Reply to "Concerns about Transmission Electron Micrographs in Recent Article by Chen and Williams"

This is in response to the letter submitted by Dr. Susan Koval (1) regarding our recently published paper in *mBio* in April 2012 entitled "Sharing of prey: coinfection of a bacterium by a virus and a prokaryotic predator" (2). Dr. Koval cites her expertise in electron microscopy (EM) and on the prokaryotic predators *Bdellovibrio* and *Bdellovibrio*-like organisms (BALOs), which include the genus *Bacteriovorax*, a focus of our report. She suggests that our conclusions based on EM observations are erroneous. In attempts to support her argument, she cites several examples. First, she states that the legend to Fig. 1 is incorrect in that the electron micrographs do not show a predator residing inside the prey cell and that the cells we refer to as bdelloplasts appear to her to be plasmolyzed *Vibrio vulnificus* cells. She bases this on her opinion (as she states in her letter) that "two things" typically observed in a bdelloblast, "the prey cell protoplast" and the growing predator or BALO, are not seen in Fig. 1 micrographs and only one structure is present. I am surprised that Dr. Koval would not be better informed on this matter, as the evidence and facts have been documented widely in the literature. During the intraperiplasmic cell cycle, BALO consumes its prey's protoplasm to obtain energy for its growth (elongation) (3). The volume of prey cell protoplasm remaining in the bdelloplast and observed by EM at any given point in time in the infection cycle depends upon the stage of the cycle. In newly infected prey when the predator cell is just beginning its growth, the protoplasm is nearly at full volume. However, as the predator growth cycle progresses, the protoplasm is observed to shrink in size with time until it is no longer visible by EM, leaving only the predator cell (more than one predator cell may be observed if multiplication has occurred). This is supported by reports from various authors (Fig. 2 in reference 4, Fig. 7 in reference 5, and Fig. 1B in our most recent paper in the *ISME Journal* [6]). Dr. Koval goes on to state, "To me, this must be the protoplast of the prey cell. . .". If these are plasmolyzed cells caused by erroneous fixation methods as she suggests, then would it not be expected that nearly all of the prey cells seen in the micrographs would be plasmolyzed? We did not find this to be the case. To the contrary, when we examined the dually infected samples multiple times with different methods, almost all prey cells were observed to be in good fixation condition, and no shrinkage was observed, as shown in Fig. 1A of our paper (2) and Fig. 1 in this reply.

Also, plasmolysis usually occurs when cells are immersed in relatively high saline environments, which causes cytoplasm shrinkage as a result of internal water loss. Dr. Koval states that the cells are plasmolyzed due to a lack of salt in our sample fixation solutions. If that were the case, lack of salt in the fixatives would cause the cell to be turgid, which is the opposite effect of plasmolysis.

Dr. Koval cites Fig. 1D in our paper and says that "a growing aseptate filament of a bacterial BALO never looks like that." This and other statements made by Dr. Koval questioning the structure of the predator cells and bdelloplast may be due to her unfamiliarity with *Bacteriovorax*. The reports of her work that she cites in support of her interpretations are studies of freshwater BALOs. We could not find reports by her on studies of the salt water

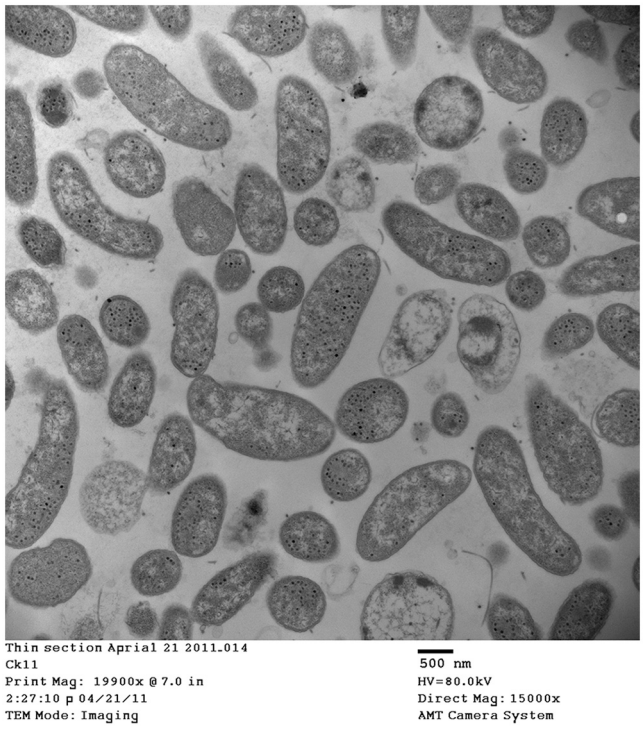

FIG 1 Example of a micrograph of fixed samples showing that prey cells were well fixed and no shrinkage was observed.

Bacteriovorax. Salt water and freshwater BALOs have major structural, genetic, metabolic, and ecological differences. One example of a physical difference is that the salt water *Bacteriovorax* are smaller than the fresh water BALOs. In addition, they do not typically infect the same bacterial species.

For the scanning EM micrographs, Dr. Koval questions whether the phages are viruses or blebs, although she previously acknowledged observing phage infections in *V. vulnificus* in our transmission EM (TEM) thin-section micrographs. Also, we used a known strain of phage, and the structures that we have described as phage particles are similar to the known particles (the heads of the phages are about 50 nm). We acknowledge Dr. Koval's point regarding the lack of arrows pointing to the phage particles (although we thought it was obvious).

Dr. Koval indicates that the phages were infecting the *Bacteriovorax* cells. The phages typically reside in the protoplasm of the

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infected cells. However, if the protoplasm is exhausted as occurs during the BALO infection cycle, we believe the phage particles would still reside inside the prey cell within the empty space which is also occupied by the *Bacteriovorax*, which is what the micrographs show. The phages do appear to be associated with the *Bacteriovorax* cell surface, but this could be due to electrostatic or other forces.

In response to Dr. Koval's comments on not understanding what a *Bacteriovorax* cluster IX is and that our ISME article was "available only by payment," we do not understand why lack of free access would prevent one from obtaining a helpful reference. As to expertise in the taxonomy of the BALOs and *Bacteriovorax* in particular, the contributions of Williams and collaborators represent major advancements (7–10) in the field.

It is also interesting that Dr. Koval cited Dr. Elio Schaechter's blog, which complemented our work. His comment that we used BALOs or more accurately called cluster IX of *Bacteriovorax* is correct. It is not "obvious" to us that he was making the point that "he did not know what a cluster IX was" in the context in which Dr. Koval asserts.

A last, but very important point is that the EM studies described in our report were from cultures consisting of only prey cells, *Bacteriovorax*, and phages. Our previous reports have confirmed the susceptibility of *V. vulnificus* FLA042 to *Bacteriovorax* cluster IX (11). Phage CK2 was chosen on the basis of its superiority in infecting strain FLA042 (12). Following inoculation of the predators into the prey suspension, the turbidity in the suspension decreased with time, as is typical of both BALO and phage infection of prey. EM studies of the culture would show the two predators and prey. According to the suppositions of Dr. Koval, infection of *Bacteriovorax* was not observed, which would beg the question as to what happened to the predator cells and the predator-infected cells in the source cultures. This is not consistent with scientific logic based on what is known about the BALOs.

In summary, Dr. Koval's points of concern about the validity of the interpretation of micrographs in our report on dual infection of a prey by phage and BALO are not supported by the available evidence. I do appreciate the opportunity to respond to Dr. Koval's assertions about our work.

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