New understanding of how bacteria build their protective cell wall

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Using a series of chemical and genetic tricks to interrogate a dizzying cast of characters involved in the process of building a cell wall, researchers believe they have discovered the hidden identity of a key enzyme involved in flipping precious cargo from the inside to the

outside of a bacterial cell.

It sounds like a hardboiled mystery, but it's the results of research published this month in *Science* from a team led by microbiologists at Harvard Medical School and Ohio State University.

The bacterial membrane is like an overinflated balloon that would burst without the cell wall, a molecular cage that surrounds the membrane and gives the membrane integrity in the face of the great osmotic pressure exerted on free-living, single-cell organisms. The building blocks of the wall are made inside the cell and need to be secreted through the membrane to the exterior to construct the wall where it's needed. The keys to the hidden passageways that export these building blocks through the membrane have remained mysterious, despite repeated efforts to bring them to light.

Cell wall construction is an important target for antibiotics such as penicillin and bacitracin. When these drugs interfere with cell wall production, bacteria burst and die. Growing concern about the increase in antibiotic resistance has fueled the search for alternative weapons in the fight against resistant bacteria. Because of its prior success as a target, researchers have been trying to learn more about cell wall assembly to discover new ways of blocking it for therapeutic development.

"The more you know about a process, the easier it is to break it," said Thomas Bernhardt, associate professor of microbiology and immunobiology at HMS.

For many years, scientists have known how the basic building blocks of the cell wall are assembled inside the cell cytoplasm, and how the blocks are stitched together on the cell surface to construct the wall. What has remained puzzling is how the bricks needed to build the cell wall are

transported across the membrane to the outside, where the wall is assembled.

The wall building blocks consist of sugar molecules linked to a lipid carrier that anchors them to the cell membrane. It has long been thought that bacterial cells possess a transport protein that promotes a flip-flop reaction to move the lipid-linked building blocks from one side of the membrane to the other. However, the identity of this transporter, known as a flippase, has remained mysterious. But now a team of scientists from HMS and OSU have found evidence that the flippase is a protein called MurJ. The researchers are hopeful that this discovery could eventually lead to a new category of antibiotics that block the flipping reaction.

A few candidates for the flippase have been considered, but researchers have been in disagreement over which candidate truly catalyzes the flip-flop. To resolve the issue, a method of detecting the reaction in living cells was needed. However, this was easier said than done.

"It's a subtle change, shifting molecules from one side of the membrane to the other," Bernhardt said. "We needed an exquisitely specific and sensitive assay."

In addition to being small in scale, the cell wall building blocks are rare. In the experiments that the researchers ran, only a few thousand of the millions of lipid molecules in the cell membrane are cell-wall related. Bernhardt's team developed a method using colicins, protein toxins that work like a molecular razor blade, slicing the sugar blocks off their lipid anchors. This releases free sugar blocks not normally produced by cells into the medium that the bacterial cells are floating in. Because the toxins can't penetrate the membrane, they can only snip sugar blocks on the outside. If the sugar blocks are outside, that shows that flipping is underway.

"The first time we were able to see the colicin product I was incredibly excited because I knew we were detecting the flipping reaction," said Lok To (Chris) Sham, a postdoctoral fellow in Bernhardt's lab.

The next step was to use a combination of genetic and chemical techniques to test what happened when the candidate flippases were switched off.

Co-author Natividad Ruiz and her laboratory at OSU had developed strains of bacteria with mutated versions of MurJ that were uniquely susceptible to a chemical that reacts with certain amino acids in proteins. When the chemical was introduced to populations of the bacteria with the mutated MurJ proteins, none of the colicin- generated sugar blocks were found in the medium. This finding indicated that flipping stopped in the absence of MurJ, revealing that MurJ was the hidden flippase.

The team performed similar experiments to test whether turning off the other proteins suspected of being the missing flippase would stop flipping, but in those tests, the bacteria continued to flip sugar blocks.

Each experiment needed to happen quickly. Sham recalled adding the reagent at his bench and running across the hall to the lab's centrifuge to harvest the cells before they could burst and thus destroy the evidence that he needed to collect.

The researchers added that finding a way to make MurJ work as a flippase in test tube models will be important in the next phase of research, as they work to purify MurJ and to monitor the mechanism of the flipping process more closely.

The experiments were done in E. coli, but the researchers suspect this process is common to all bacteria with cell walls.

"We now know what protein is involved in the process," Bernhardt said. "Next we need to delve into the mechanics of the operation, the structure of MurJ and how it promotes transport so that we can plug it with small drug molecules to interfere with the flipping process."

As is often true in the complex world of microbiology, solving one case only leads to new mysteries.

More information: Lok-To Sham, et al. "MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis." *Science* 11 July 2014: Vol. 345 no. 6193 pp. 220-222. DOI: 10.1126/science.1254522

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