Insight into a Deadly Disease

COMPARED with plague and anthrax, tularemia is less well known to the general public, but recent outbreaks and its potential as a bioterrorism agent have brought the disease into the limelight. Sometimes called rabbit fever, tularemia primarily infects small- to medium-size mammals such as hares, prairie dogs, and rodents. However, the disease can be spread to humans through contact with infected animals, bites from ticks and deerflies, or inhalation of the airborne bacteria. Early symptoms of the disease are similar to the flu but can develop into serious, acute conditions of the glands, intestines, and respiratory system, including life-threatening pneumonia. To make matters worse, although antibiotics can be used to effectively treat the disease, the amount of time available for therapeutic intervention can be fairly short, typically three to five days if bacteria are inhaled.

Tularemia is caused by the bacterium *Francisella tularensis*. Four subspecies of *F. tularensis* are currently recognized, and several strains within these subspecies are highly virulent, with as few as ten organisms causing infection. Tularemia's virulence and ability to be aerosolized raise concerns that the bacterium could be used as a bioterrorism agent. To combat this potential threat, scientists have ramped up efforts to develop a licensed vaccine for the disease, which would be especially important for military personnel. However, before a vaccine can be produced, scientists must first understand how the bacterium infects cells and what causes it to be so virulent. With funding from Livermore's Laboratory Directed Research and Development Program and the National Institutes of Health, Livermore immunologist Amy Rasley, in collaboration with scientists at Livermore and several other research institutions across the U.S., has made substantial progress toward deciphering the complex cross talk that occurs between *F. tularensis* and host cells.

F. tularensis can infect an extremely broad range of hosts, including more than 200 different animals, but how the bacterium is able to persist in a natural host environment is not well understood. As part of their research, Rasley and colleagues set out to identify the mechanisms by which a potential bacterial reservoir—the amoeba *Acanthamoeba castellanii*—supports the different strains of *F. tularensis*. In doing so, they gained insight into how amoebas and the bacteria

interact and the process by which the bacteria survive. The team also identified several novel *F. tularensis* proteins that may be key to the bacteria's survival in the environment as well as in human immune cells.

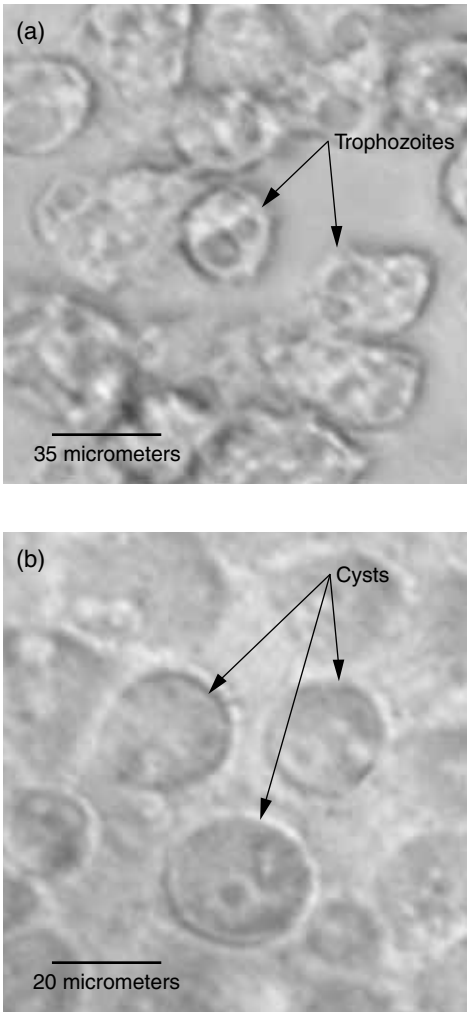
Survival of the "Cyst-est"

Reservoirs are organisms that can harbor bacteria without being killed by them, thus allowing the bacteria to exist in the environment. *A. castellanii* is a known reservoir for a number of pathogens and typically exists in an aqueous host environment. One of the ways an organism, such as the amoeba, can protect itself from adverse environmental conditions is through a process called encystment. When an amoeba (or other unicell organism) encounters unfavorable conditions, such as lack of nutrients or shifts in pH or temperature, it undergoes a morphological change. Trophozoites (protozoan) within the organism transform from mobile forms into immobile, compact cysts—a process that requires extensive protein degradation.

These cysts allow the amoebas to remain in a dormant state until more favorable conditions return. "Once encysted, amoebas are incredibly resistant to temperature and osmolarity shifts, chemical disinfectants, and antibiotics," says Rasley. The Livermore team hypothesizes that the ability of *F. tularensis* to induce amoebic encystment is an essential mechanism ensuring the bacterium's survival in the environment.

In previous studies, an attenuated strain of *F. tularensis* was shown to infect *A. castellanii*, but these studies excluded pathogenic strains of the bacterium. Rasley's team took this research one step further, infecting amoebas with subspecies of *F. tularensis* having varying degrees of virulence. The team observed that fully virulent strains induced rapid encystment within *A. castellanii*. "Typically the encystment process takes 12 to 72 hours," says Rasley. "We found that when we infected amoebas with virulent *F. tularensis*, encystment occurred within just 2 hours."

