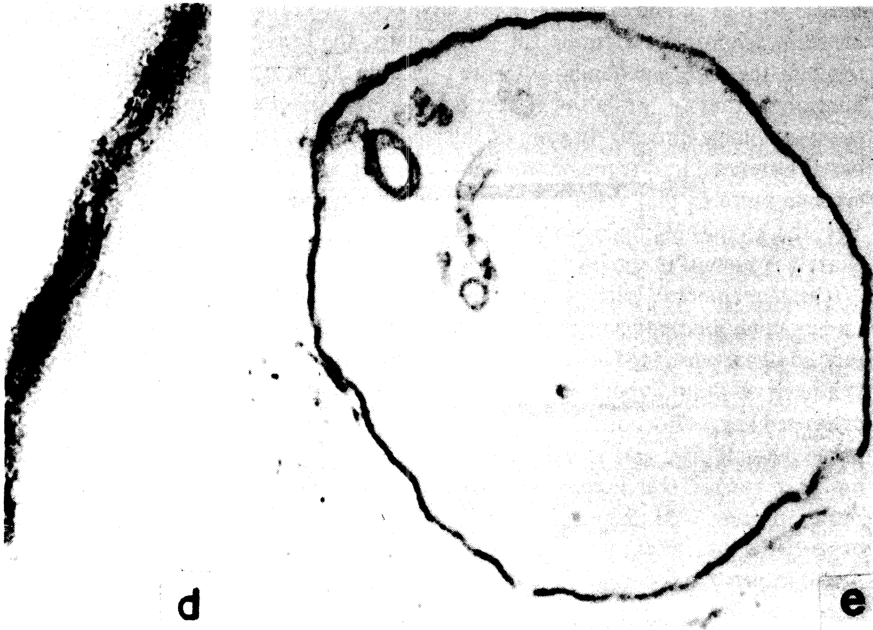


Figure 13 (Continued)

of a freshly induced protoplast L-form requires the addition of an unknown growth factor in the form of 5-10% horse serum to the culture medium. Established protoplast L-forms can then be adapted to life without serum.

1. Heterogeneity of the Membranous Structures

Thin sections of the stable protoplast L-forms reveal a great variety of different membranous structures [38] and this heterogeneity is also reflected in the complex pattern obtained when the membranes are fractionated by density gradient centrifugation [38] according to the technique of Osborn et al. [44]. The reason for this heterogeneity is not known. Separation into cells of different sizes and analysis of the isolated fractions might clarify the situation.

When submitted to fractionation by density gradient centrifugation, the membranes of the protoplast L-forms (of *P. mirabilis*) do not yield predominant banding at that low density where a cytoplasmic membrane is expected. Instead, they give rise to a multiple-banded pattern of "heavy" and "light" fractions,

similar to that obtained with the cell envelopes of normal proteus or the unstable spheroplast L-forms (in which cases, the heavy band essentially contains the outer membrane with the typical major proteins, lipoproteins and lipopolysaccharide, while the lighter bands are considerably enriched in the cytoplasmic membrane). In spite of this unexpected behavior, all heavy and light bands generated by the membranes of the protoplast L-forms have protein and enzyme patterns that essentially are those of a cytoplasmic membrane [38]. Yet, the 15,000 M_r lipoprotein is found in the heavy band and lipopolysaccharide is present in the light bands.

Outer membrane (and periplasmic) proteins are known to be produced in membrane-bound polysomes in the form of a precursor that contains a characteristic signal peptide. This signal peptide is processed during or shortly after synthesis of the polypeptide. Depending on the proteins, evidence has been presented suggesting cotranslational or posttranslational transfer across the cytoplasmic membrane, and, in the case of the 7200 M_r lipoprotein, the possibility has been evoked that maturation can occur only on a glyceride-containing prolipoprotein [45]. Why among all the major proteins and lipoproteins that are exported to the outer membrane in *Proteus* only the 15,000 M_r lipoprotein is found sequestered (perhaps in a nonprocessed form) in the membranes of the stable protoplast L-forms, is not known.

In the gram-negative enterobacteria, the O-side chains of the lipopolysaccharide are synthesized in the plasma membrane independently of the other portions of the polymer. This synthesis involves the same C55 undecaprenyl–phosphate lipid carrier as that involved in peptidoglycan synthesis and the reactions sequence is analogous to that leading to the formation of the peptidoglycan disaccharide peptide units. Transfer of the polymerized O-side chains to the preformed core-lipid A then takes place within the plasma membrane itself. After the two components are joined, the resulted complete lipopolysaccharide molecules are translocated into the outer membrane through the adhesion sites mentioned above and distributed over the entire surface of the cell within the outer leaflet of the outer membrane [6]. In the protoplast L-forms, the above synthesis and assembly reactions are still in operation but with defects so that incomplete lipopolysaccharide molecules with short side chains largely predominate. In addition, these molecules can no longer be exported and necessarily remain associated at their site of assembly. Their exact location is unknown, but they apparently do not give the membrane an asymmetrical character similar to that found in the outer membrane of the normal bacteria.

