Bacterial protein secretion – a target for new antibiotics?

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The heavy use of antibiotics over recent decades has resulted in widespread resistance of bacteria to many drugs. Overcoming resistance requires new approaches to antibiotic development, including the exploitation of new targets in the bacterial cell. Protein secretion is essential for bacterial cell growth and virulence, so it could be a suitable target for new therapeutic agents.

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The first 'miracle drugs', the sulfonamides and penicillins, were introduced in the late 1930s and the 1940s to subdue bacterial pathogens. It was commonly believed that the use of this type of drug could soon lead to the permanent control of bacterial disease. That optimism now seems sadly naive. Although morbidity and mortality from bacterial diseases are much lower than in the pre-antibiotic era, bacterial diseases still kill tens of thousands of people in the United States each year and remain the major cause of death in underdeveloped nations. Microorganisms have survived billions of years through rapid adaptation and evolution, and the intense selective pressure exerted globally by the heavy use of antibiotics has led to widespread dissemination of resistance mechanisms among common pathogens [1]. Drug-resistant bacterial strains for which there are few chemotherapeutic options are a particular danger in institutional settings (e.g. hospitals and nursing homes) where there are large populations of immunocompromised individuals, such as those with AIDS. There is an urgent need for new antimicrobial drugs to address these challenges.

Antibiotics that are currently in widespread clinical use target diverse cellular processes such as protein synthesis (targeted by the aminoglycosides, tetracyclines and macrolides), cell-wall synthesis (β lactams and glycopeptides), DNA supercoiling (quinolones) and folate synthesis (sulfonamides). The antibiotics introduced to clinical practice in the past 20 years have been almost exclusively structural variants of members of established drug families. The newer variants generally have improved efficacy or have broader action spectra than established drugs, or are able to overcome some resistance mechanisms. Although this approach to drug development has been reasonably successful, microbes have made their ability to evolve resistance to each new modification abundantly clear. Consider the situation with antibiotics that are inhibitors of cell-wall synthesis. Clinical isolates of penicillin-resistant *Streptococcus pneumoniae*, the most frequent cause of bacterial pneumonia, increased 60-fold in the United States in 1987–1992 [2]. Hospital-acquired infections with methicillin-resistant *Staphylococcus* and vancomycin-resistant *Enterococcus* have increased dramatically worldwide [3]. These observations suggest that identifying completely new classes of antimicrobial compounds which act on new targets may be necessary to cope with pathogens in the future [4]. A potential target within the bacterial cell should be essential for cell survival or growth, and should be broadly distributed and highly conserved in bacteria but not in humans. Alternatively, functions that are necessary only for pathogenic behavior could be targeted. To illustrate the type of physiological processes that fit these criteria, we will focus on the export of proteins across cellular membranes.

The pathways of bacterial protein secretion

Cells interface with their environment through proteins that are displayed on, or translocated through, the cytoplasmic membrane. Functions that rely on extra-cytoplasmic proteins include nutrient uptake, chemosensing, motility, adhesion and cell-wall biosynthesis. Roughly one fifth of the proteins in the average bacterial cell are located partially or completely outside the cytoplasmic membrane [5]; the ability to localize these proteins is essential for growth and viability. As purified phospholipid membranes are impermeable to polar, charged polymers such as proteins, the most fundamental issue in protein export is to understand how the hydrophobic interior of the lipid bilayer is traversed. Most extra-cytoplasmic proteins initially have an amino-terminal signal peptide of 20–30 residues and cross the cytoplasmic membrane via the general secretory pathway (GSP) [5,6]. The GSP has been studied most extensively in *Escherichia coli*, a gram-negative bacterium; bacteria of this class have a physically distinct outer membrane surrounding the cytoplasmic membrane. The two membranes are separated by a thin peptidoglycan layer and a compartment called the periplasm. The GSP of gram-negative bacteria is responsible for translocating proteins into the periplasm; other mechanisms are then required for crossing the outer

