BACTERIAL OUTER MEMBRANES

Biogenesis and Functions

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Preface

In the last few years research interest in the outer membrane, especially the outer membrane proteins of gram-negative bacteria, has become extremely active. One can easily see this trend in a rapid survey of publications in the *Journal of Bacteriology*: During the years 1971 and 1972, only one paper concerning outer membrane proteins was published, and in the next three years four to five papers appeared per year. However, in 1976 and 1977, the number of publications increased fourfold, and in 1978 more than 20 papers were published.

One of the reasons for this outburst of activity in outer membrane research is that the outer membrane contains several major proteins, which can be easily purified and characterized. Studies on the structure, function, and biogenesis of these proteins have broad implications in many different areas of membrane biochemistry and molecular biology. Furthermore, investigations into the nature of the lipopolysaccharide-covered outer surface of gram-negative bacteria are also important for an understanding of the interaction between bacteria and their environment, such as host animal tissues.

In this book, efforts have been made to illustrate the dynamic aspects of the outer membrane, how its individual constituents are synthesized and assembled in the outer membrane and how they function. It is our good fortune that those scientists in the front line of outer membrane research have contributed chapters to this book. It is my hope that this book will be useful, not only for those who are engaged in outer membrane research, but also for those who have a general interest in membrane biogenesis and function.

Finally, I would like to express my gratitude to Dr. Hiroshi Nikaido for his valuable advice in editing this book and to Dr. Stanley F. Kudzin of State University of New York, New Paltz for his help in the production of this book.

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Chapter 1 What is the Outer Membrane?

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1 INTRODUCTION

At the end of the last century, Christian Gram, a Danish histologist, developed a method of staining bacteria in tissues that is now one of the most valuable and most widely used procedures for bacterial staining (see review by Bartholemew and Miltwer, 1952). Bacteria that are stained with the I_2–crystal violet complex by this method are classified into two groups; gram positive and gram negative. The affinity of crystal violet for bacteria in this staining procedure appears to be associated with the nature of the bacterial envelope, since gram-negative bacteria are known to be more

resistant that gram-positive bacteria to the actions of certain dyes, chemicals, and antibiotics.

In this chapter, I describe the structure and functions of the envelope of gram-negative bacteria and discuss its unique features.

2 STRUCTURE OF THE ENVELOPE OF GRAM-NEGATIVE BACTERIA

As shown in Figure 1, *Escherichia coli*, a gram-negative bacterium, is surrounded by five electron dense tracks in contrast to the typical double-

