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The Outer Membrane Took Center Stage

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Abstract

My interest in membranes was piqued during a lecture series given by one of the founders of molecular biology, Max Delbrück, at Caltech, where I spent a postdoctoral year to learn more about protein chemistry. That general interest was further refined to my ultimate research focal point—the outer membrane of *Escherichia coli*—through the influence of the work of Wolfhard Weidel, who discovered the murein (peptidoglycan) layer and biochemically characterized the first phage receptors of this bacterium. The discovery of lipoprotein bound to murein was completely unexpected and demonstrated that the protein composition of the outer membrane and the structure and function of proteins could be unraveled at a time when nothing was known about outer membrane proteins. The research of my laboratory over the years covered energy-dependent import of proteinaceous toxins and iron chelates across the outer membrane, which does not contain an energy source, and gene regulation by iron, including transmembrane transcriptional regulation.

Contents

INTRODUCTION

I was born on July 18, 1938, in Ravensburg, a rather small town with medieval towers located in beautiful surroundings in the south of Germany. Our family lived in a rural area, and World War II made life difficult. The shortages of food, clothing, and fuel were lasting experiences that influenced my later choice of study. After nearly complete destruction during the war, the rapid and extensive development of the chemical industry offered a solid career. Fortunately, chemistry captivated me from my first hour at secondary school, with a fascinating demonstration of the transformation of matter: My teacher heated sawdust, and out came a clear liquid with the wonderful smell of acetic acid ethyl ester! I went on to study chemistry at the universities of Freiburg and Munich, finishing my undergraduate studies in 1962. For my doctoral studies, I was attracted to the Max Planck Institute of Biochemistry in Munich, directed at that time by Adolf Butenandt, who at the age of 36 had received the Nobel Prize in Chemistry, jointly with Leopold Ruzicka, for his discovery of human sex hormones. Only a small number of chemistry students from the university chemistry department were allowed to complete a doctoral thesis at this institute, and I considered myself fortunate to be one of them.

EVOLUTION OF HEMOGLOBINS

I started my doctoral thesis in the group of Gerhard Braunitzer, which had just determined the complete amino acid sequence of human hemoglobin. Hemoglobin was an outstanding model protein. Comparison of hemoglobin sequences was the first approach to study molecular evolution

to derive a molecular clock to measure the pace at which molecules evolve (121). A mutated form of this protein represented the first example of a molecular disease (sickle cell anemia). The structures of hemoglobin and myoglobin were the first to be determined by X-ray crystallography, providing insights into the three-dimensional structure of proteins (121). Hemoglobin displays allosteric properties, which earned it the moniker honorary enzyme. All in all, working with hemoglobins seemed to be worthwhile. The Braunitzer group had started a comparative analysis of hemoglobin sequence. I was given hemoglobin from an insect of the genus *Chironomus*, which was at the bottom of the evolutionary tree. This midge was a preferred subject of cytogenetic studies because of its giant chromosomes. Hemoglobin could be found dissolved in the hemolymph of the larvae of the midge. I needed grams of protein for sequence analysis but received only tiny amounts from a pet shop, as it was normally given to customers as fish food. As luck would have it, I met someone who had captured animals in Africa for zoos and could understand the strange needs of a scientist. He provided me with the address of a midge larvae supplier. Finally, with sufficient material, I was able to use chromatographic and electrophoretic methods to reveal an unusually large hemoglobin polymorphism in a single species: 12 hemoglobins, in monomeric and dimeric forms (19, 27, 40, 41). This extreme evolution of various hemoglobin sequences might have been promoted by the very low oxygen supply in the mud of the lakes where the larvae live. Together with Helmut Formanek, we were able to crystallize one of the hemoglobins (21), and its X-ray structure was determined by Robert Huber (83), who went on to become a famous protein crystallographer (Nobel Prize in Chemistry, 1988). The secondary and tertiary structures of the *Chironomus* hemoglobin were almost identical to those of whale myoglobin and similar to those of human hemoglobins. Braunitzer's group continued to study the evolution of *Chironomus* hemoglobins for another 20 years and showed that hemoglobin sequences differ among *Chironomus* species as much as they do between humans and finfish. After the basic design of the insect hemoglobins was unraveled I left the field and moved on to bacterial membranes.

LIPOPROTEIN OF THE BACTERIAL OUTER MEMBRANE

After spending a postdoctoral year in 1964–1965 in the biology department of the California Institute of Technology in Pasadena, I returned to Germany and joined the lab of Ulf Henning, who had just succeeded the late Wolfhard Weidel at the Max Planck Institute of Biology in Tübingen. Henning proposed that I isolate and characterize succinate dehydrogenase of *Escherichia coli*, for which he thought that he had mutants. Isolation of the enzyme turned out to be impossible. The activity declined faster than purity increased. This *E. coli* enzyme was structurally characterized only much later by applying recombinant DNA technology (151). In experiments aimed at releasing the enzyme or part of it from the cytoplasmic membrane by proteolytic cleavage, the optical density of the isolated cell envelopes decreased most strongly with trypsin, the most specific enzyme used. Electron microscopy of trypsin-treated cell envelopes showed dissociation of the outer membrane from the cytoplasmic membrane (33). This indicated a particularly sensitive trypsin bond that seemed to be important for the structural organization of the cell envelope. I began to identify the trypsin bond, a challenge as demanding as finding a needle in a haystack.

Cell envelopes of gram-negative bacteria consist of an outer membrane and a cytoplasmic membrane. In between is the periplasm, where the murein (peptidoglycan) is located. Murein is a bag-shaped macromolecule of the size and form of the cell. It is composed of an oligosaccharide of repeating units of *N*-acetylmuramic acid (MurNAc)-*N*-acetylglucosamine (GlcNAc), which are cross-linked by short peptide bridges (L-Ala-D-Glu-*meso*-Dap-L-Ala). The published procedure for murein isolation included treatment of cell envelopes with NaOH and hot phenol, followed by digestion of the cell envelope with a protease mixture (pancreatin) and pepsin. Murein purified

in this way is mainly composed of the murein constituents but also contains various amounts of contaminating proteins (149). Therefore, I isolated murein from exponentially growing *E. coli* cells with and without treating cell envelopes with trypsin. Lipopolysaccharide, phospholipids, and proteins were dissolved in hot 4% sodium dodecyl sulfate (SDS). The resulting insoluble murein of trypsin-treated cell envelopes contained lysine as the sole additional amino acid. The protein that remained on the murein of cell envelopes not treated with trypsin contained all common amino acids except histidine, proline, glycine, cysteine, phenylalanine, and tryptophan. These results together indicated that the protein material was not a mixture of contaminating envelope proteins but instead represented a single defined protein with a terminal lysine (33). Sequencing of the protein was difficult because it was rather resistant to cleavage by proteases used to isolate peptides for sequencing. Use of proteases with low specificity and partial acid hydrolysis resulted in a set of peptides, whose amino acid sequence could be derived. The sequence was very peculiar in that it was highly repetitive. In addition, nonpolar amino acids occurred consistently along the sequence at intervals of four or three residues. In a helical structure, the nonpolar residues would be located along one side of the helix (15, 16). Indeed, this lipoprotein was later shown by us to have a high helical content (34), and it forms a trimeric coiled coil in the lipid-free crystal structure (141). It was the first sequence determination of a coiled-coil protein (103).