Figure 1
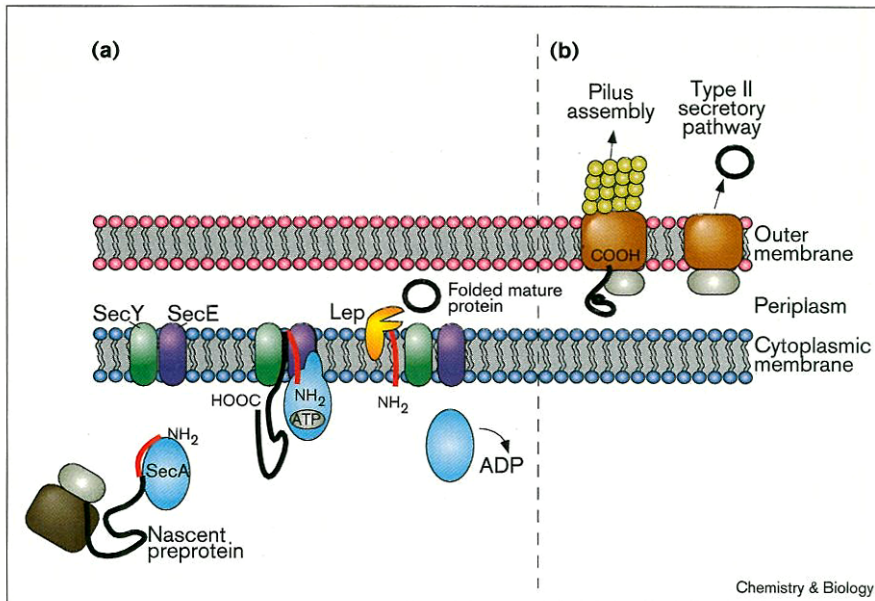
Protein-secretion pathways of the outer and inner (cytoplasmic) membranes of gram-negative bacteria. **(a)** An outline of the general protein-secretion pathway of *E. coli*. SecA binds to the amino-terminal signal peptide of preproteins as they are translated. In the presence of ATP, SecA is able to associate with the membrane-pore complex, SecE–SecY, and to partially insert into the membrane, allowing the hydrophobic region of the signal peptide to insert. The remainder of the preprotein is then threaded through the SecE–SecY pore. The membrane-associated leader peptidase (Lep) cleaves the signal peptide, releasing the mature protein into the periplasm. ATP hydrolysis allows SecA to be released from the membrane complex to recycle. A more complete description of the *E. coli* GSP can be found in [6]. **(b)** Two supplementary pathways used by gram-negative bacteria for outer-membrane export of proteins that have been delivered to the periplasm by the GSP. See references [5] and [21] and text for details.

membrane (see below). In contrast, gram-positive bacteria lack an outer membrane so the GSP is sufficient for the release of proteins from the cell.

The core of the GSP in *E. coli* consists of three proteins — SecA, SecE and SecY — each of which is necessary for viability and for reconstitution of protein secretion *in vitro* [5,6]. A simplified outline of the *E. coli* GSP is shown in Figure 1a. SecA binds to the signal peptide of a preprotein as it exits the ribosome [7,8]. SecA then partially inserts into the cell membrane, driving the signal peptide into the membrane at or near a pore comprised of SecE and SecY [9,10]. SecA is an ATPase, and ATP binding and hydrolysis stimulate the insertion and release of SecA from the SecE–SecY membrane-pore complex [11–13]. The pore, the structure of which has not yet been determined, facilitates the passage of export-competent (i.e. non-folded) preproteins through the membrane. Translocation of pre-proteins appears to be driven across the membrane by energy derived from SecA-mediated ATP hydrolysis and the transmembrane proton gradient [14]. ATP hydrolysis is clearly involved early in the process and can suffice for translocation *in vitro* but a transmembrane proton gradient dramatically increases the rate of translocation *in vitro* and is necessary for secretion *in vivo* [15]. As the preprotein crosses the membrane, the signal peptide is cleaved by a peptidase (leader peptidase) located on the outer face of the membrane. Signal-peptide cleavage is not required for membrane translocation *per se* but *E. coli* strains lacking leader peptidase die quickly, presumably because an uncleaved signal-peptide anchor interferes with the function of essential extra-cytoplasmic proteins [16].

Secreted proteins are often important pathogenic 'virulence factors' that are intimately involved in host colonization [17]. Cholera toxin, diptheria toxin and tetanus toxin are well-known examples of secreted bacterial virulence factors. These proteins are typically not necessary for growth of the pathogen outside the host organism [18,19]. Secreted virulence factors, like cell-surface proteins, have often been used or proposed as vaccine antigens; less appreciated, however, is the possibility that the systems used for virulence-factor secretion could be targets for therapeutic agents to prevent colonization or aid in clearance of specific pathogens. Some virulence factors, such as the *Neisseria gonorrhoeae* IgA protease, are released into the periplasm by the GSP and subsequently cross the outer membrane without assistance from any additional secretory system [20]. More commonly, specialized outer-membrane channels facilitate the release of periplasmic intermediates (Figure 1b). One such system is used for the assembly of pili in enteric bacteria [21]. Pili are fibrous structures extending from the cell surface that are used for attachment to host cells. Another pathway (often termed type II) is used by the plant pathogen *Erwinia* for secretion of the enzymes pectate lyase and cellulase which are used in plant-cell-wall degradation; this pathway is also used by the mammalian pathogen *Pseudomonas aeruginosa* to secrete cytotoxin A, lipases and proteases [5].