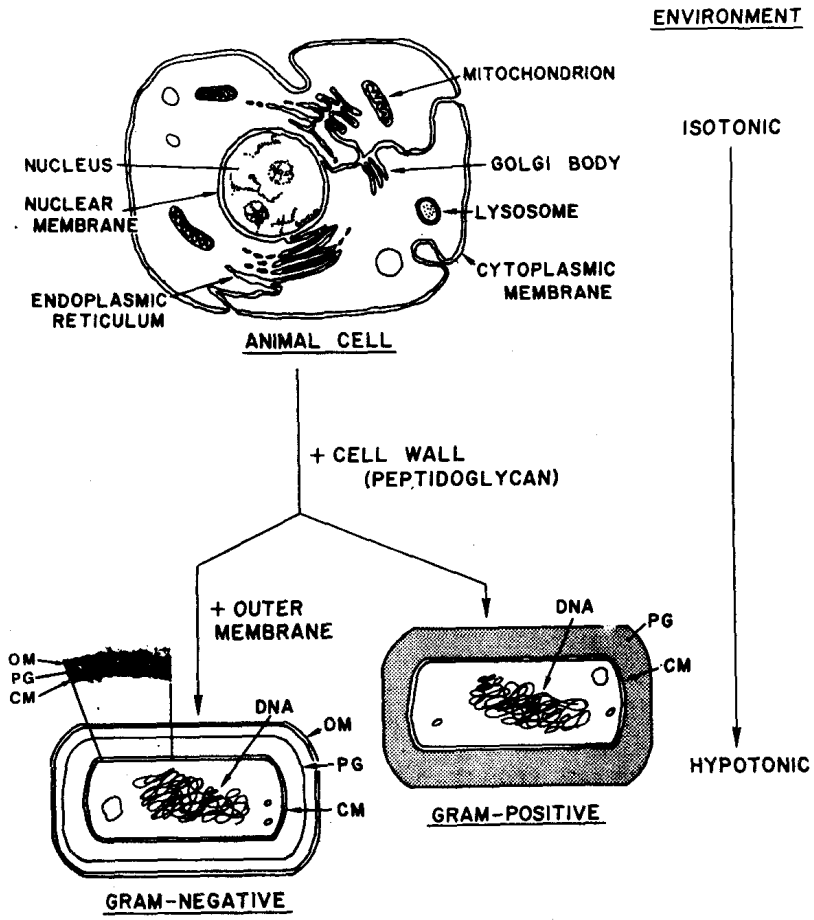

Figure 1 Schematic illustration of the membrane structures of eukaryotic and prokaryotic cells. CM, cytoplasmic membrane; CW, cell wall; OM, outer membrane; PG, peptidoglycan. The ultrastructure of the envelope of the gram-negative cell, in which there are five election dense layers, is shown in the insert.

tracked layer of a unit membrane (Murray et al., 1965; dePetris, 1976). Based on the fact that the central track disappeared after lysozyme treatment of cells, it was concluded that the cell wall or the peptidoglycan layer exists between the two double-track layers, which represent two distinct membrane systems. Both systems consist of a typical unit membrane having a thickness of about 75 Å (Figure 1) (see also review by Glauert and Thornley, 1969). These membranes are called the outer membrane and the inner, or cytoplasmic, membrane. It should be noted that adhesion sites between the outer membrane and the cytoplasmic membrane have been found and their important roles in the assembly of outer membrane components have been demonstrated (see Chapter 6 by Bayer).

On the other hand, gram-positive bacteria such as *Bacillus subtilis* are surrounded by a cytoplasmic membrane and a thick electron dense layer representing the cell wall or the peptidoglycan (about 200 Å), which is exterior to the cytoplasmic membrane (Figure 1).

3 FUNCTIONS OF THE OUTER MEMBRANE

Why is there such a drastic difference in the envelope structure between gram-negative and gram-positive bacteria? Why do gram-negative bacteria have a thin peptidoglycan layer and an outer membrane instead of a thick peptidoglycan layer as in gram-positive bacteria? What are the functions of the outer membrane? To answer these questions let us consider the structure of the envelope of gram-negative bacteria in relation to the membrane systems of animal cells. Also, let us discuss the significance of the cell envelope in regard to the natural habitats.

As shown in Figure 1, animal cells require only the cytoplasmic membrane (plasma membrane) because they live in osmotically isotonic environments. On the other hand, bacteria usually live in hypotonic environments that would cause the lysis of animal cells. To prevent cells from lysing in the hypotonic environment, the bacteria cytoplasmic membranes are surrounded by the cell wall, a rigid network composed of the peptidoglycan. The peptidoglycan is highly cross-linked and the cells are probably surrounded by a single supermacromolecule comprised of peptidoglycan. As can be seen in Figure 1, gram-positive bacteria have very thick multilayered cell walls, whereas gram-negative bacteria have a single layer of peptidoglycan (see Chapter 5 by Mirelman). In both cases, the peptidoglycan layers can be removed by lysozyme, resulting in the formation of spherical cells in an isotonic medium (these spherical cells are called protoplasts and spheroplasts for gram-positive and gram-negative bacteria, respectively). They will lyse, as do animal cells, if they are placed in a hypotonic medium.

