Translocation of an Outer Membrane Protein into Prey Cytoplasmic Membranes by Bdellovibrios

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Within minutes of *Bdellovibrio bacteriovorus* attack on prey cells, such as *Escherichia coli*, the cytoplasmic membrane of the prey is altered. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified invited prey cell (bdelloplast) membranes revealed the appearance of a noncytoplasmic membrane protein. This protein is not observed in preparations of noninvaded *E. coli* membranes and migrates in a manner similar to that of *E. coli* OmpF. Isoelectric focusing and two-dimensional gel electrophoresis of bdelloplast cytoplasmic membrane preparations also revealed the presence of a protein with electrophoretic properties similar to those of OmpF and the major *Bdellovibrio* outer membrane proteins. The protein appears in cytoplasmic membrane preparations within minutes of attack and persists throughout most of the intraperiplasmic developmental cycle. The appearance of this protein is consistent with our hypothesis that bdellovibrios translocate a pore protein into the bdelloplast cytoplasmic membrane to kill their prey and to gain access to the cytoplasmic contents for growth.

A critical feature of bdellovibrio intraperiplasmic growth is the event which renders the membrane-bound contents of the prey cytoplasm available to the bdellovibrios. The mechanism by which bdellovibrios gain access to prey cytoplasmic constituents has been linked to an alteration of the cytoplasmic membrane of the prey moments after attachment. Damage to the cytoplasmic membrane renders it permeable to small hydrophilic molecules and ions and disrupts the proton gradient, thereby inhibiting respiration and killing the prey cell (9, 16). Electron microscopy has shown that the prey cytoplasmic membrane remains physically intact during bdellovibrio penetration and growth (1, 3). The damage to the membrane, therefore, appears to compromise its functional integrity without extensively altering its physical construction.

A potential model for cytoplasmic membrane alteration is the bdellovibrio-mediated insertion of a pore protein into the cytoplasmic membrane of the prey cell. A channel-forming protein inserted nonspecifically into the phospholipid bilayer of the cytoplasmic membrane of the prey cell would permit passage of cytoplasmic components into the periplasm and disrupt the proton gradient without significantly impairing the physical integrity of the membrane. Parasite-mediated insertion of proteins into the cell membranes of their hosts has already been demonstrated for both bacterial and eukaryotic pathogens (2, 12, 18, 23). The translocation of a porin from the outer membrane of either the bdellovibrio or the prey cell into the prey cytoplasmic membrane could effectively initiate the events that sponsor bdellovibrio growth within the prey periplasm.

**MATERIALS AND METHODS**

Organisms and culture conditions. *Bdellovibrio bacteriovorus* 109J (21) was propagated and maintained on *Escherichia coli* K-12 (4) in dilute nutrient broth (20) at 30°C with shaking at 240 rpm. *E. coli* W7M5, a diaminopimelic acid (DAP) and lysine auxotroph (21), was used as the prey for all experiments involved in the isolation of invaded prey membranes. Prey cultures were grown overnight in nutrient broth (Difco) supplemented with 0.2 M lysine and 0.1 M DAP at 30°C. Cells were harvested by centrifugation, washed in fresh nutrient broth, and suspended in nutrient broth supplemented with 0.2 M lysine but not DAP. Cultures were incubated at 30°C with shaking at 300 rpm for 60 to 90 min to weaken the cell walls before interaction with *B. bacteriovorus* 109J. The cell walls were weakened so that bdelloplasts could be broken by treatment that would not break uninvaded *E. coli* or free bdellovibrios.

**Synchronous cultures and isolation of bdelloplasts.** Synchronous cultures were prepared as previously described (22) by combining 2 x 10¹⁰ *B. bacteriovorus* 109J organisms and 1 x 10¹⁰ *E. coli* W7M5 organisms with weakened cell walls (see above) in 3 mM N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES)–1 mM CaCl₂–0.1 mM MgCl₂ adjusted to pH 7.2 with NaOH (21).