Time and effort were needed to show that lipoprotein is attached via the epsilon amino group of the C-terminal lysine to the carboxyl group of the optical L-center of diaminopimelate (Dap) of murein, where it replaces D-alanine. This lysine residue was part of the sought after most sensitive trypsin cleavage site (sequence Arg–Lys) (15, 16). Since individual lipoprotein molecules with two murein units were released with lysozyme, lipoprotein does not form clusters but is instead evenly distributed over the murein. Approximately every 10th murein subunit carries the lipoprotein substitution, which determines the spatial distribution of the protein molecules in the cell envelope (33), occurring approximately every 100 Å along the oligosaccharide chain.

When I started work on lipoprotein, the term outer membrane was still rarely used. Even I preferred the term cell wall in my first publications, despite having observed via electron microscopy two well-separated membranes after release of lipoprotein from murein by trypsin—the cytoplasmic membrane and outer membrane (33). These two membranes are attached to each other partly via the lipoprotein fixed to the outer membrane by the N-terminal lipid and anchored to the murein with its C-terminal lysine.

In 1970, I was asked to become an independent research group leader (Associate Professor level) at the Max Planck Institute of Molecular Genetics in Berlin. There my group determined the structure of the lipid part of the lipoprotein and its attachment to the protein. We showed that the lipid remains associated with the murein–protein complex, and with the protein after degradation of the murein with lysozyme or after release of the protein from murein with trypsin. Treatment with hot 4% SDS and with various organic solvents did not remove the lipid. Since the protein could not be sequenced from the N terminus but all other amino acid positions could be determined by conventional sequence analysis, it was likely that a lipid was not bound to internal sites of the protein but instead covalently attached to the N terminus. Indeed, an isolated and highly purified N-terminal peptide contained lipid. An acid hydrolysate of the lipopeptide contained an unknown compound, which was determined to be glycerylcysteine by using chemical reagents and chemical synthesis, incorporating radiolabeled precursors, and using chromatographic methods. Fatty acid analysis of acid hydrolysates of lipopeptide yielded 3 mol of fatty acids per 2 mol of serine, which was the major amino acid in the lipopeptide sample. The N-terminal cysteine was substituted at the amino group mainly by palmitic acid and at the SH group by diacylglycerol with a fatty acid composition similar to that of phospholipids (69) from which it is derived (39). This

novel structure turned out to be typical for all bacterial lipoproteins (10). Immunological methods revealed ten times more lipoprotein in the outer membrane than in the cytoplasmic membrane (8).

Murein–lipoprotein was the first protein characterized with a covalently linked lipid. It is the most abundant protein in *E. coli*, amounting to several hundred thousand molecules per cell. It is fixed by its C terminus to murein, and with its N-terminal lipid, it is embedded in the outer membrane (25). Additional lipoprotein not bound to murein makes up twice the amount of murein-bound lipoprotein (86, 87).

E. coli lipoprotein started out as a curiosity (33) but later became a model system for unraveling signal-sequence-dependent secretion of proteins across the cytoplasmic membrane (85), release from the cytoplasmic membrane, translocation across the periplasm, and insertion into the outer membrane (110, 111). Over 2.2 million lipoproteins in over 80 million nonredundant bacterial protein sequences (~3%) have been identified to date [LipoP 1.0 server (89); Jens Baßler, personal communication]. They occur in both gram-positive and gram-negative bacteria, mostly in triacylated forms but also in monoacylated and diacylated forms (5, 113). In gram-positive bacteria nearly all substrate binding proteins of ABC transporters are lipoproteins. Lipoproteins serve numerous functions. They stimulate B lymphocytes (108), act as adhesins and antigens, and are constituents of systems involved in membrane biogenesis, biofilm formation, nutrient uptake, sensing and signaling, conjugation, germination, and spore maturation. Murein lipoprotein bound to Toll-like receptor 2 stimulates the innate immune system (2, 111, 113). In the literature, the murein–lipoprotein is referred to as Braun's lipoprotein, a term coined by Masayori Inouye, the discoverer of the free form.

FHUA: OUTER MEMBRANE VIRUS RECEPTOR AND ENERGY-DEPENDENT IRON TRANSPORTER

While we were working on the lipoprotein, we also began to investigate the unknown outer membrane receptor of phage T5 (14). No protein as phage receptor was identified when we started the project. A cell envelope fraction inactivated phage T5 by causing the release of DNA from the phage head, as shown by electron microscopy. Although previously a membrane fraction was isolated by a lengthy treatment with a mixture of pancreatic enzymes, mainly proteases, it was believed that the receptor was proteinaceous because it was sensitive to phenol, in contrast to the phenol-resistant lipopolysaccharide receptors of phages T3, T4, and T7 (148). We purified the receptor using the phage T5 inactivation assay and identified a polypeptide with a molecular weight of 85,000 (36). We were unable to isolate the protein from *tonA* mutants, which were known to be T5 resistant. Thirty years after the seminal studies of Luria & Delbrück on the nature of mutations (104), including phage-resistant *E. coli tonA* mutants, our work proved that the *tonA* gene encodes a protein.

In 1974, my group moved to the University of Tübingen, where I was offered a position as chair of microbiology in the biology department. I became part of the existing Microbial Collaborative Research Center (SFB, Sonderforschungsbereich). Such centers, in which various natural science and medical research groups work together on a major topic, were established by the German Research Foundation (Deutsche Forschungsgemeinschaft) to support excellent research at universities. I received this special funding throughout my entire university career. At the SFB, Hans Zähner had isolated iron-complexing antibiotics, designated sideromycins, which belong to a group of compounds now called siderophores. We used one of the antibiotics, albomycin, to isolate iron-transport-deficient *tonB* mutants. It was known that *tonB* mutants display a tenfold higher K_m of ferric iron uptake and an unaltered V_{max} (147). We obtained an albomycin-resistant *tonB* mutant as expected, but we also completely unexpectedly obtained many *tonA* mutants. *tonB*

is related to *tonA* in that all processes that require TonA also require TonB, except for sensitivity to phage T5, which only requires TonA. All our albomycin-resistant mutants except the *tonB* mutant were resistant to phage T5. The *tonA* mutants were also unable to transport [$^{55}Fe^{3+}$]-ferrichrome, a structural analog of albomycin. TonA, the virus receptor, was therefore a transport protein (70). As TonA transports ferrichrome, a siderophore of the hydroxamate type, we renamed it FhuA (ferric hydroxamate uptake) (90).

FhuA turned out to be an energy-dependent transport protein in a membrane without an obvious energy source. Phages T1 and φ80, which both require FhuA and TonB for infection, adsorbed reversibly to $FhuA^+TonB^-$ cells and isolated FhuA but adsorbed irreversibly to $FhuA^+$ $TonB^+$ cells. The energy source was the electrochemical potential of the cytoplasmic membrane (63). Both phages adsorbed to an energized conformation of FhuA elicited by the energized cytoplasmic membrane; DNA release from the phage head was triggered, and phage DNA entered the cytoplasm. We identified both an energized and an unenergized FhuA conformation based on the complete inhibition of phage T5 adsorption to a *tonB* mutant by ferrichrome and only a partial inhibition of phage T5 adsorption to $tonB^+$ wild-type cells by a 1,000-fold higher ferrichrome concentration (71). Over the next 30 years, my laboratory and several other laboratories elucidated the structure and function of FhuA, as outlined below.