2. Physical Stability and Fluidity

Central to the problem of the protoplast L-forms is how the membrane has enough physical stability to permit vigorous growth under conditions of minimal

osmotic stabilization and, at the same time, has a sufficient degree of fluidity in order to maintain normal "plasma membrane" functions [46].

It is known that lipopolysaccharide exerts a stabilizing action on phospholipid model membranes. Lipopolysaccharide thus may also exert a protective function in the membranes of the protoplast L-forms (and impart to them an increased viscosity). In addition, preferential synthesis of the more hydrophobic lipopolysaccharide molecules with short O-side chains may also provide for increased stability of the membrane.

In turn, membrane fluidity probably occurs by a shortening of the saturated fatty acids in both the lipopolysaccharide and phospholipid molecules (and not by an increased incorporation of unsaturated fatty acids, which would be the alternative for gaining more fluidity). Thus, while in the lipopolysaccharide of the normal bacteria, tetradecanoic, hexadecanoic, and 3-hydroxytetradecanoic acids occur in the molar ratio of 5:1:6, the same fatty acids in the lipopolysaccharide of the protoplast L-forms occur in the ratio 5:0.1:6 [46]. Similarly, while the quantitative and qualitative composition of the phospholipids is very similar in the normal bacteria and the protoplast L-forms (the range of mole percentages of phospholipid species is 76–80 for phosphatidylethanolamine, 10–13 for phosphatidylglycerol, 4–5.5 for diphosphatidylglycerol, and 1–2 for lysophospholipid), all phospholipid species in the L-form differ from those of the normal bacteria by a lower content of long-chain fatty acids and a higher content of short-chain fatty acids [46].

The effect of horse serum as a "growth factor" in the early propagation of freshly induced protoplast L-forms is not understood. Short fatty acids occur to a lesser extent in the lipids of the protoplast L-forms grown in the presence of serum than in its absence and lipids present in the serum appear in the membranes of the protoplast L-forms [46]. Cholesterol, for example, is taken up from the serum and incorporated in the membranes. However, it is unlikely that the incorporated cholesterol fulfils any essential role since cholesterol-containing protoplast L-forms do not show any increased sensitivity to amphotericin B or digitonin [46]. It may be that some of the serum lipids are temporarily needed for the initial conversion of the bacteria to the L-form life. However, none of them is essential for the maintenance of established protoplast L-forms.

3. PBPs and Inability to Revert to Normal

It has been reported (W. Hammes; cited in Ref. 34) that proteus protoplast L-forms synthesize the peptidoglycan cytoplasmic precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide. In addition, their membranes are known to possess a normal PBP pattern [32,47] (Fig. 11) except for the lack of PBP4 which (as shown with *P. vulgaris*) is excreted in the culture medium during growth. Finally, PBP4 and PBP5 have been isolated and shown to perform the same

enzyme activities and to exhibit the same sensitivities to β-lactams as the corresponding PBPs of the normal bacteria [32,33,48,49].

We have seen that the benzylpenicillin-induced unstable spheroplast L-forms continue to manufacture a cross-linked wall peptidoglycan although they lack free PBP4. In addition, mutants of *E. coli* lacking PBP4 grow normally under a wide range of laboratory conditions. It thus follows that the defect for the hereditary and permanent inability of the proteus stable protoplast L-forms to reverse to walled bacteria is unlikely to be the lack of integration of PBP4 in the plasma membrane.

These protoplast L-forms might have all the enzymes required for peptidoglycan synthesis but, because of lack of wall "primer," glycan strands with uncross-linked peptide substituents might be excreted into the culture medium. This situation would be similar to that found with glycosidase (lysozyme)-induced protoplasts of gram-positive bacteria. Alternatively, the proteus protoplast L-forms might be unable to manufacture the disaccharide peptide units from the nucleotide precursors because of defects at the level of the membrane lipid cycle. In support to this view, one should bear in mind that preferential synthesis of incomplete lipopolysaccharide molecules with short side-chains in these organisms might be due (among other possibilities) to defects in the functioning of the undecaprenyl phosphate carrier and that the same carrier is involved in peptidoglycan synthesis. One may also note that defects in the synthesis of the nucleotide precursors and translocation into the plasma membrane have been reported [50] in the case of some stable L-forms of gram-positive bacteria.