The envelope structure of gram-negative bacteria is more differentiated than that of gram-positive bacteria, having an extra membrane system outside the peptidoglycan layer (Figure 1). A similar differentiation of membrane systems can also be seen in animal cells that contain the lysosomal membrane, mitochondrial membrane, endoplasmic reticulum, and nuclear membrane in addition to the cytoplasmic membrane as shown in Figure 1. The functions of the outer membrane are, in part, very similar to those of the eukaryotic lysosomal membrane. The lysosomes are organelles surrounded by a single membrane and are sometimes called "suicide bags" because they contain many hydrolytic enzymes, such as phosphatases, glycosidases, nucleases, proteases, and lipases, are able to hydrolyze all classes of essential components released inside the cells. These enzymes are used to digest that foreign material taken in by the cell, such as bacteria. Bacteria also need these kinds of hydrolytic enzymes for their survival because they have to digest macromolecules or low molecular weight organic compounds that are utilized as nutrients. However, because bacteria do not contain lysosomes, these enzymes must be maintained separately from other components within the cell by other means in order to prevent self-digestion. Gram-positive bacteria simply excrete these enzymes outside the cell, whereas gram-negative bacteria elaborately house these enzymes in the space between the outer and the cytoplasmic membranes. This space is called the periplasmic space and appears to play vital roles in cell growth. The space may include as much as 20–40% of the total cell mass (Stock et al., 1977; see also Chapter 11 by Nikaido) and contains other important proteins, such as specific amino acid and sugar transport binding proteins. These proteins are involved in transport of specific amino acids and sugars through the cytoplasmic membrane. Thus the periplasmic space would appear to be more sophisticated, at least on a functional basis, than the eukaryotic lysosome. As is seen above, one of the functions of the outer membrane is to confine the periplasmic enzymes and proteins to the periplasmic space. At the same time, the outer membrane provides specific and nonspecific channels for those nutrients and ions required for growth (see Chapter 10 by Konisky and Chapter 11 by Nikaido). It should be noted that these nutrients are transported through the outer membrane channels by passive diffusion. After they are passively transported into the periplasmic space they are then actively incorporated into the cytoplasm across the cytoplasmic membrane. The active transport systems for these nutrients are exclusively located in the cytoplasmic membrane. Nonspecific diffusion pores for small hydrophilic molecules have been well characterized and have been shown to consist of a class of major outer membrane proteins (matrix proteins or porins) (see Chapter 11 by Nikaido).

On the other hand, the outer membrane also serves as a selective barrier to the cell exterior. As is described earlier, gram-negative bacteria are more resistant than gram-positive bacteria to the actions of certain dyes, chemicals, enzymes, and antibiotics. This is because gram-negative bacteria have an outer membrane that prevents toxic compounds from entering the cells. It is important, especially for those gram-negative bacteria that comprise the normal intestinal flora of animals (*Enterobacteriaceae*, enteric bacteria), that the outer membrane protects the cytoplasmic membrane from direct exposure to bile salts that would lyse the cells. The most important component in the outer membrane in this regard is the lipopolysaccharide that exists exclusively in the outer membrane (see Chapter 2 by Osborn). Its long polysaccharide chains, extending toward the outside of the cell, not only prevent certain compounds (mostly hydrophobic; see Chapter 11 by Nikaido) from penetrating into the cell, but also play an important role by interacting with natural environments such as animal tissues. Furthermore, lipopolysaccharide is known as endotoxin, the major toxin of pathogenic enteric bacteria.

It should be pointed out that the outer membrane contains many specific receptors for phages and colicins; however, their primary roles have been revealed to be specific receptors and channels for nutrients required for growth (see Chapter 10 by Konisky). Moreover, the outer membrane plays important roles in cell–cell interaction during conjugation (see Chapter 12 by Manning and Achtman) as well as indirect roles in chemotaxis (see Chapter 13 by Hazelbauer).

4 COMPONENTS OF THE OUTER MEMBRANE

Methods to separate the outer membrane and the cytoplasmic membrane have been developed (Miura and Mizushima, 1969; Osborn et al., 1972). In these methods the outer membrane is separated from the cytoplasmic membrane because of its greater density due to the presence of lipopolysaccharide. Other purification methods employ detergents that solubilize the cytoplasmic membrane but not the outer membrane (see review by DiRienzo et al., 1978).