Some secreted proteins cannot start their journey via the GSP because they lack an amino-terminal signal peptide. These proteins use alternative secretory pathways to traverse the gram-negative cell envelope (Figure 2). The alternative pathways include the type I secretory pathways,

Figure 2

Non-GSP-dependent protein-secretion pathways of gram-negative bacteria. The type I and type III secretory pathways are shown. The ATPase component (the ABC transporter; purple) of the type I pathway is located in the cytoplasmic (inner) membrane and recognizes substrates via a carboxy-terminal recognition sequence. Substrates of the type I pathway cross the entire envelope in a single step via the ABC transporter, periplasmic linker (light orange) and outer-membrane pore (green) so there is no free periplasmic intermediate. The cytoplasmic membrane component of the type III pathway (yellow) is probably composed of several subunits. Type III pathways have a cytoplasmic ATPase subunit that is thought to recognize substrates via a signal located at or near the amino terminus.
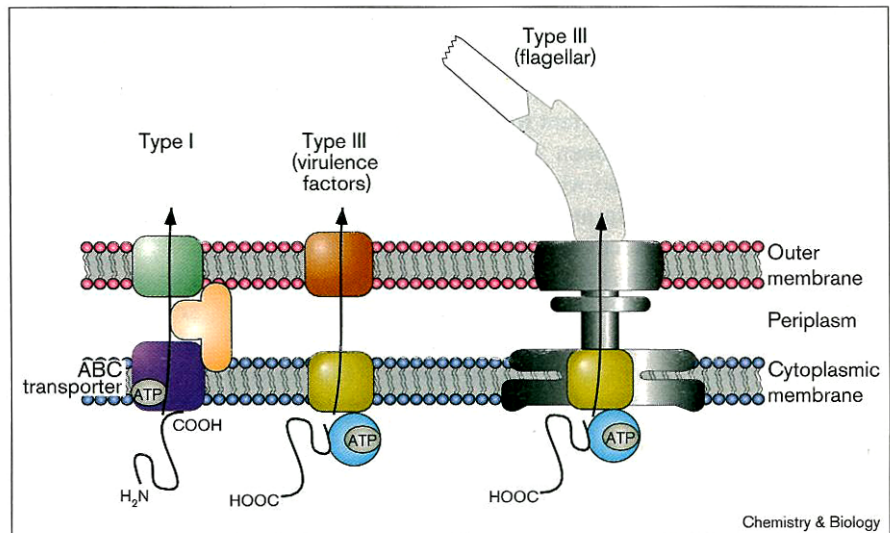
which have three major components — an inner-membrane channel, a periplasmic linker and an outer-membrane pore [22]. Proteins secreted by type I pathways have a non-cleavable carboxy-terminal secretion signal which is recognized a protein of the inner membrane. This inner-membrane protein has an ATP-binding cassette that is characteristic of a large family of membrane proteins called ABC transporters [23]. These transporters are widely distributed in both prokaryotes and eukaryotes, and are involved in processes as diverse as sugar uptake (the maltose transporter in *E. coli*), antigenic peptide transport (the TAP transporter in the endoplasmic reticulum of human lymphocytes) and drug efflux (the mammalian multidrug resistance protein). How the ABC-transporter protein of type I protein secretion systems couples ATP hydrolysis to the translocation of substrates across the inner membrane is unknown but, as with the GSP, the transmembrane proton motive force is also required for secretion [24]. It has been proposed that translocation of proteins by type I secretory pathways across both membranes of the Gram-negative cell envelope is a concerted process [24].

A second non-GSP-dependent secretory pathway is the 'type III' pathway that is used by some gram-negative pathogens to inject virulence factors directly into mammalian cells [25]. For example, this type of pathway is involved in the secretion of antiphagocytic proteins by *Yersinia* species (including *Yersinia pestis*, the agent of bubonic plague), and in the secretion of host-cell invasion factors by bacteria of the enteric pathogenic genera *Shigella* and *Salmonella* [25]. This type III pathway is more complex than the type I pathway; it includes a cytoplasmic ATPase, seven probable inner-membrane proteins and at least one outer-membrane protein. The outer-membrane component

is homologous to the outer-membrane channel of the type II pathway (Figure 1) [26], and is therefore an attractive target for inhibitors that could block two distinct secretory pathways without ever crossing the cytoplasmic membrane.