Assuming that the observations made with proteus apply to other bacterial species, then the presence of defined PBP patterns in the stable protoplast L-forms may have important, practical applications. It may help to identify the parental bacterial species. In addition, it may help to distinguish the L-forms from the mycoplasmatales (see Sect. I) since, as shown with *Acholeplasma laidlawii* and several *Mycoplasma* species, these organisms do not contain, even in trace amounts, any protein having the ability to bind penicillin with high affinity [47].

V. CONCLUSIONS

Bacteria have developed several mechanisms to withstand β-lactam antibiotic therapy by raising the antibiotic concentration necessary for killing beyond the level that can be achieved clinically (for recent information, see Ref. 51).

Plasmid-mediated and chromosomally determined β-lactamases very effectively degrade susceptible β-lactams into biologically inactive metabolites by hydrolyzing the β-lactam amide bond.

In the gram-negative bacteria, the β-lactamase molecules are "concentrated" in the periplasmic region. Diminishing the rate of penetration of the β-lactams through the porin channels of the outer membrane is an important mechanism of resistance, at least against those β-lactams that show high susceptibility to β-lactamase. Indeed, their extremely rapid degradation must be counteracted by bringing in new molecules at a very rapid rate.

Alterations of the PBPs is another mechanism of resistance. The case of *Neisseria gonorrhoeae* has already been mentioned. In *Streptococcus pneumoniae*, the shift in PBP pattern involves loss of some PBPs and acquisition of others. Methicillin-resistant staphylococci, when compared with sensitive ones, show, depending on the cases, either a major decrease in the affinity of PBPs 1, 2, and 3, or an affinity change of only PBP3. Changes in PBPs have also been shown in β-lactam-resistant strains of *Pseudomonas aeruginosa*.

The above mechanisms result, in one way or another, in a decreased effectiveness of β-lactam action at the level of the primary targets, the PBPs. However, there is also very strong experimental evidence for "an indirect mode of β-lactam action" in which PBP inactivation is not lethal per se but initiates a chain of secondary events that abolish protein (and RNA/DNA) synthesis and affect the specific activity and/or cellular control of peptidoglycan hydrolases (autolytic enzymes). Alterations at the level of presumed "effector molecules" that normally link the synthesis of the wall peptidoglycan and the synthesis and/or functioning of other essential biopolymers may result to tolerance to β-lactams. Tolerance, as first seen in pneumococci, can be defined as diminished and/or delayed killing by growth-inhibiting concentrations of the antibiotic.

Persistence, a widespread phenomenon among penicillin- and ampicillin-sensitive bacteria including *E. coli*, relates to the survival of a small fraction (about 1 in 10^6 cells) of bacteria that remain viable despite prolonged exposure to bacteriocidal doses of β-lactams. Neither resistance, tolerance, impaired growth, nor reversion of spheroplasts account for persistence. The recent recognition of a gene (*hipA*) in *E. coli* that affects the frequency of persistence after inhibition of peptidoglycan synthesis may shed light on the underlying mechanism [52].

Finally, as shown in the present article, environmental conditions are susceptible to influence profoundly survival of bacteria from β-lactam action. *Proteus* is able to survive in the presence of high β-lactam antibiotic concentrations in the form of wall-deficient spheroplast L-forms if it finds an environment of suitable osmolality. Life under these conditions may not be practicable forever, but these L-forms keep the capacity of reverting to normal bacteria once the inducing β-lactam is removed from the medium. Alternatively, *Proteus* can shift to a permanent life in the form of wall-less protoplast L-forms (whether the β-lactam remains present or not) providing that it finds appropriate lipids in the environment until it can redirect its own lipopolysaccharide and lipid

syntheses to manufacture a plasma membrane with both sufficient physical stability and fluidity.

Studies on these *Proteus* L-forms (remarkably conducted by H. H. Martin, J. Gmeiner, and their colleagues at the Technische Hochschule of Darmstadt) have also greatly contributed to the establishment of important (and "nonorthodox") concepts with respect to β-lactam action. Thus, peptidoglycan exhibiting a correct average extent of peptide cross-linking, but with defects in physical strength, can be synthesized by bacteria possessing only a minor proportion of PBPs left in a free form. Sharp decrease in peptide cross-linking by β-lactam action may be achieved only through the combined action of "complementing" β-lactams. PBP inactivation can induce (directly or indirectly) lesions in components of the bacterial cell envelope other than the peptide moiety of the peptidoglycan. Simple chemical substituents in the peptidoglycan, as the O-acetyl groups in proteus, may be markers of great physiological significance.

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