The outer membrane thus isolated is composed of protein, phospholipid, and lipopolysaccharide. In comparison to the cytoplasmic membrane, the outer membrane contains a small variety of proteins in rather large quantities. Therefore, it is easy to purify and characterize these major proteins. The content of phospholipid in the outer membrane is much less than that in the cytoplasmic membrane, and the outer membrane appears to be

enriched in phosphatidylethanolamine in comparison with the cytoplasmic membrane (see Chapter 3 by Cronan). In the wild-type *Salmonella typhymurium* the phospholipid content was found not to be large enough to cover even one side of the lipid bilayer (Smit et al., 1975; Kamio and Nikaido, 1976; see Chapter 3 by Cronan and Chapter 11 by Nikaido). On the other hand, the outer membrane contains a specific component, lipopolysaccharide (see the structure in Figure 1 of Chapter 1 by Ocborn and Figure 2 of Chapter 11 by Nikaido). In the case of *S. typhimurium* it has been calculated that there are approximately 2.5×10^6 molecules of lipopolysaccharide per cell (Smit et al., 1975). These molecules are localized exclusively in the outer leaflet of the outer membrane and occupy about 45% of the surface of the outer membrane.

It should be pointed out that the composition of the outer membrane changes rather drastically as a result of mutation. In "deep-rough" mutants of the lipopolysaccharide, the phospholipid content increases significantly, whereas the protein content decreases drastically without changing the number of lipopolysaccharide molecules per cell (Smit et al., 1975).

Besides those components described above, the peptidoglycan is closely associated with the outer membrane (see Chapter 5 by Mirelman). Some outer membrane proteins are shown to have specific interactions with the peptidoglycan (see Chapter 4 by Halegoua and Inouye), and a part of the lipoprotein, one of the major outer membrane proteins, is actually covalently linked to the peptidoglycan, as is described in the next section.

5 OUTER MEMBRANE PROTEINS

Recently, the outer membrane proteins were extensively reviewed by DiRienzo et al. (1978). Therefore, I present here only a brief description of these proteins to help the reader better understand the other chapters of this book.

5.1 Major Proteins

The definition of major proteins is rather arbitrary. A minor protein may become a major protein when its production is fully induced. However, the outer membrane of wild-type *E. coli* K-12 contains at least three classes of major proteins; matrix proteins, ompA protein (tolG protein), and lipoprotein. Although at the present time a universal nomenclature for the outer membrane proteins has not been agreed upon, the designations matrix protein, ompA protein, and lipoprotein are used throughout this book (the

reader is referred to DiRienzo et al. (1978) for a cross-reference of other nomenclatures).

5.1.1 Matrix Proteins—Porins Matrix proteins are characterized by their tight, but noncovalent, association with the peptidoglycan. One of these proteins was first isolated by an elegant method described by Rosenbusch (1974). He purified, to homogeneity, matrix protein Ia from *E. coli* B. Its molecular weight was calculated as 36,500 and it consisted of a single polypeptide of 336 amino acid residues. On the other hand, *E. coli* K-12 contains matrix protein Ib in addition to matrix protein Ia (see review by DiRienzo et al. (1978); see also Chapter 8 by Reeves). These two proteins appear to be coded for by independent genes, and their relative amounts vary greatly with growth conditions.

A striking features of matrix proteins is their extremely high content of β-structure in contrast to many other "intrinsic" membrane proteins, which show high contents of α-helix (Rosenbusch, 1974; Nakamura and Mizushima, 1976). Electron micrographs of the negative-stained matrix protein–peptidoglycan complex show that the matrix protein molecules are arranged as a hexagonal lattice layer with a 7.7 nm repeat (Rosenbusch, 1974; Steven et al., 1977; see also Chapter 11 by Nikaido). There are about 1.5×10^5 molecules of matrix proteins per cell and the hexagonal lattice structure covers 60% or more of the outer surface of the peptidoglycan (Steven et al., 1977).

Nakae showed that the incorporation of the matrix protein into artificial lipopolysaccharide–phospholipid vesicles greatly enhances their permeability to sucrose (Nakae, 1976a, 1976b; see Chapter 11 by Nikaido). Those vesicles that were reconstituted with the matrix protein showed almost the same molecular sieving properties as the intact outer membrane, which excludes oligo- and polysaccharides with molecular weights higher than 900 (Nakae and Nikaido, 1975). These results indicate that the function of the matrix proteins is to form passive diffusion pores and because of this property they are also called "porin" (Nakae, 1976b).