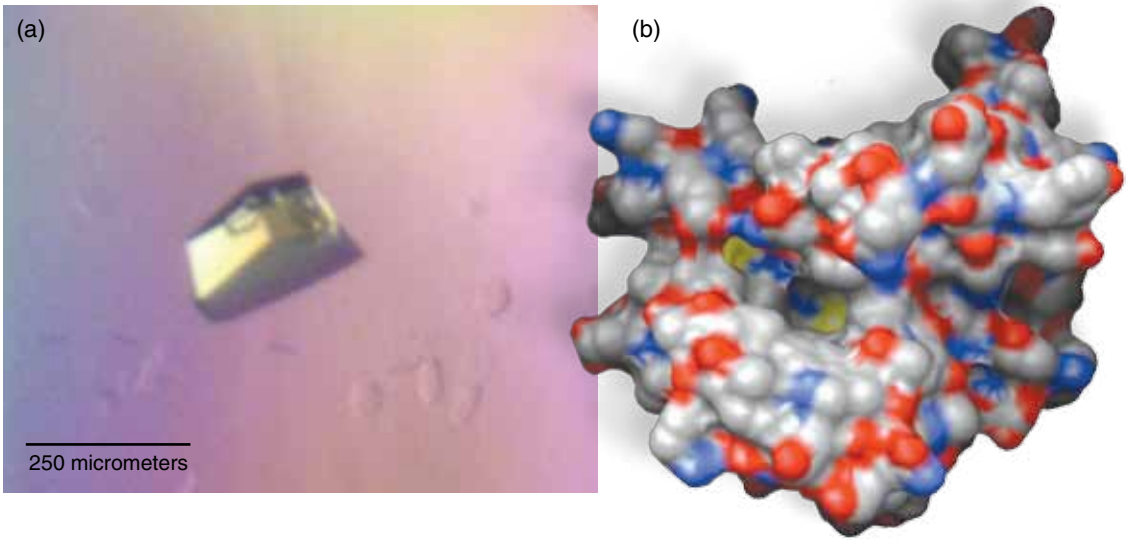
The team's next step was to determine the physical processes that allowed encystment to occur. Amoebas and bacteria were placed in a specialized transwell plate with a filter that separated the bacteria from the amoeba and prevented direct contact between them. Similar to the way a spaghetti strainer allows water to pass through but keeps the pasta contained, the filter permits anything soluble to traverse the boundary, but stops the bacteria. Interestingly, encystment occurred despite the amoeba's relative isolation from the bacteria, suggesting that cell-to-cell contact was not required and that likely secretions from the amoeba or the bacteria were traversing the filter and causing the encystment. To test this theory, the team added some of the spent culture medium from amoeba–*F. tularensis* cocultures to unexposed amoebas, yielding similar results.



(a) *Acanthamoeba castellanii* trophozoites move freely in culture under favorable conditions. (b) However, within two hours of infection with virulent strains of *Francisella tularensis*, amoebas become immobile and form cysts.

Identifying the Culprit

Each experiment provided another piece of the puzzle as Rasley and her team came closer to seeing the big picture of how *F. tularensis* manages to infect and survive within host cells. "After results showed that some agent within the liquid medium was causing the encystment, we wanted to know if the agent was proteinlike in nature," says Rasley. Spent media from amoeba–*F. tularensis* cocultures were then boiled or treated with an enzyme to break down protein structure. This process changed the properties of proteins within the organisms, rendering them inactive. "We then ran the same test as before, and no encystment occurred," says Rasley. "Therefore, we suspected that the agent must be a protein."



Novel *Francisella tularensis* proteins may provide insight into how the bacteria survive in host cells. (a) A Livermore team is characterizing a crystal of one of the proteins believed to be involved in amoeba encystment. (b) The crystal's x-ray diffraction pattern was used to solve the protein's structure. Yellow depicts the active site pocket.

Using high-performance liquid chromatography, the team separated the medium into four fractions based on size. "We identified the fractions that had activity and analyzed a sample of the peptide mixture with mass spectrometry to distinguish which proteins were present," says Rasley. In total, five amoeba proteins and seven novel *F. tularensis* proteins were identified. "Interestingly," says Rasley, "the seven *F. tularensis* proteins were present only in the amoeba– *F. tularensis* coculture and were secreted only in the presence of the amoeba."

The seven *F. tularensis* proteins are now undergoing further tests to characterize their structure and properties, and the team has already obtained a complete crystal structure for one of them. Through this research, the team has identified key genes encoding the proteins that may be responsible for the formation of amoebic cysts. When tested, genetically mutated bacteria missing these genes were unable to induce encystment and failed to survive in human immune cells.

Building the Foundation

Inside the human body, *F. tularensis* attacks macrophages, immune cells that function as a first line of defense against infection. Amoebas can be thought of as an early environmental macrophage. Thus, amoeba–*F. tularensis* interactions may give us some information about how virulence evolved. Altogether, Rasley and her group tested 13 strains of *F. tularensis*, five of which had high levels of encystment. "We did have some variability among the individual strains, which suggests more than one environmental reservoir may exist for *F. tularensis*," says Rasley.

Although it can take decades to create a licensed vaccine for commercialization, this research provides some fundamental data regarding how *F. tularensis* and host cells interact, which is the first step in the process. "Before a vaccine can be developed for clinical trials, we have to fully understand the pathology of the disease and determine at what stage to intervene," says Rasley. Research like this, although time-consuming, is definitely worth it, providing insight into the progression of a deadly disease and helping to safeguard the nation against potential bioterrorism threats.

—Caryn Meissner

Key Words: Acanthamoeba castellanii, amoeba, bioterrorism, encystment, Francisella tularensis, host cell, pathogen, tularemia, vaccine.

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