The N terminus of FhuA contains a pentapeptide that we called the TonB box. The TonB box is similarly found in all TonB-dependent outer membrane transporters and colicins (see below) that are taken up by a TonB-dependent import mechanism (140). The TonB box forms the interface to TonB, as we showed by disulfide cross-linking and the inactivity of FhuA TonB–box point mutants that are suppressed by point mutations in TonB (20, 109, 114), and as others elucidated based on crystal and NMR structures of FhuA with bound TonB fragments (119, 120).

In FhuA, 22 antiparallel β strands form a β barrel with an internal pore that is closed by a globular plug. The N-terminal plug enters the pore from the periplasm (56, 100). Deletion of the plug inactivates FhuA and converts it into an open pore that facilitates diffusion of hydrophilic compounds. We cloned the plug and the barrel, which together form an active FhuA, separately. The plug and barrel spontaneously reconstituted in the periplasm (9).

The antibiotics albomycin (54) and rifamycin CGP 4832 (57), a synthetic derivative of rifamycin, occupy the same site as ferrichrome in the crystal structures of FhuA. The three-dimensional structures of the antibiotics were particularly interesting, as albomycin assumes both an open and a closed conformation, and the chemical structure of rifamycin CGP 4832 does not resemble that of ferrichrome. Active transport of rifamycin CGP 4832 across the outer membrane, followed by uptake into the cytoplasm by diffusion, lowers the minimum inhibitory concentration 200-fold as compared to rifamycin.

The *fhuA* gene is part of an operon: *fhuA fhuC fhuD fhuB* (44, 53, 90, 96). The latter three genes encode an ABC transporter, with the periplasmic protein FhuD, the cytoplasmic membrane protein FhuB, and the ATPase FhuC. Only ferrichrome uptake and albomycin uptake require all four gene activities. For the uptake of the other FhuA ligands, FhuA (and TonB) is sufficient. Albomycin is composed of an iron carrier similar to ferrichrome and an antibiotic thioribosyl-pyrimidine moiety that inhibits seryl-tRNA synthetase. After active transport into cells, the carrier is separated from the antibiotic by peptidases A and N. The antibiotic remains inside cells, whereas the carrier is released (24, 76).

It was long known that certain phage T5 lines propagated on *E. coli* F much better than on *E. coli* K-12. The reason remained mysterious until we showed that *E. coli* F has a particular lipopolysaccharide O-antigen consisting of polymannose, to which phageT5 binds reversibly via the pb1 protein of its L-shaped tail fibers (80). Reversible binding accelerated adsorption 15-fold. Movement along the cell surface by adsorption/desorption reduced the search for FhuA from a

three-dimensional diffusion to a two-dimensional diffusion and thus increased the detection rate of FhuA.

The phage T5 genome encodes a lipoprotein that binds to and inactivates FhuA. Cells expressing this lipoprotein are resistant to all tested FhuA ligands (28). It has not been shown whether the lipoprotein covers FhuA at the cell surface or whether it interacts with FhuA at the periplasmic side and triggers a conformational change in FhuA that abolishes its receptor function. Since inactivation of FhuA by the lipoprotein develops slowly after infection, lipoprotein probably does not exclude superinfection but prevents inactivation of released phage T5 when cells lyse (46).

ENERGY COUPLING BETWEEN THE OUTER MEMBRANE AND THE CYTOPLASMIC MEMBRANE

FhuA and the other outer membrane proteins of the FhuA type (β barrels with N-terminal plugs) display energy-coupled transport, but the outer membrane contains no energy source. After we had shown that the electrochemical potential of the cytoplasmic membrane serves as an energy source (63), it was of interest how this is transmitted to the outer membrane. We selected FhuA as a model transporter. Energy is provided by the cytoplasmic membrane mediated by three proteins: TonB, ExbB, and ExbD (termed the Ton system). We showed interactions between the proteins in vivo; ExbB reduced the spontaneous degradation of TonB and ExbD (58). Others considered ExbB and ExbD to be auxiliary proteins because e*xbB* and *exbD* mutants displayed residual TonB-dependent activities. As we considered ExbB and ExbD essential for TonB-dependent outer membrane import processes, we determined the sequences of the *exbB* and e*xbD* genes (47) and the membrane topology of the ExbB and ExbD proteins. The leaky phenotype was explained by the homology of *exbB* and e*xbD* to *tolQ* and *tolR*, respectively; the proteins encoded by these latter genes are constituents of another energy-coupled system that is important for outer membrane integrity and the uptake of a specific group of colicins (see below) and infection by filamentous phages. *E. coli exbB* mutants are sensitive to phages T1 and φ80, but *exbB tolQ* double mutants are completely resistant (12). ExbB and ExbD function with TolA, the equivalent of TonB, and TolQ and TolR are active with TonB. However, TolA and TonB show no sequence similarity and cannot replace each other. TonB and ExbD (91) are anchored in the cytoplasmic membrane by their C termini and extend into the periplasm. ExbB folds three times through the cytoplasmic membrane, with the N terminus in the periplasm (93). The membrane topologies of TolQ and TolR are very similar to those of ExbB and ExbD (92). Sequence similarity, similar membrane topology, and functional cross-complementation between ExbB-ExbD and TolQ-TolR suggest that the two import systems evolved from a single import system (13, 26).

ExbB was isolated by us as an oligomer of 4–6 monomers (131). We copurified ExbD with ExbB, forming a complex with a ratio of 6:1, as deduced by laser-induced liquid bead ion-desorption mass spectrometry (129). The X-ray structure recently determined by Celia et al. (45) shows an ExbB pentamer with a central transmembrane pore, in which an ExbD helix is contained. Our isolates did not contain TonB, and TonB was not seen in the X-ray structure (45). It remains to be elucidated how TonB interacts with the ExbB-ExbD complex, how the complex responds to the proton-motive force of the cytoplasmic membrane, and how TonB converts the outer membrane proteins into active transporters.