Another interesting aspect of the matrix proteins is the regulation of their production. Gene expression of both the *ompF* gene (for matrix protein Ia) and the *ompC* gene (for matrix protein Ib) is controlled by another independent gene called *ompB*. Furthermore, in mutants that lack both matrix proteins Ia and Ib, new proteins having properties similar to those of the matrix proteins are produced (see Chapter 8 by Reeves).

5.1.2 ompA Protein (tolG Protein) This protein also exists in large quantities in the outer membrane and is known to show an anomalous mobility

on SDS gels (see review by DiRienzo et al., 1978). The molecular weight is approximately 30,000 and the protein has a high β-structure content (Nakamura and Mizushima, 1976). One important function of the ompA protein is its requirement in F pilus-mediated conjugation (see Chapter 12 by Manning and Achtman). It has been suggested that ompA protein also forms pores (Manning et al., 1977); however, this conclusion may have to be reconsidered (see Chapter 11 by Nikaido).

*5.1.3 **Lipoprotein*** The lipoprotein of the outer membrane is one of the most thoroughly investigated membrane proteins. This protein is the most abundant protein in the cell in terms of number of molecules and has many unique features. Recently, an extensive review on the lipoprotein was published (Inouye, 1979).

In 1969, Braun and Rehn first reported the existence of a lipoprotein, covalently linked to the peptidoglycan, with a molecular weight of about 7000. The complete chemical structure of this protein has been determined by Braun and his co-workers as shown in Figure 2 (Braun and Bosch, 1972; Hantke and Braun, 1973). The lipoprotein consists of 58 amino acid residues and lacks histidine, tryptophan, glycine, proline, and phenylalanine. It is linked by the ϵ-amino group of its *C*-terminal lysine to the carboxyl group of every tenth to twelfth *meso*-diaminopimelic acid residue of the peptidoglycan. The *N*-terminal portion of the lipoprotein consists of glycerylcysteine [*S*-(propane-2′,3′diol)-3-thioaminopropionic acid] to which two fatty acids are attached by two ester linkages and one fatty acid is attached by an amide linkage. The fatty acids bound as esters are similar to the fatty acids found in the phospholipids, while the amide-linked fatty acids consist of 65% palmitate, the rest being mainly monounsaturated fatty acids.

Inouye and his co-workers (Inouye et al., 1972; Hirashima et al., 1973) independently found that the lipoprotein also exists in the *E. coli* membrane without covalent bonds to the peptidoglycan (i.e., free instead of bound). The free, as well as the bound, form of the lipoprotein exclusively exists in the outer membrane. There are about 2.4×10^5 molecules of the bound form per cell, and about twice as much of the free form, that is, about 4.8×10^5 molecules/cell. The total free and bound lipoprotein molecules, 7.2×10^5, makes this lipoprotein the most abundant protein, numerically, in the cell (see review by Inouye, 1979).

The free form lipoprotein was extensively purified and paracrystallized (Inouye et al., 1976). The purified protein has a very high α-helical content and, from the known sequence of the lipoprotein, a three-dimensional molecular model for the assembly of the lipoprotein have been proposed (see reviews by DiRienzo et al., 1978; Inouye, 1979). Although the exact

