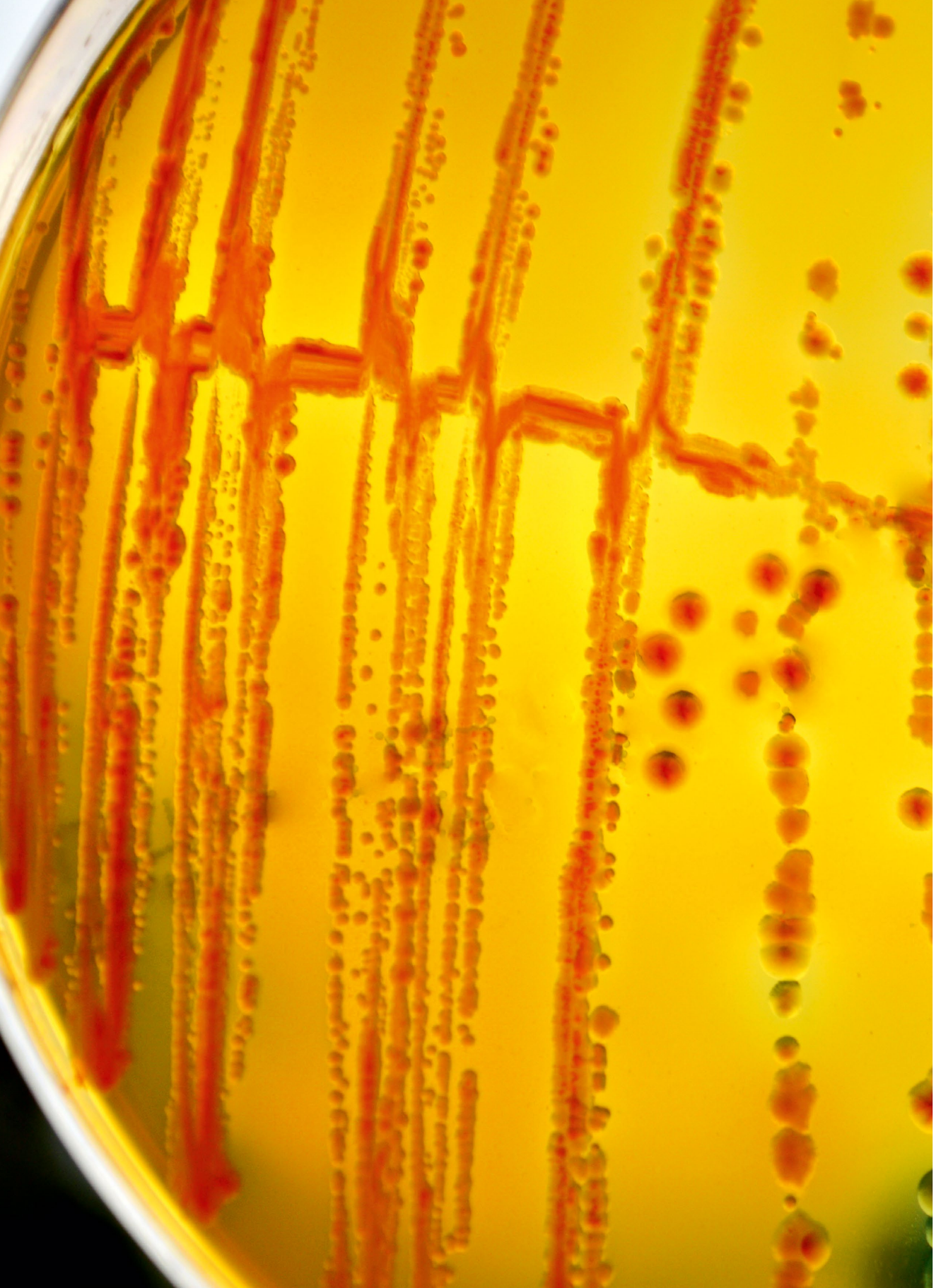


Working to win the war against Gram-negative pathogens

With the rise of the antimicrobial resistance era, it is becoming more important than ever to find new ways to combat pathogens. **Professor Kathleen Postle's** work at the Pennsylvania State University does just this. Her research group focuses on using iron transport and signal transduction in Gram-negative bacteria as targets for the development of novel antibiotics.