TRANSMEMBRANE TRANSCRIPTION CONTROL (SURFACE SIGNALING) OF THE FERRIC CITRATE TRANSPORT SYSTEM

Citrate does not enter *E. coli* cells growing aerobically. We questioned whether ferric citrate does. We found that not ferric citrate but rather iron alone is transported into cells (85). Study

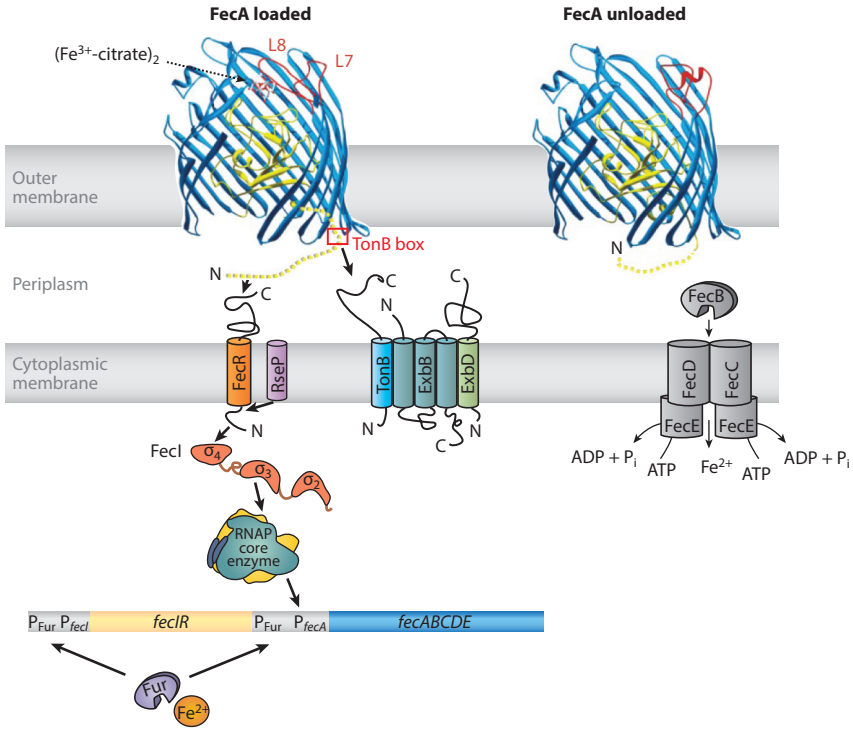

Figure 1

$(Fe^{3+}\text{-citrate})_2$ transport and regulation. $(Fe^{3+}\text{-citrate})_2$ binds to the outer membrane protein FecA and causes a large structural change in loops L7 and L8 (*red*) (54, 55, 153), which is transmitted to the signaling domain (*yellow*). The FecA signaling domain interacts with the C-terminal domain of FecR, which transmits the signal into the cytoplasm, where it activates the FecI sigma factor. FecI binds to RNA polymerase (RNAP) and initiates transcription of the *fecABCDE* transport genes. Fur, activated by binding of Fe^{2+}, represses transcription of *fecIR* and *fecABCDE*. Signaling and translocation of $(Fe^{3+}\text{-citrate})_2$ across the outer membrane is energized by the electrochemical potential of the cytoplasmic membrane mediated by the TonB, ExbB, and ExbD proteins (Ton system). FecA interacts via the TonB box with TonB. The signaling domain is essential for the regulatory response to $(Fe^{3+}\text{-citrate})_2$ but is dispensable for transport. FecB binds $(Fe^{3+}\text{-citrate})_2$ and delivers it to the FecC and FecD transmembrane proteins. Transport across the cytoplasmic membrane is energized by ATP through the FecE ATPase (29, 30). The transport model (*right*) is typical for siderophore-mediated iron transport across the outer membrane and the cytoplasmic membrane. Adapted with permission from Reference 29.

of this system revealed a new type of gene transcription control in which the inducer does not enter the cytoplasm. Instead, the outer membrane protein FecA binds the inducer ferric citrate, which triggers a signal that is transferred across the outer membrane, periplasm, and cytoplasmic membrane into the cytoplasm, where it induces transcription of the ferric citrate transport genes *fecABCDE* (29–31, 43, 156) (**Figure 1**). FecA is a typical Ton-dependent outer membrane transporter that transports ferric citrate into the periplasm. Induction and transport require the Ton system, which suggests that similar structural changes in FecA occur for induction and transport. However, transport of ferric citrate is not required for induction, as induction still occurs in transport-deficient *fecA* missense mutants (74) and all iron-transport-negative *fecBCDE* mutants of the ABC transporter in the cytoplasmic membrane (**Figure 1**) are fully inducible. FecA missense mutants constitutively express *fec* genes (without inducer). When ferric citrate is present in high

concentrations in the medium, it diffuses through the porins and leads to abundant ferric citrate in the periplasm, but *fec* transcription is not induced (74). In addition, intracellular iron and citrate concentrations exceeding 10- to 100-fold the outside concentrations required for induction (1 mM citrate, 1 μM Fe^{3+}) do not induce *fec* gene transcription (85). Lack of induction by intracellular citrate indicates the presence of a signaling cascade that starts at FecA in the outer membrane upon binding of ferric citrate.

X-ray structures determined by others revealed that binding of ferric citrate induces strong long-range structural transitions in FecA (55, 153). Two surface loops moved 11 and 15 Å, respectively, and cover the entry of the surface cavity, preventing the escape of ferric citrate back into the external milieu (**Figure 1**). In the FecA region exposed to the periplasm, a short helix unwinds. Our analysis showed that compared to other, nonsignaling, Ton-dependent outer membrane transporters, FecA contains an N-terminal extension of 80 residues (signaling domain) that is exposed to the periplasm and is required for signaling but is dispensable for transport (94). Nuclear magnetic resonance spectroscopy revealed a unique fold of the signaling domain (59). Signaling-defective mutants generated by random mutagenesis contain amino acid replacements along one surface of the signaling domain, which indicates an interface with the FecR protein, i.e., the reaction partner of FecA (42).

In addition to the *fecABCDE* transport genes, the *fec* operon contains upstream two regulatory genes, *fecI* and *fecR* (**Figure 1**) (144). Our determination of the FecI sequence showed that it differs from σ^{70} such that standard similarity searching methods did not identify FecI as a sigma factor (144). Mark Buttner at the John Innes Center drew our attention to his finding that the FecI amino acid sequence derived from the *fecI* gene (144) shows similarity to a new subfamily of σ^{70}–like sigma factors (102), which were at that time designated extracytoplasmic function (ECF) sigma factors because they responded to environmental changes. This property of FecI prompted us to investigate FecIR function in detail. FecI is required for *fecABCDE* transcription. FecR regulates FecI activity. FecR has a hydrophilic 84-residue N terminus in the cytoplasm, a single transmembrane stretch in the cytoplasmic membrane, and the C terminus in periplasm (150). This arrangement qualifies FecR as a signaling device across the periplasm and the cytoplasmic membrane. FecR receives the signal from FecA via the signaling domain (50). The FecR C-terminal domain was shown to interact with the FecA signaling domain in the periplasm (52, 150). In addition, the functions of negative mutants in one domain are restored by mutations in the other domain. Both findings indicate the path of the signal from FecA to FecR (50). Cytoplasmic N-terminal fragments of FecR induce *fecABCDE* transcription in the absence of FecA. Ferric citrate induction might trigger FecR proteolysis, as has been shown for activation of σ^E in response to stress (1). Indeed, a mutation in the RseP protease involved in σ^E activation strongly reduces the amount of FecA (31). RseP seems to release the N-terminal fragment of FecR that binds to FecI and triggers *fecABCDE* transcription. RseP does not influence constitutive synthesis of FecA by FecR N-terminal fragments.

FecI is a *fec*-specific sigma factor that binds to the RNA polymerase and activates only *fecABCDE* transcription (105). Our genetic and biochemical investigations have shown that FecR binds via its N terminus to regions 4.1 and 4.2 of FecI (106) and that the FecI–RNA polymerase complex binds to the promoter of *fecA* (51). *E. coli* with FecI mutations in domain 2.2 are impaired in binding to the β′ subunit of RNA polymerase (156).