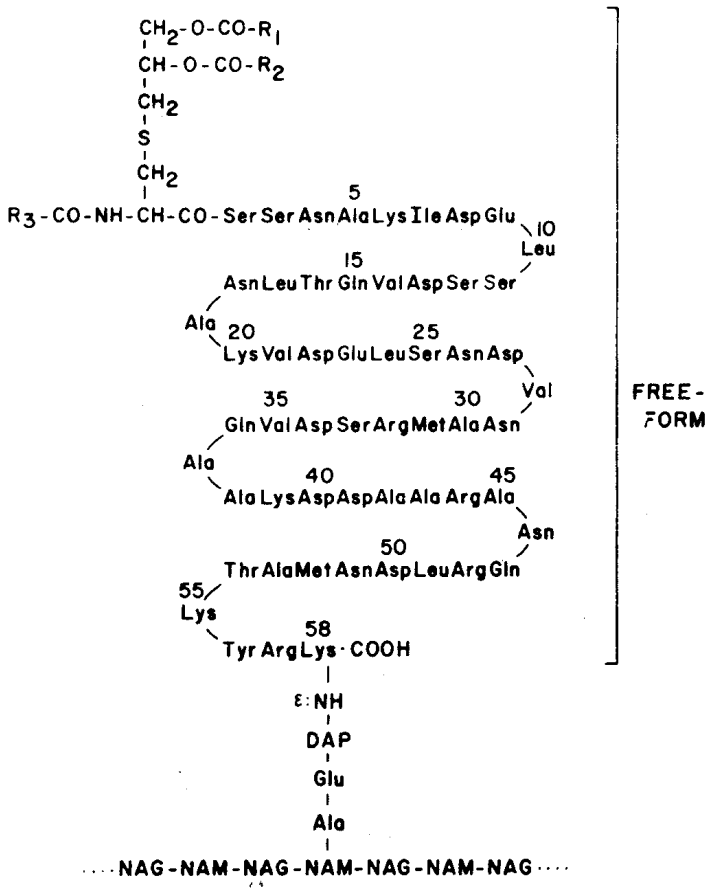

Figure 2 The complete chemical structure of the bound form of the lipoprotein (Braun and Bosch, 1972; Hantke and Braun, 1973). DAP, diaminopimilic acid; NAM, *N*-acetylmuramic acid; NAG, *N*-acetylglucosamine. R_1, R_2, and R_3 represent hydrocarbon chains of fatty acids.

function of the lipoprotein in the outer membrane is still obscure, analysis of lipoprotein mutants has revealed that the lipoprotein at least plays an important role in maintaining the integrity of the outer membrane structure (see reviews by DiRienzo et al., 1978; Inouye, 1979). The mechanism of biosynthesis and assembly of the lipoprotein has been extensively investigated and is discussed in Chapter 4.

An intriguing aspect of lipoprotein research is the examination of the existence of the lipoprotein in various gram-negative bacteria. When the homology of the lipoprotein gene between *E. coli* and other gram-negative bacteria was examined with ^{32}P-labeled purified lipoprotein mRNA from *E. coli* it was found that there are three different groups classified according to the degree of homology with the *E. coli* lipoprotein: (*E. coli*, *Shigella dysen-*

teriae, *Salmonella typhimurium*, *Citrobacter freundii*) > (*Enterobacter aerogenes*, *Klebsiella aerogenes*, *Serratia marcescens*, *Erwinia amylovora*) > (*Proteus mirabilis*, *Proteus morganii*) (Nakamura and Inouye, 1979). DNAs from bacteria outside the family *Enterobacteriaceae* described above (*Pseudomonas aeruginosa*, *Acinitobacter* sp. H01-N, *Caulobacter crescentus*, *Myxococcus xanthus*) did not hybridize with the *E. coli* lipoprotein mRNA. However, it is highly possible that these bacteria also contain the lipoprotein in the outer membrane. In this regard, it should be noted that the lipoprotein was purified recently from *Pseudomonas aeruginosa* and was found to lack proline, valine, isoleucine, phenylalanine, tryptophan, and cysteine (Mizuno and Kageyama, 1978). The *Pseudomonas* lipoprotein contained only 0.89 mole % of fatty acid although it appeared to have a glycerol group, suggesting that the lipoprotein lacks ester-linked fatty acids.

5.2 Minor Proteins

About 10–20 minor proteins are present in the outer membrane. The term "minor protein" may be misleading since under certain growth conditions some of these proteins are made in quantities almost as great as that of "major protein." Many minor proteins, in addition to the major outer membrane proteins described above, have been identified as receptors for phages and colicins. Most of them, as well as additional proteins with no known receptor functions, are now known to have vital roles in the growth of the cell, such as the uptake of nutritional substrates through the outer membrane (see review by DiRienzo et al., 1978; see also Chapter 10 by Konisky).