There are many paths by which bacteria can develop antibiotic resistance, an increasing problem across the world. To combat this problem, Professor Kathleen Postle is working to understand a molecular mechanism that is crucial to the survival of Gram-negative bacteria. If the complex interactions of this pathway can be uncovered, it would not only add to our understanding of the biology of bacteria but may also lead to the development of new antibiotics.

THE DILEMMA OF HAVING AN OUTER MEMBRANE

All Gram-negative bacteria display an outer membrane that protects them from degradative enzymes, environmental agents and antibiotics. They are more resistant to antibiotics than Gram-positive bacteria because of their cell envelope structure, consisting of a peptidoglycan layer sandwiched between an inner cell membrane and a concentric bacterial outer membrane. Indeed, 2 out of 3 infectious agents on the Center for Disease Control Urgent Threat list are Gram-negative bacteria. However, this outer membrane comes at a cost. Many molecules and complexes that the bacteria need to acquire exceed the standard diffusion limit of the outer membrane and are present at such low concentrations that, in any case, diffusion would not supply sufficient amounts to sustain growth. Gram-positive bacteria, named for their ability to show positive on a Gram-stain test, have a thicker peptidoglycan layer but lack the protective outer membrane and also the dilemma imposed by it.

THE FIGHT FOR IRON

Iron is an essential nutrient for virtually all bacteria and other forms of life. Iron is the fourth most abundant element on the Earth's surface, but inside the human body, there is a constant tug-of-war for iron between host and pathogenic bacteria. As a type of innate immunity, hosts keep free serum iron levels low by binding elemental iron to host proteins such as transferrin. To be an effective pathogen, bacteria must work out a way to get sufficient iron from the host that they can survive.

GRAM-NEGATIVE BACTERIA AND TonB

Almost all Gram-negative bacteria synthesise molecules, called siderophores (Greek for 'iron carrier') with very high affinities for iron. The siderophores are sent out to find and bind iron atoms in the environment. The class of outer membrane proteins known as TonB-dependent transporters (TBDTs) are the solution to the dilemma imposed by the outer membrane. Their binding sites, which have very high affinities for their siderophores, populate the external surface of the Gram-negative bacteria. While they are efficient collecting systems for the iron-bearing siderophores, energy is required to break the tight bond between Fe-siderophores and the TBDTs. This energy comes from the inner membrane and must be supplied to the outer membrane, which lacks the ability to generate its own energy.

It is the molecular mechanism of this entirely novel type of transport that fascinates Prof Postle. Energy generated in the cytoplasmic membrane as an ion (proton) gradient is somehow converted into a mechanical response at the outer membrane. Current information indicates that energy is transduced by a series of conformational changes in the

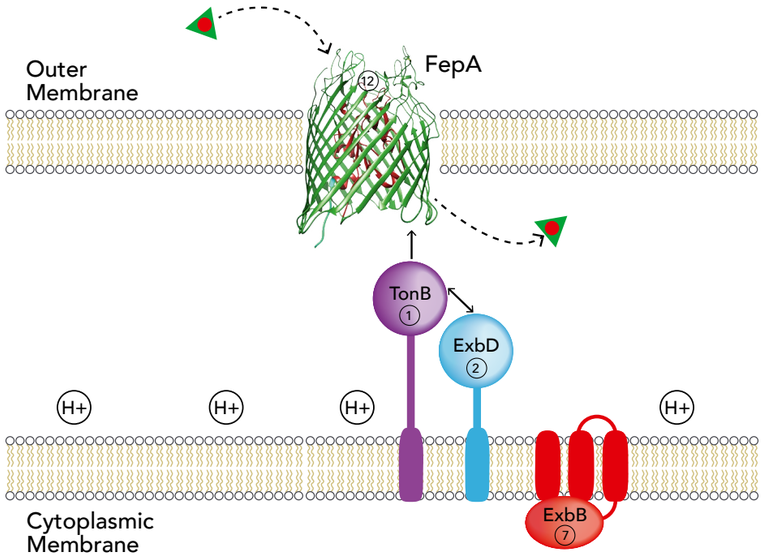
cytoplasmic membrane TonB protein, which are transmitted to the outer membrane transport proteins by direct physical contact.