Transcription of the *fecIR* regulatory genes is not autoregulated but is repressed by the Fe^{2+}-Fur protein (3, 156). If cells contain enough iron, the lack of FecIR avoids wasteful synthesis of the ferric citrate transport system. However, it takes some time until FecIR proteins are diluted to an extent that no transcription is induced. Transcription of *fecABCDE* is immediately inhibited

by binding of Fe^{2+}-Fur to the promoter upstream of *fecA* (3). Binding of Fe^{2+} converts Fur to a repressor (65, 66, 68). The reason for the development of this regulatory device may be that the high affinity of the FecA receptor for the ferric citrate inducer is in the nanomolar range, making regulation extremely sensitive.

IutA: THE RECEPTOR OF CLOACIN AND TRANSPORTER OF AEROBACTIN

It was known that certain pColV plasmids that specify synthesis of colicin V [which is in fact a microcin (32)] increase the virulence of *E. coli* through an iron transport system. We identified the siderophore—aerobactin (11)—which consists of two residues of 6-(*N*-acetyl-*N*-hydroxy)lysine linked to citrate. We characterized the uptake of Fe^{3+}-aerobactin and synthesis of aerobactin. Transport across the outer membrane is catalyzed by the IutA protein (7), and transport across the cytoplasmic membrane is catalyzed by the FhuB, FhuC, and FhuD proteins (18). Three genes are responsible for the synthesis of aerobactin: *aerA*, *aerB*, and *aerC* specify enzymes that catalyze *N*-hydroxylation and acetylation of lysine and condensate the fully modified lysine with citrate (62). Synthesis and transport are regulated by iron through the chromosomally encoded Fur protein (17).

IRON TRANSPORT ACROSS THE CYTOPLASMIC MEMBRANE

Since Fe^{3+} is virtually insoluble at pH 7, iron transport could not be determined until the discovery of bacterial iron ligands, which solubilize Fe^{3+} and transport it into cells. These ligands, designated siderophores, are structurally derived from hydroxamates, hydroxyphenolates, or citrate. After transport via highly specific outer membrane transport proteins, transport across the cytoplasmic membrane is usually less selective. For example, the FhuBCD proteins transport not only ferrichrome but also a variety of Fe^{3+}-hydroxamates (97). The iron complexes are most frequently taken up by ABC transporters (see **Figure 1** for an example). The ferrichrome-recognizing receptor FhuA is found in all *E. coli* strains. The source of this typical fungal siderophore in the gut is unknown, or another unknown hydroxamate iron complex is the true substrate. In *Serratia marcescens* we characterized an Fe^{3+}-transport system for which we could not identify a siderophore (4, 155). Fe^{3+} supplied as a phosphate salt was transported independent of the Ton system.

Fe^{2+} is soluble and taken up by the Feo transport system, which consists of three proteins, FeoA, FeoB, and FeoC; FeoB is essential. Feo was discovered in *E. coli* by Klaus Hantke and was later found in many bacteria (67).

REGULATION OF IRON TRANSPORT SYSTEMS BY IRON

In the beginning we determined iron regulation by growth rates and SDS-polyacrylamide gel electrophoresis to quantify outer membrane proteins. Later the insertion of phage Mu(Ap *lac*) with *lacZ* as a reporter helped a lot to find and study the genes of interest. In these operon fusions, expression of β-galactosidase is under the control of the gene promoter and determination of β-galactosidase activity results in quantitative data of the gene transcription rate. Using this procedure, Klaus Hantke discovered the *fur* gene, which encodes a repressor that regulates all of the iron transport genes (65, 66, 68). Fur is activated by binding of Fe^{2+}. Fur mutants no longer respond to the cellular iron concentration and express the iron-controlled genes constitutively. When we transferred cells from an iron-sufficient medium to an iron-deficient medium, it took 1 h, i.e., one to two generations, before cells began to synthesize increasing amounts of β-galactosidase. The highest level was obtained after 3 h, which indicates substantial intracellular amounts of

available iron. When iron-starved cells were transferred back into a high-iron medium, they transported iron for an hour until they resumed growth (75). Proteins of the Fur family regulate uptake of other divalent cations, e.g., zinc (118), manganese, and nickel, and regulate a variety of cellular functions, such as photosynthesis, energy metabolism, and the redox state.

Ton-DEPENDENT SUGAR TRANSPORT

Ton-dependent transport was first recognized with ferric siderophores and vitamin B_{12}. In nature, these substrates are available in micromolar concentrations, and they are too large to diffuse through the porins at growth-promoting rates. Their binding affinity to the outer membrane transporters is in the nanomolar range. It could be foreseen that not only ferric siderophores, heme, and Fe^{3+} delivered by lactoferrin and transferrin are energy-dependent imported across the outer membrane but also other scarce substrates and substrates too large to pass through the porins.

The genome sequence of *Caulobacter crescentus* predicts 67 Ton-dependent outer membrane proteins and no protein of the *E. coli* OmpC/OmpF porin type. The large number of Ton-dependent proteins suggests that substrates distinct from those known are taken up via a Ton-dependent mechanism. *C. crescentus* thrives mainly in freshwater lakes, ponds, and streams with low nutrient content. Therefore, we examined whether *C. crescentus* grows on chitin and starch, the most abundant carbon sources. The bacteria do not grow on the polysaccharide polymers but do grow on the degradation products, namely maltodextrins and chitin oligosaccharides. We identified an outer membrane protein, designated MalA, responsible for the uptake of maltose and maltodextrins (112). MalA supports growth on maltodextrins up to maltotetraose and requires in addition the Ton system for the larger maltodextrins. Although *malA* mutants grow on maltose, the maltose transport rate is only 1% of the rate of the *malA*$^+$ parent strain. Bypass of MalA is inefficient. MalA-supported maltose transport requires TonB, ExbB, and ExbD. Not only transport but also initial binding of the substrates to MalA is facilitated by the proton-motive force of the cytoplasmic membrane. Of the three predicted TonB proteins in *C. crescentus*, only TonB1 is active (101). MalA has the novel property that it functions both as a pore through which maltose and the smaller maltodextrins diffuse and as a Ton-dependent energy-coupled transporter that strongly enhances the transport rate and is required for larger maltodextrins.

The *malY* gene near the *malA* locus encodes a transporter of the H^+, Na^+ cotransporter type and is required for maltose uptake into the cytoplasm (101). An additional gene encodes a glucoamylase in the periplasm, and two genes encode amylases in the cytoplasm that might degrade larger maltodextrins to maltose. *mal* gene transcription is controlled by MalI, a protein homologous to the *lacI* repressor. Maltose transport is induced by maltose and is constitutive in a *malI* mutant (101, 112).

We further investigated whether chitin, the second-largest carbon source in nature, serves as a nutrient for *C. crescentus*. Chitin does not support growth, which indicates lack of a secreted chitinase that degrades chitin to GlcNAc and larger homologs [$(GlcNAc)_n$]. GlcNAc, $(GlcNAc)_3$, and $(GlcNAc)_5$ support growth of the wild-type strain but fail to serve as nutrients for an *nagA* deletion mutant; the NagA protein is localized in the outer membrane (48). Transport of GlcNAc requires NagA but is independent of TonB, ExbB, and ExbD. However, growth of an *exbB exbD* mutant is slower on GlcNAc and much slower on $(GlcNAc)_3$ and $(GlcNAc)_5$. NagA contains a TonB box close to the N terminus, as in MalA, which is typical for Ton-dependent outer membrane transporters. NagA functions as an efficient GlcNAc-specific pore but also functions as a Ton-dependent transporter of chitin oligosaccharides.