6 CONCLUSION

In this chapter, I have attempted to described the structure and functions of the outer membrane as a unique membrane system. One may use the outer membrane as a model system for the study of the biosynthesis, and assembly of the outer membrane components may be directly applicable to eukaryotic membrane systems. Studies on the secretory mechanism of outer membrane proteins across the cytoplasmic membrane may provide valuable information as to how hormones and proteins are secreted across the eukaryotic cytoplasmic membrane (see Chapter 4 by Halegoua and Inouye, and Chapter 7 by Silhavy et al.).

The outer membrane also provides unique and excellent systems for the

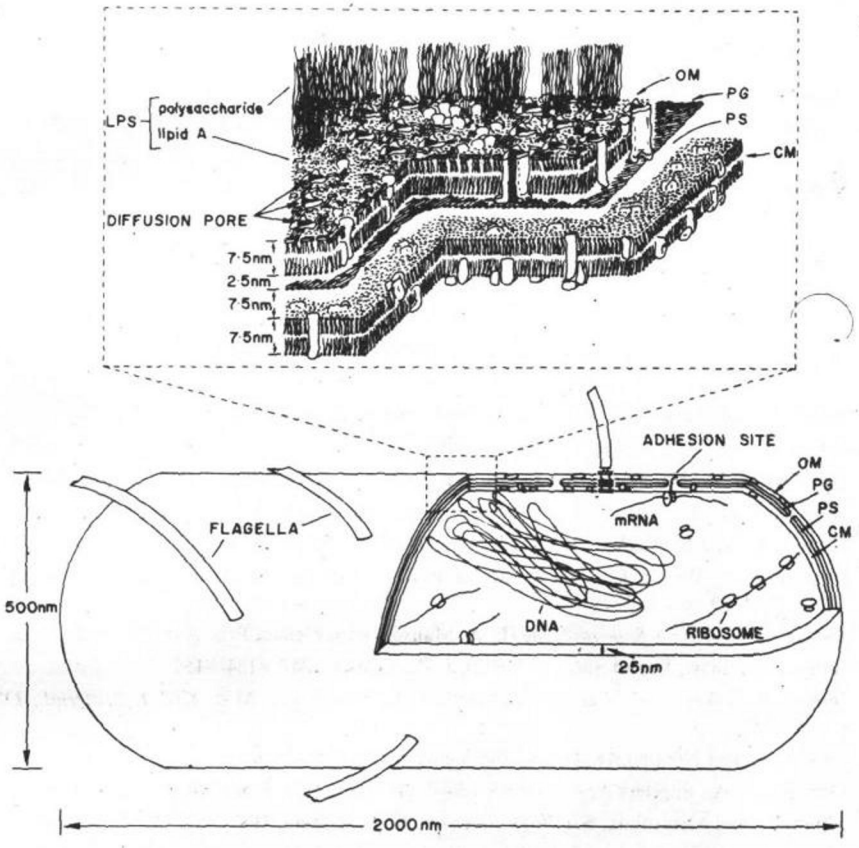

Figure 3 Molecular architecture of the *E. coli* envelope. OM, outer membrane; PG, peptidoglycan; PS, perisplasmic space; CM, cytoplasmic membrane. The structure of the basal end of the flagellum is from Chapter 13 by Hazelbauer.

investigation of many other problems associated with membranes, such as regulation of the biogenesis of membranes (see Chapter 8 by Reeves; Chapter 9 by Ohki), the genetics of membrane proteins (see Chapter 8 by Reeves), membrane receptors and diffusion pores (see Chapter 10 by Konisky and Chapter 11 by Nikaido), cell–cell interactions (see Chapter 12 by Manning and Achtman), and the interaction between pathogenic bacteria and animal tissues (see Chapter 14 by Buchanan and Pearce).

On the basis of the current knowledge described in this chapter, a schematic representation of the possible molecular architecture of the *E. coli* envelope in perspective to the whole cell is shown in Figure 3.