Cultures were shaken at 400 rpm, and samples were taken at various times, centrifuged at 5000 x g for 10 min, and suspended in a 0.05 x culture volume of 0.25 M sucrose in 50 mM HEPES buffer (pH 7.2). Chloramphenicol (final concentration, 40 μg/ml) was added to stop further development of the bdellovibrios. Bdelloplasts were separated from uninvaded *E. coli* and free *Bdellovibrio* cells by layering the suspension onto Percoll (Sigma) gradients (density = 1.10 in 0.25 M

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FIG. 1. Percoll gradient showing the band of bdelloplasts in the top 25% of the gradient, with uninvaded *E. coli* cells and free bdellovibrios in a band at the bottom of the gradient.
Isolation of prey and Bdellovibrio membranes. Prey membranes from uninvaded E. coli W7M5 cells and attack-phase bdellovibrios were isolated from overnight cultures. Cells were broken at 16,000 lb/in² with a French pressure cell. Cytoplasmic and outer membranes were isolated by the procedure of Diedrich et al. (7).

Gel electrophoresis and Western blot (immunoblot) analysis. Membrane proteins were precipitated with 10% trichloroacetic acid at 4°C, washed twice in cold acetone, and suspended in loading buffer (3% sodium dodecyl sulfate, 15% glycerol, 6 mM dithiothreitol, 30 mM Tris-Cl [pH 8.0]). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the membrane proteins was carried out by the method of Laemmli (11) with a 10% acrylamide separating gel and a 3% polyacrylamide stacking gel. Isoelectric focusing and twodimensional gel electrophoresis were carried out by the methods of O'Farrell (14). Gels were stained with Faststain (Zoion Research, Allston, Mass.).

SDS-polyacrylamide gels were electroblotted onto Immobilon membranes (Millipore Corp., Bedford, Mass.) and probed with a monoclonal antibody directed against E. coli OmpF (AMAC, Inc., Westbrook, Maine).

RESULTS

Isolation of bdelloplasts and bdelloplast cytoplasmic membranes. The first requirement for the study of the bdellovibrio-mediated insertion of a porin into the cytoplasmic membrane of the invaded cell is the development of reliable methodologies for the isolation and purification of bdelloplast cytoplasmic membranes. Following penetration by bdellovibrios, attacked cells generally become round and swell, resulting in the formation of bdelloplasts that are less dense than either the bdellovibrio cells or the E. coli cells used as the prey. By taking advantage of this density difference, we reproducibly separated bdelloplasts from uninvaded E. coli cells and free attack-phase bdellovibrios by using Percoll gradients established by centrifugation at 18,000 × g for 30 min. Because of the decreased density of the bdelloplasts compared with the uninvaded E. coli cells, bdelloplasts banded at or near the top of the Percoll gradient, while the bdellovibrios and E. coli cells banded at or near the bottom (Fig. 1). The bdelloplast layer was easily removed from the gradient without contamination from the other cells. Phase-contrast microscopic examination of material from the top band confirmed the presence of concentrated bdelloplasts without any observable uninvaded E. coli or free Bdellovibrio cells.
Bdelloplasts derived from *E. coli* W3M2 had weakened walls because the prey cells (DAP auxotrophs) had been cultured in the absence of DAP for 90 min prior to bdellovibrio attack. When isolated bdelloplasts with weakened walls were broken at 6,000 lb/in² with the French pressure cell, almost all of the resident, intraperiplasmic bdellovibrio cells remained intact, as determined by a microscopic count of bdellovibrios remaining following pressure treatment and by measurement of the release of radioactivity from labeled bdellovibrios during the procedure. Both of these methods confirmed that less than 5% of intraperiplasmic bdellovibrios were lysed or released labeled material at the pressure used to break the bdelloplasts. This finding is consistent with that of Galdiero (9) using similar methods.

**PAGE and Western blot analysis.** *E. coli*, *Bdellovibrio*, and bdelloplast cytoplasmic membranes and *E. coli* and *Bdellovibrio* outer membranes were analyzed by PAGE to determine whether a new protein appeared in the cytoplasmic membranes of invaded *E. coli*. SDS-PAGE of bdelloplast cytoplasmic membranes revealed the presence of a protein not found in uninvaded prey cytoplasmic membranes (Fig. 2, arrow), as well as a number of other minor changes in the protein composition of the bdelloplast cytoplasmic membranes. This protein migrated in a manner similar to that of *E. coli* OmpF, with an apparent molecular weight of 36,000. Major *Bdellovibrio* outer membrane proteins also migrated in a similar manner in standard preparations. The occurrence of this apparent outer membrane protein in bdelloplast cytoplasmic membrane preparations was detectable within 5 to 10 min following penetration of the bdellovibrios into the periplasm of the prey (data not shown). It persisted in detectable amounts for at least 150 min, steadily decreasing in amount as the prey protoplast diminished in size.