The *nag* gene cluster contains seven genes that are sufficient for GlcNAc transport and metabolism. Transport across the cytoplasmic membrane occurs via the phosphotransferase

system. Transcription is induced by GlcNAc and controlled through the predicted GntR repressor (48).

BACTERIAL PROTEIN TOXINS

Colicin M

Colicins are monomeric proteins produced and released by nearly 50% of *E. coli* strains. They intrigued me because they are the only proteins that are actively imported by *E. coli* and related bacteria. Their uptake must be efficient since a few colicin molecules are sufficient to kill cells. Bacterial membranes are usually impermeable to proteins. Colicins are encoded on plasmids together with an adjacent immunity gene that confers colicin resistance to the producing cells. Another adjacent lysis gene renders cells permeable for colicin release.

I began investigating colicin M because it was known that colicin M uses FhuA as the entry site, as indicated by the colicin M resistance of FhuA mutants. In addition, we realized that the mode of action of colicin M differed from that of other colicins in that cell lysis was the primary action (35, 134) and not, as observed with other colicins, lysis as a consequence of pore formation in the cytoplasmic membrane, or DNA or RNA degradation. Colicin M has the peculiarity that it is encoded together with colicin B on a medium-sized plasmid without lysis genes but with immunity genes (115). Transcription of the colicin M activity gene is controlled by a promoter upstream of the colicin B activity gene (115). As there is no lysis protein, most colicin M remains cell bound (35). Working with colicin M was difficult until we realized that loss of activity could be prevented by adding Triton X-100. Analysis of mutants identified the active site; one of the active site amino acids, aspartate, was of particular importance as its conversion to asparagine or glutamate inactivates colicin M (125). The crystal structure (154) revealed that the aspartate residue is exposed at the surface of colicin M and is surrounded by residues that when mutated inactivate the colicin. Unlike other colicins, colicin M has a unique compact structure and is organized in three functional domains that are structurally not obvious. Nevertheless, genetic and biochemical studies revealed a domain organization with an N terminus required for translocation across the outer membrane, a central region involved in receptor binding, and a C-terminal region that contains the activity domain (77, 154). The sequence of the activity domain of colicin M is conserved in other bacteria, but receptor binding and the translocation domains differ, which reflects the particular import requirements of these organisms (79, 154).

The same four genes required for import of ferrichrome are also needed for the import of colicin M across the outer membrane—*fhuA*, *tonB*, *exbB*, and *exbD*. Mutations in these genes render cells resistant to colicin M. A fifth type of mutation, in the *tolM* gene, maps close to the *rpsL* gene, but no function could be assigned (135). Much later, in a search for periplasmic chaperones that assist FhuA incorporation into the outer membrane, a specifically colicin M–resistant chaperone mutant was identified that was identical to the previously isolated *tolM* mutants (84). The mutation was in the *fkpA* gene, which encodes a periplasmic peptidyl prolyl *cis-trans* isomerase. Among the isolated *tolM* mutants was a temperature-sensitive mutant that is sensitive at 30°C but resistant at 42°C. In this mutant, colicin M is reversibly translocated across the outer membrane. When the temperature is increased from 30°C to 42°C, shortly before cells lyse after 20 min exposure to colicin M, cells are rescued and colicin M again becomes accessible to degradation by trypsin. After lowering the temperature to 30°C, trypsin-untreated cells lyse. The remarkable reversible membrane translocation could also be achieved with ethylene glycol tetraacetic acid (EGTA), which binds Ca^{2+} required for colicin M activity. Addition of EGTA prior to cell lysis stops lysis, and cell-bound colicin M is inactivated by trypsin, SDS, and antibodies (125, 135). Colicin M

bound to FhuA becomes trypsin sensitive; colicin M in the free form does not. FkpA is required for colicin M activity regardless of whether it is imported from the cell surface into the periplasm or exported across the cytoplasmic membrane into the periplasm by a fused signal peptide. Whenever colicin M crosses a membrane, it is unfolded and has to be refolded to be active (79). This does not apply to the in vitro activity of colicin M; colicin M released by cells into the medium does not require FkpA. Release probably does not involve a specific membrane translocation step but occurs through partial cell lysis. All mutations that inactivate FkpA are located in the isomerase. We identified Phe-Pro-176 as the most likely bond that is isomerized when colicin M refolds (79). No prolyl bond has been identified for any other natural isomerase substrate.

Studies on *tolM* revealed another gene, *cbrA*, that mediates partial resistance to colicin M (78). Overexpression of *cbrA* confers full resistance to colicin M and to osmotic shock. It is not known how CbrA affects outer membrane permeability and sensitivity to colicin M. Transcription of *cbrA* is controlled by the CreBC two-component regulatory system. Colicin M may be a suitable tool to unravel CbrA activity and regulation. The data collected resulted in the following import scenario. Colicin M binds to FhuA, and both proteins assume an import-conductive conformation, which requires interaction with TonB through the N-terminal TonB boxes of both proteins (125) in response to the energy input from the electrochemical potential of the cytoplasmic membrane. Translocation across the outer membrane is accompanied by unfolding. Colicin M refolds in the periplasm with the aid of FkpA and cleaves the murein precursor. Colicin M kills cells by inhibiting regeneration of the lipid carrier of murein biosynthesis (72). It cleaves the phosphate bond between undecaprenol and PP-*N*-acetyl-muramylpentapeptide-*N*-acetyl-glucosamine (49), which results in cell lysis. Colicin M is a unique phosphatase because it shows no sequence similarity to known phosphatases. It also inhibits biosynthesis of lipopolysaccharide O-antigen, which uses the same lipid carrier.

Strains producing colicin M are protected from its lethality by the immunity protein, which is contained in the periplasm with its N terminus anchored in the cytoplasmic membrane (61). Lack of immunity protein in cells that produce colicin M does not kill cells, provided they do not contain a functional FhuA receptor protein (73). Wild-type colicin M must enter cells from the outside to be toxic. The requirement for FhuA and TonB is bypassed when colicin M is osmotically shocked into cells (22). We used this osmotic shock procedure extensively to differentiate between uptake mutants and activity mutants.

Colicin B

My lab studied colicin B because it is cotranscribed with colicin M on the same plasmid. Its structure and mode of action differ entirely from those of colicin M. The pure protein is monomeric and has a molecular weight of 55,000. It forms stable voltage-dependent single channels in artificial lipid bilayer membranes. Pore formation in cells leads to dissipation of the proton-motive force and inhibition of proline transport (132). Colicin B contains a TonB box (142) that is inactivated by the amino acid replacements V20R and V21E (109). Mutation Q160K in TonB restores sensitivity of cells with the mutant colicin B derivatives. The same TonB mutation restores TonB box mutations in FhuA and the TonB-dependent receptors BtuB and Cir (13). Site-specific suppression of mutations suggests interaction of the interacting proteins along these sites.