UNDERSTANDING THE TonB SYSTEM

Escherichia coli is the most well studied Gram-negative pathogen in the world; therefore, it provides an ideal tool for the Postle team to work with. The TonB system involves several proteins (TonB, ExbB and ExbD) that harvest energy from the inner cytoplasmic membrane and transmit it to specialised proteins in the outer membrane (TBDTs). Whilst other Gram-negative bacteria may have multiple sets of TonB proteins, *E.coli* only has one, making it much easier to elucidate the functions of these proteins and the genes that encode them.

To investigate the mechanism of TonB-dependent energy transduction, Prof Postle examined the dynamics of TonB homodimerisation (the process of converting of two identical single molecules to a homodimer) during an energy transduction cycle. Postle and her team discovered that if a particular residue in the transmembrane domain of TonB is mutated at one end of the protein, then homodimerisation can no longer occur at the opposite end, and TonB activity is prevented. This sits alongside her previous work to show that this homodimeric TonB is required to form a TonB-ExbD heterodimer, which renders the TonB ready for interaction with a TBDT in the outer membrane.

Determination of protein crystal structures has been informative in many fields. It was, therefore, a surprise to the Postle team to discover that while TonB is undoubtedly a homodimer, the conformations seen in the solved dimeric crystal structures of TonB do not exist *in vivo*. As more became known, this was perhaps to be expected, given the dynamic set of interactions that TonB must go through. During an energy transduction cycle, the periplasmic domain of TonB has to bind to itself, change and bind to ExbD, and then change again to bind to FepA. To accomplish this, it may be that ExbD helices need to move apart or rotate, as those of a similar protein, TolR, appear to do and that this process is accomplished by the interaction of



The known proteins in the TonB system. FepA is the OM transporter (TBDT) for the siderophore enterochelin (also called enterobactin) (triangles). Enterochelin is synthesised and excreted by *E. coli* to capture iron. TonB, ExbD, and ExbB are integral CM proteins that harness the proton gradient (H+) of the CM for the active transport of ferric enterochelin across the OM through FepA. The numbers in circles on each protein represent the per-cell ratios of the proteins under iron limiting conditions. The relatively thin layer of peptidoglycan between the OM and CM is not shown. Based on an illustration from the *American Society for Microbiology's Journal of Bacteriology*, Volume 197 Number 21, 2015, 3433 – 3445, doi:10.1128/JB.00484-15.

ExbB domains with other, as yet unidentified proteins. There is good evidence for the presence of one or more mystery proteins in this system. This, in addition to the complexity of the system – two membranes and at least four different proteins – means that Prof Postle and her team carry out the majority of their experiments in whole bacteria. They engineer mutations in each of the four TonB system proteins, and use results from several different mechanistically informative assays to understand this novel system at the molecular level.

THE TonB SYSTEM AS A NEW ANTIBIOTIC TARGET

Development of antibiotic resistance is a continuing arms race. The drug industry has mostly been producing antibiotics that are variations on previously developed antibiotics due to the difficulty of identifying new viable targets. Almost as fast as antibiotics are re-engineered, the bacteria become resistant to them. New targets for antibiotics are desperately needed – few if any have been implemented for Gram-negative bacteria in the past ~ 30 years. Importantly, Gram-negative pathogens lacking a functional TonB system are widely compromised in their ability to cause disease (see the 2016 review by Sheldon et al. PMID:27227297).

The role of the TonB system is not just limited to iron-siderophore complexes. TonB systems in other species of Gram-negative bacteria are also used to transport molecules such as zinc, nickel, or complex sugars, all of which can support the growth of different pathogens in human hosts. Furthermore, some of the most effective Gram-negative pathogens use the TonB system to steal iron right out of transferrin, the very protein humans use to protect their iron stores.

The TonB system is an especially attractive target for a novel antibiotic since many of its components reside in the periplasmic space. An antibiotic targeting the TonB system needs only the chemical properties necessary to traverse the outer membrane (hydrophilicity) and not inner membrane, (hydrophobicity) as well. The Postle group is actively working to develop leads for novel antibiotics. Since it is never clear where exactly the best targets will be, they are also working to broadly understand the molecular mechanism of TonB-dependent energy transduction, and discover unique insights into all signal transduction processes.

TonB protein is an attractive target because it is the bottleneck through which all of the high-affinity iron transport in *E. coli* flows

Q&A

How do Gram-positive pathogens deal with iron transport?