Since the newly acquired bdelloplast cytoplasmic membrane protein had a mobility similar to that of *E. coli* OmpF, we tested its reactivity to a monoclonal antibody specific for OmpF to determine whether the protein was *E. coli* derived. Western blots were inconclusive but indicated the presence of at least some OmpF protein or OmpF-cross-reactive protein in the bdelloplast cytoplasmic membrane. Faint bands were visible on blots of polyacrylamide gels of bdelloplast cytoplasmic membranes at a position consistent with that of the OmpF protein. More conclusive data could not be obtained, since the monoclonal antibody that was used (purchased commercially) reacted only weakly with OmpF on Western blots of *E. coli* outer membrane preparations.

To aid in the determination of whether this newly acquired bdelloplast cytoplasmic membrane protein was *E. coli* OmpF,
a major *Bdellovibrio* outer membrane protein, or some other protein, isoelectric focusing and two-dimensional gel electrophoresis were carried out on bdelloplast and *E. coli* cytoplasmic membrane and *E. coli* and *Bdellovibrio* outer membrane preparations. Figures 3 and 4 show the results of the isoelectric focusing and two-dimensional gel electrophoresis, respectively. The bdelloplast cytoplasmic membrane preparations contained a protein that migrated in a manner similar to that of OmpF and the major *Bdellovibrio* outer membrane protein in both analyses (Figs 3 and 4). This protein was not detected in preparations of *E. coli* cytoplasmic membranes. The major *Bdellovibrio* outer membrane protein migrated too closely to *E. coli* OmpF in both analyses to definitely determine the identity of the protein appearing in bdelloplast cytoplasmic membranes.

**DISCUSSION**

SDS-PAGE, isoelectric focusing, and two-dimensional gel electrophoresis have shown that the cytoplasmic membrane of the prey cell acquires a new protein following attack by *B. bacteriovorus* 109J. This acquired protein appears to migrate with an apparent molecular weight and isoelectric point similar to those of *E. coli* OmpF and/or a *Bdellovibrio*-specific outer membrane protein. Whether this protein is indeed a porin (and, if so, whether it is OmpF) has not yet been determined. A model in which the invading bdellovibrios are capable of translocating a porin into the prey cytoplasmic membrane is, however, consistent with what is now known about bdellovibrio attack and growth within the periplasm of its prey.

It has long been known that the bdelloplast boundary layers are altered early during bdellovibrio attack, that the respiratory potential of the prey cell ceases, and that the prey cytoplasmic membrane becomes permeable to lactose very early following bdellovibrio attack (16, 17). Additionally, Galdiero (9) demonstrated that there was almost complete cellular potassium loss from the prey cell protoplasts during the first 10 to 20 min following the bdellovibrio-prey interaction. Cover et al. (4) have also shown that the prey cytoplasmic membrane becomes freely permeable to small hydrophilic molecules within minutes of bdellovibrio attack. The almost immediate death of the prey cytoplasmic membrane is also due to the introduction of a pore protein into the cytoplasmic membrane, eliminating respiratory potential and ATP production. The disruption of the osmotic potential of the prey cytoplasmic membrane could initiate degradative enzymes within the protoplasts of the prey (i.e., nucleases and proteases, etc. [13]). The resulting breakdown products could then diffuse through the pores that have been created in the cytoplasmic membrane into the periplasmic space and be rapidly taken up by the resident bdellovibrio. This mechanism would result in semicontrolled release and uptake of the cytoplasmic contents of the prey during the intraperiplasmic growth phase of the bdellovibrio.

Given the fact that bdellovibrios are known to acquire OmpF upon invasion of *E. coli* (5, 6, 8, 10), it is conceivable that the protein appearing in the bdelloplast cytoplasmic membrane is translocated OmpF. Since *B. bacteriovorus* has its own porin (15), the significance of its acquisition of OmpF during prey cell invasion has been questioned. Acquiring the major porin during penetration for the purpose of translocating it into the cytoplasmic membrane would provide an explanation for this unusual activity.

Given the unusual metabolic capabilities of bdellovibrios while inside the prey periplasm and the obvious alteration of the prey cytoplasmic membrane, the insertion of a porin into the cytoplasmic membrane as a channel between the cytoplasm and the periplasm seems to be a model of significant possibility.

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**REFERENCES**