Colicin D

Colicin D was of interest because it shares uptake, receptor specificity, and TonB dependence with colicin B but shares target specificity with ribonuclease colicins. The amino acid sequence

deduced from the nucleotide sequence of the activity gene revealed its identity to colicin B in the 313 N-proximal amino acids, with only 14 deviations (133). This segment determines receptor specificity and uptake. Colicin D has a unique target and mode of action, which explains the lack of sequence similarity with known nuclease colicins. It cleaves four arginine isoaccepting tRNAs. The sequence reflects the common import pathway with colicin B and the unique toxicity. The colicin B–colicin D pair clearly demonstrates the assembly of colicin genes from three DNA fragments that encode receptor specificity, uptake mechanism, and mode of action. This proposal based on comparison of the colicin B and colicin D activity gene sequences (133) was further supported by additional colicin sequences.

Comparison of Colicins 5, 10, K, S4, and U

To further identify regions in colicin sequences involved in receptor binding, uptake, and activity, we investigated additional colicins. Uptake of colicins 5, 10, K, S4, and U is dependent on the Ton system or the Tol system, but the functions of their outer membrane receptor proteins are not coupled to either system. These colicins form pores in the cytoplasmic membrane and kill cells by dissipating the proton-motive force. Accordingly, their amino acid sequences have a high level of identity in the C-proximal activity regions. Entry of colicins 5, 10, and K into cells requires the Ton system (colicins 5 and 10) or the Tol system (colicin K). They attach to the same receptor protein, Tsx, which is not coupled to either system. Tsx is not an active transporter but facilitates diffusion of nucleosides across the outer membrane (64, 98). Colicins 5 and 10 display the unique property that they require the TolC protein, a constituent of the type I secretion system across the outer membrane. The regions that bind to Tsx are 78 and 99% identical, and the regions involved in Ton-dependent uptake are 100% identical, but the Ton- and Tol-dependent regions differ by 80% (123, 124, 140). The immunity and lysis genes of colicins 5 and K, which are adjacent to the activity genes, are 99% and 100% identical, respectively. Uptake of colicins S4 and U depends on the Tol system (127, 142), but the functions of the outer membrane receptor proteins OmpW and OmpA do not require the Tol system (or Ton system). The amino acid sequence of colicin S4 contains a duplication in the predicted receptor binding site (127); these duplicated sites are exposed in the crystal structure. Both sites bind OmpW, but one site is sufficient for killing cells (6).

Immunity proteins of pore-forming colicins are located in the cytoplasmic membrane. Mutagenesis and exchange of DNA fragments between the most closely related colicins, 5 and 10, and their immunity genes identified the region of the colicin 5 immunity protein that binds to colicin 5 (122). It is located in the cytoplasmic loop and the inner leaflet of the cytoplasmic membrane, which indicates that the immunity protein does not prevent entry of the colicin into the cytoplasmic membrane but rather inactivates colicin 5 shortly before the lethal pores are opened.

The structure and function of these colicins strongly supports evolution from a few DNA fragments that were mixed to specify new colicins with regard to receptor specificity, uptake via the Ton or the Tol system, pore formation, immunity, and cell lysis. It is remarkable that the DNA fragments encode functional domains.

PESTICIN

Yersinia pestis contains a small plasmid that encodes a toxin of the colicin type. Pesticin uses the FyuA outer membrane receptor protein to enter cells. Both pesticin and FyuA contain a TonB box, and uptake depends on the Ton system. The mode of action of pesticin differs from that of all other colicins in that it hydrolyzes the oligosaccharide strands of murein in the periplasm with a

lysozyme specificity (muramidase) (145). Pesticin-producing cells form an immunity protein that inactivates pesticin in the periplasm (126). The crystal structure of pesticin reveals two distinct structural domains connected by a short peptide linker (117). The N-terminal region contains two domains, one for receptor binding and one for translocation across the outer membrane, and the C-terminal domain is the active center. The latter is peculiar among colicins as it is the only activity domain with a structure similar to a known enzyme. The activity domains of all other colicin enzymes (nucleases, phosphatase) have no overall structural similarity to any known enzyme. The pesticin activity domain folds similarly to phage T4 lysozyme, although the sequence identity is only 13%. The pesticin activity domain can be replaced by T4 lysozyme, and the resulting chimera protein kills cells (117). The receptor binding and translocation domain of pesticin carries T4 lysozyme into the periplasm, where it hydrolyzes murein. We tested the unfolding of pesticin for translocation across the outer membrane by using introduced disulfide bridges. The cross-linked pesticin derivatives do not kill cells but retain in vitro murein hydrolysis activity. Cleavage of the disulfide bonds by reduction restores cell killing (114). The disulfide bridges prevent unfolding of pesticin during import across the outer membrane. Amino acid replacement of residues predicted to be located in the active center inactivate pesticin. The active center of pesticin is similar but not identical to the active center of T4 lysozyme (117).

HEMOLYSIN OF *SERRATIA MARCESCENS*

When I became interested in specific bacterial protein secretion systems, no secreted protein other than hemolysin was known in *E. coli* (60, 143). Colicins were released by partial lysis of cells. No specific colicin secretion system was uncovered by us or others. By contrast, species of the genus *Serratia* secrete a number of hydrolases. Therefore, we started to investigate *S. marcescens* protein secretion, and selected a protease for study (37, 138). Using spectroscopy, we tested for protease activity in culture supernatants of *S. marcescens* in a convenient assay of the release of hemoglobin from erythrocytes lysed by the protease without knowing that erythrocytes are resistant to most proteases. We observed lysis, but it was not caused by the protease. In earlier medical literature, hemolytic activity around colonies of *S. marcescens* had been rarely noted. Indeed, if hemolysis zones occurred, they were very small and were caused, as we now know, by small amounts of hemolysin, low stability, degradation by exoproteases, and limited diffusion. Next we examined whether the *S. marcescens* hemolysin was similar to the *E. coli* hemolysin, because we did not want to unnecessarily duplicate the advanced *E. coli* hemolysin studies (60, 143). The first indication of a difference was the Ca^{2+} independence of *S. marcescens* hemolysis (23), in contrast to *E. coli* hemolysis, which requires Ca^{2+}. Further studies demonstrated that the two hemolysins are completely different with regard to structure, activity, modification, and secretion (38). The *S. marcescens* hemolysin is determined by two genes arranged in tandem—*shlA* and *shlB* (128). *shlA* encodes the hemolysin, and *shlB* encodes an outer membrane protein responsible for ShlA secretion. Both proteins have N-terminal signal sequences for transport across the cytoplasmic membrane by the Sec system.

ShlB not only secreted ShlA but also activated ShlA. In an *shlB* deletion mutant, ShlA remains in the periplasm in an inactive nonhemolytic form (128, 137). Activation can be uncoupled from secretion by in vitro incubation of isolated periplasmic ShlA with purified ShlB, which results in hemolytic ShlA (81, 116). Uncoupling can also be achieved by ShlB mutants that secrete inactive ShlA. Secreted inactive ShlA is activated in vitro by wild-type ShlB. These data demonstrate that secretion and activation are two distinct functions of ShlB.