The secretion of the protein subunits that are used to build the bacterial flagellum also depends on a type III pathway. The bacterial flagellum acts as a screw propeller that drives cells through aqueous environments [27,28]. The majority of eubacterial species can build flagella, so the flagellar secretory system is widely distributed. Flagellar assembly and function often have an important role in bacterial infections, because many pathogens require motility for host colonization [29]. The flagellum is assembled outwards from the cytoplasmic membrane [30]; subunits are secreted via a gated type III channel thought to be located in the center of the ring at the base of the flagellum [28] (Figure 2). Secreted subunits pass through a central channel in the growing axial structure(s) to be assembled at the tip. Because subunits traverse the outer-membrane via the axial channel, there is no need for the separate outer-membrane channel found in other secretory pathways.

Bacterial pathogens have clearly evolved diverse attack strategies involving secreted virulence factors [17]. Drugs targeting secretory pathways other than the GSP would be suitable primarily for use against gram-negative pathogens, but such specialized antimicrobial agents could nevertheless be valuable, as can be illustrated by considering the cases of *Pseudomonas aeruginosa* and *Helicobacter pylori*. *P. aeruginosa* is an opportunistic pathogen that can cause fatal wound and burn infections, and chronically infects the lungs of cystic fibrosis patients, eventually resulting in death [30]. It is notoriously resistant to standard antibiotics

and secretes important virulence factors through all the pathways described above. *H. pylori* colonizes the mucosal lining of the stomach, and is the ultimate cause of most gastric and duodenal ulcers. Treatment of *H. pylori* infection typically requires a combination of three antibiotics; clinical isolates that are resistant to one or more of the component antibiotics have been isolated. The hundreds of millions of people infected with *H. pylori* worldwide represent a huge market for improved therapeutic agents. The importance of protein secretion to *H. pylori* pathogenesis is demonstrated by observations that virulence is correlated with a unique cluster of genes encoding components of multiple secretory pathways [31]; the critical VacA cytotoxin is a secreted protein; and flagellar motility (and hence flagellar subunit secretion) is necessary for establishment of *Helicobacter* infection in animal models [32]. Narrow spectrum antibiotics targeting *P. aeruginosa* or *H. pylori* could have profound effects on human health.

Developing drugs that inhibit protein secretion
What are the prospects for developing antimicrobial agents that target the GSP? The components of the GSP that have relatively defined biochemical activities, such as leader peptidase and SecA, may offer the best targets for *in vitro* approaches to drug development. Because the active site of leader peptidase is outside the cytoplasmic membrane, agents directed against this enzyme need not cross the membrane to be effective. Leader peptidase is a novel type of serine protease [33], and one group of compounds that inhibit the catalytic activity of the *E. coli* enzyme has already been described [34]. The structures of SecA and leader peptidase have not been solved, so there is no opportunity at present for rational drug design. Nevertheless, functional assays can be adapted to high-throughput screening for inhibitors. Synthetic chemical libraries, natural product libraries and combinatorial chemistry could provide a vast array of compounds for testing.

Two compounds that inhibit SecA function are known — sodium azide and phenylethyl alcohol [35,36]; azide affects ATPase activity but has little future as an antibiotic because of its general toxicity. SecA ATPase activity is insensitive to inhibitors of phosphorylation and vacuolar-type ATPases and the F_0F_1 ATPase; if highly specific inhibitors of the SecA active site could be identified generalized toxicity might be avoidable. The signal peptide is involved in several stages of the secretion process. Stable peptide analogs might be developed that could interfere with the binding of SecA (or the SecE–SecY complex, or even the leader peptidase) to the natural signal peptides of preproteins.

As the core components of the GSP are known and secretory activity can be reconstituted using *E. coli* GSP components in a defined cell-free system, there is the potential to use this assay to identify secretion inhibitors. Alternatively,

screening strategies have been proposed that use intact *E. coli* cells which have been genetically engineered to show inhibition of GSP activity or which may be used to positively select for compounds that reduce secretion [37]. The need for new drugs that are active against gram-positive pathogens is especially pressing, so it would probably also be useful to develop screens using a well-characterized and genetically manageable gram-positive species such as *Bacillus subtilis* or *Staphylococcus aureus*.

Developing approaches that target alternative protein-secretion pathways will require a greater understanding of the structure and function of these pathways. Essentially, the only components for which functions have been tentatively assigned are the putative outer-membrane channels and the proteins with ATP-binding motifs such as the ABC transporter of the type I system. What the other conserved components of the pathways do is as yet unknown. How are the inner- and outer-membrane channels formed and regulated? Is passage through both membranes a concerted process with no periplasmic intermediate? How are secretion signals in substrate proteins recognized? How are substrates driven through membranes? To answer these questions, and to develop strategies for blocking protein secretion in general, will require a combination of biochemical and genetic analysis, and an integration of cellular and molecular biology. Protein secretion is only one of many possible targets for the development of new antibiotics; our ability to deal with bacterial diseases in the 21st century may depend on the enthusiasm and ingenuity we apply to the task of drug development around these novel targets.

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