The name of the game for both Gram-positive and Gram-negative pathogens is herding an iron atom or an iron-containing molecule to the cytoplasmic membrane, where it can subsequently be actively transported into the cytoplasm, energised by ATP-dependent transporters. Gram-positive bacteria do not have or need a TonB system. Their main problem is diffusion of nutrients through the rather thick but porous layer of peptidoglycan, that surrounds their cytoplasmic membrane. One strategy pathogenic Gram-positives use is to lyse red blood cells, grab the iron-containing heme molecules that spill out into their environment, and escort them through the peptidoglycan using a series of proteins with increasing affinities for the heme until they reach the cytoplasmic membrane. Unlike the TonB system, the escort process does not require energy.

If a therapeutic was designed to target the TonB system, how likely is it that bacteria would eventually evolve resistance to this too?

TonB protein is an attractive target because, unlike the individual TBDTs, it is the bottleneck through which all the high-affinity iron transport flows in *E. coli*. Thus, knocking out TonB activity in *E. coli* or any other Gram-negative bacterium where it is the sole such protein, effectively knocks out the ability of the bacteria to obtain iron through any of the TBDTs (*E. coli* has 7-9). If the bacteria developed a resistance to an antibiotic targeting TonB, it could be because the TonB protein was not functional. However, by the very act of protecting themselves from the antibiotic in this way, they would be cutting off their ability to compete with the host for iron. We imagine that we'd get them either way.

What approach did you use to sequence the first TonB gene and how have you seen technologies, such as DNA sequencing change over the years?

We used a technique called Maxam and Gilbert sequencing. It was elegant but laborious, requiring the ability to separate radioactively labelled, single strands of DNA, and then treat them chemically with four different protocols to ultimately determine the sequence of bases by gel electrophoresis and autoradiography on X-ray films. After that, my lab moved to Sanger's dideoxy chain termination method of sequencing – much easier to do but similar analysis. I used to make everyone determine their mutant DNA sequences in our lab. It has now become so inexpensive and routine that we send our Sanger samples off to the Huck Life Sciences Genomics Core Facility at Penn State. DNA sequencing of genomes – a remarkable achievement given where we started – has moved to Next Generation sequencing with Illumina.

Do you aim to deduce the in vivo crystal structure of TonB and its partners, if time and funding allow?

Our talents lie in teasing apart the *in vivo* mechanism of this highly dynamic system. We are not crystallographers, and leave that analysis to others. Considering that the proton gradient of the inner membrane is required for correct interaction between TonB and the TBDTs, or TonB and ExbD, it may be that crystallography cannot reveal the *in vivo* crystal structure of TonB and its partners, since, by its very nature, crystallisation occurs with purified proteins in the absence of any proton gradient. Crystallography has, however, already provided valuable information about the structure of FepA and monomeric ExbB.

Is the same mechanism used to transport vitamin B12 across the outer membrane, as is used for iron siderophore transport?

Essentially yes. Vitamin B12 binds to a TBDT that is specific to it. After that, the protein partners (TonB, ExbB, ExbD) are the same. A different, but related, ATP-dependent transporter is used to move the vitamin B12 from the periplasm across the inner cell membrane into the cytoplasm.

Detail

RESEARCH OBJECTIVES

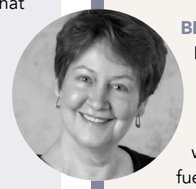
Dr Postle's research group is studying a form of signal transduction in *Escherichia coli*: how cytoplasmic membrane energy (protonmotive force) is transduced by the TonB protein to customised transport proteins in the outer membranes that characterise Gram-negative bacteria. Current information indicates that energy is transduced by a series of conformational changes in the cytoplasmic membrane TonB protein, which are transmitted to the outer membrane transport proteins by direct physical contact. An understanding of the molecular mechanism of TonB-dependent energy transduction provides a tantalising target for the development of novel antibiotics that can prevent pathogens from obtaining iron. It will also provide unique insights into all signal transduction processes.

FUNDING

National Institutes of Health (NIH)

COLLABORATORS

- Scott Showalter, Penn State University, Pennsylvania
- Ray A. Larsen, Bowling Green State University, Ohio



BIO

Kathleen Postle cloned and sequenced the DNA of the first *tonB* gene ever, back when that was difficult to accomplish. Her work in this system continues, fuelled, as it has been throughout her career, by a dedicated and brilliant cohort of enthusiastic lab members. She is a Fellow of the American Academy of Microbiology and of the American Association for the Advancement of Science.

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