An ShlA N-terminal fragment of 242 residues (ShlA consists of 1,578 residues) complements inactive periplasmic ShlA to hemolytic ShlA when ShlA242 is provided in *trans*. ShlA242 must be secreted and activated by ShlB for functional complementation (81, 139). ShlA242 purified from

the periplasm of an *shlB* mutant cannot activate periplasmic ShlA. ShlA242 mimics secretion and activation of ShlA by ShlB but is not hemolytic since the C-terminal region of ShlA is required for pore formation in erythrocyte membranes (136, 137, 140). A deletion derivative of ShlA lacking residues 4 to 117 is complemented by the N-terminal ShlA fragment to active hemolysin. It is not activated by ShlB because it lacks the region required for hemolysis, which is provided by the ShlA fragment (116).

ShlA and ShlA242 change conformations when they are secreted and activated by ShlB. Secreted ShlA and ShlA242 are largely trypsin resistant. By contrast, cytoplasmic and periplasmic ShlA and ShlA242 are cleaved by trypsin to small fragments (146, 152). ShlA242 isolated from the periplasm, cytoplasm (ShlA242 without a signal sequence), and culture supernatant of an *shlB* mutant that secretes but does not activate ShlA242 has low electrophoretic mobilities in native polyacrylamide gels. By contrast, secreted and activated ShlA242 has a much higher mobility. In addition, circular dichroism spectra revealed structural differences (137, 146). The data indicate that inactive ShlA and inactive ShlA242 are less structured than the hemolytic isoforms. Furthermore, activation most likely does not involve a chemical modification through ShlB as activation does not change the molecular weights (137, 146). ShlA is secreted and folds into a hemolytic structure through its N terminus, which is secreted first. Folding into the active conformation probably thermodynamically drives secretion of ShlA through ShlB. Unfortunately, we have no structural data for these two proteins.

S. marcescens hemolysin secretion was the first example of the type Vb or two-partner secretion mechanism. The ShlB structure is predicted to be similar to that of FhaC (130), the outer membrane exporter of the *Bordetella pertussis* filamentous hemagglutinin, which was extensively studied as a type Vb model protein (88). ShlB is predicted to consist of a β barrel of 16 β strands with a central pore filled with an α helix and two polypeptide transport–associated (POTRA) domains in the periplasm (130).

Only active hemolysin firmly integrates into erythrocyte membranes (136) and forms pores of a defined size. It is cleaved by trypsin mainly at a single C-proximal site from the inside of the erythrocytes.

S. marcescens is involved in urinary tract infections, bacteremia, endocarditis, keratitis, arthritis, and meningitis. The hemolysin/cytolysin is a major virulence factor that is cytotoxic for eukaryotic cells and elicits inflammatory responses in leukocytes (82, 95, 107). ShlA is essential for *S. marcescens* pathogenicity in an animal model (99).

Regardless of the subject in which I became interested, it somehow strangely ended up to be an outer membrane protein problem. It was rewarding to actively participate in the transition from the once vague term cell wall to the now structurally precise outer membrane, with appreciation for its vast repertoire of functions.

PERSONAL NOTES

Although I devoted my adult life to science, life also took place outside of the laboratory. I married Marlies Arndt while still a doctoral student. We have two daughters and two grandchildren. Although some of our plans for our years together were at first shattered by the early outbreak of my wife's multiple sclerosis, which restricted social life and our passion for hiking in the mountains, we found solace in classical music concerts and theater performances. I appreciate very much her support over the years and her understanding of the long working hours of a scientist. With Friedrich Götz I played table tennis once a week, and with students I played volleyball. I was fortunate to partake in a yearly one-week excursion with coworkers and students to visit one of the Swiss pharmaceutical companies and to hike in the southern Swiss Alps.

For both key positions in my scientific career, namely, leader of an independent research group at the Max Planck Institute of Molecular Genetics in Berlin (associate professor level) and chair of the microbiology department at the University of Tübingen, I was asked to apply. Both positions were very advantageous because they provided a stimulating atmosphere and sufficient financial support and space. My scientific achievements were honored by the Robert Koch Prize, the most prestigious award in Germany, and memberships in Leopoldina (the German National Academy of Sciences) and the Heidelberg Academy of Sciences. The Medical Faculty of the University of Würzburg awarded me the title Dr. med. hc.

For most of my time at the university, microbiology was the only subject for students interested in cell biology because genetics, botany, and zoology research was conducted on the organismic level. Therefore, there was no shortage of gifted doctoral students and postdocs who were interested in my research projects. After completion of the doctoral thesis, most students went into industry. A number, however, remained in academia and became university professors, namely, Andreas Bäumler, Albrecht Bindereif, Valerie Bosch, Roy Gross, Anton Hartmann, Ralf Koebnik, Wolfgang Köster, Tobias Ölschläger, Roland Schönherr, Avijit Pramanik, Elmar Schiebel, Uwe Stroeher, and Renee Tsolis. The highly successful coworkers and postdocs in academia were Karl-Heinz Altendorf, Wolfgang Bessler, James Coulton, Robert Hancock, Knut Heller, Keith Poole, David Smajs, and Peter Wookey. As long-term guests on sabbatical, Jorge Crosa, Peter Howard, and Robert Kadner shared their great scientific experience with us. In particular, Robert Kadner taught us bacterial genetics. I thank them all for substantial contributions while working in my lab. Klaus Hantke accompanied me through most of my scientific life, and through his masterly and imaginative work he contributed much to the scientific success of the department.

When I retired from my university position in 2007, the Max Planck Society offered me the newly created position of Max Planck Fellow, which allowed me to concentrate on research without responsibilities related to teaching, administration, and grant applications. I am indebted to the German Science Foundation for continuous support. I thank the Max Planck Society for giving me the opportunity to continue research after my retirement from the university, and I am especially thankful to Andrei Lupas for his generous hospitality at the excellent Department of Protein Evolution at the Max Planck Institute for Developmental Biology. I am very grateful to the many colleagues who accompanied me during my scientific journey. I am indebted to my colleagues Friedrich Götz, Wolfgang Wohlleben, and Hans Zähner for their cooperative teamwork over the years.

SUMMARY POINTS

1. *E. coli* lipoprotein was a discovery of much wider significance than originally was anticipated. The specific lipid structure is contained at the N terminus of a multitude of proteins in most bacteria and serves to tether the proteins to membranes to fulfill a large variety of functions. Lipoprotein-derived lipopeptides stimulate the immune system and are examined as immune therapeutics.
2. A major effort was devoted to the energy-consuming translocation of substrates, iron compounds, sugars, and protein toxins across the outer membrane, which is devoid of an energy source. Structure and function of the common energy coupling device between the outer membrane and cytoplasmic membrane were extensively studied.

3. A new type of transcription control, starting at the cell surface, was uncovered. The complete signaling cascade transfers the signal across the outer membrane, the periplasm, and the cytoplasmic membrane. The receiver in the cytoplasm was first fully elucidated in the *E. coli* Fec system.
4. Sequence determination of colicins revealed structures composed of three functional domains that are intermixed to form new colicins. Peptidoglycan was the new target of two colicins, and a periplasmic chaperone activated an imported colicin.
5. The first example of a type Vb secretion system was found where an outer membrane protein secretes and activates a hemolysin.
6. The findings described here offer attractive targets for the development of new antibacterials.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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