JOURNAL OF BACTERIOLOGY, May 1974, p. 663-680

Vol. 118, No. 2
Printed in U.S.A.

Penetration of *Bdellovibrio bacteriovorus* into Host Cells

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Received for publication 6 August 1973

Electron microscopy reveals that, in *Bdellovibrio* infection, after the formation of a passage pore in the host cell wall, the differentiated parasite penetration pole is associated with the host protoplast. This firm contact persists throughout the parasite penetration and after this process is completed. In penetrated hosts this contact is also apparent by phase microscopy. The association between the walls of the parasite and the host at the passage pore, on the other hand, is transient. *Bdellovibrio* do not penetrate hosts whose protoplast and cell walls are separated by plasmolysis, or in which the membrane-wall relationship is affected by low turgor pressure. It is concluded, therefore, that for penetration to occur it is essential that the host protoplast be within reach of the parasite, so that a firm contact can be established between them. A penetration mechanism is proposed that is effected by forces generated by fluxes of water and solutes due to structural changes in the infected host envelope. These forces cause a differential expansion of the host protoplast and cell wall and their separation from each other around the entry site, while the parasite remains firmly anchored to the host protoplast. Consequently, the parasite ends up enclosed in the expanded host periplasm. The actual entry, therefore, is a passive act of the parasite.

The sequence of events in the life cycle of parasitic *Bdellovibrio bacteriovorus* has previously been established (19, 20, and references cited therein). The mechanisms involved in the first phase of the infectious cycle, i.e., from the attachment until the enclosure of the parasite in the host periplasm, are not fully understood. Several proposed penetration mechanisms, which stress the role of forces generated by the motion of the parasite, so far lack direct evidence (3, 19-21, 24). It has been shown, however, that an enzymatic activity is involved in the penetration, since in the presence of protein synthesis inhibitors this process is prevented, whereas the rapid movement of the parasites or their capacity to attach to host cells remains unchanged (24). Parasites rendered nonmotile by various substances or by anaerobic conditions (24) do not attach to susceptible hosts. On the other hand, in a viscous medium, such as semisolid agar, although the motion of the parasites is slowed down considerably, both attachment and penetration still occur. In addition, attachment is prevented in the presence of ethylenediaminetetraacetic acid (EDTA), at concentrations that do not affect *Bdellovibrio* motility (7, and Abram, unpublished data). These observations indicate that the rapid motion of *Bdellovibrio* and the violent impact upon collision between parasite and host are neither sufficient nor absolute prerequisites for attachment or penetration. They are not consistent with the hypothesis that weakening of the host cell wall and the formation in it of the passage pore are caused by a force generated during collision (29). Other proposed mechanisms relate the formation of the penetration pore to a drilling action by the rotation or swivelling of parasites already attached to the host surface (3, 20). Phase microscopy reveals, however, that attached parasites usually are nonmotile before penetration occurs. Moreover, parasites are immobilized as soon as they attach to heavily capsulated but readily penetrated host organisms (*Aerobacter*, *Klebsiella*, *Xanthomonas*, and *Acinetobacter*) and can be seen to be partially or completely embedded in the hosts viscous capsules (unpublished data). Finally, there is also no direct evidence for the proposed hypotheses (3) that the active motion of *Bdellovibrio* is necessary throughout its entry to counteract pressure exerted by the edges of the passage pore, or that the growth of new wall at the parasite attachment tip causes the physical breaking of the host cell wall.

This study was undertaken to further elucidate the penetration process. It will be shown that, during its penetration, *Bdellovibrio* is firmly attached to the host protoplast, and that for penetration to occur, the host protoplast

must be within reach of the parasite. A penetration mechanism previously not considered will be proposed and discussed.

MATERIALS AND METHODS

B. bacteriovorus strains 109 and D (obtained from M. Shilo), ATCC 15143 and 6-5-S (obtained from S. F. Conti, University of Kentucky, Lexington, Ky.), *Escherichia coli* B and ATCC 15144, *Pseudomonas fluorescens* NCTC 10038, and *Spirillum serpens* NCIB 9150 and LVH (obtained from S. F. Conti) were employed.

Trypticase-yeast extract media (TY) were used as previously described (1). *Bdellovibrio* lysates were obtained from two-membered cultures in TY/5 broth; *E. coli* B, *E. Coli* ATCC 15144, and *S. serpens* LVH served as hosts for the parasite strains 109 and D, ATCC 15143, and 6-5-S, respectively. Parasites were obtained from fresh lysates, i.e., within 2 h after more than 99.9% of the host cells had lysed, or 6 to 8 h later, from old lysates. Prior to harvesting, lysates were filtered through a double layer of Whatman no. 41 paper to remove some host debris. The host organisms were obtained at the mid-exponential, or at 6 to 8 h into the stationary phase, from cultures in TY broth at 30 C with shaking. Parasite and host cells were harvested by centrifugation at 10,000 to 12,000 × *g* for 10 min at 5 C. To obtain washed host cells, the pellets were suspended in a volume of distilled water equal to that of the broth culture and recentrifuged. Except for *S. serpens* NCIB 9150, which is cryosensitive and was kept at room temperature for no longer than 30 min, the sedimented cells were kept before use at 2 C for no longer than 2 h. Cells suspended in water, 0.02 M KCl, and TY/5 broth not supplemented with Ca^{2+} were exposed to osmotic upshocks by using NaCl and KCl at final concentrations of 0.1 to 0.2 M and sucrose at final concentrations of 0.1 to 0.4 M. Turbidity changes, known to occur during plasmolysis (11), were measured and expressed in percentage, E', as previously described (10): $E' = [(Es - Ec)/Ec] \times 100$, where Es and Ec are the optical densities (OD) of the suspensions in the solute and in the suspending medium, respectively. A Coleman Universal spectrophotometer model 14 was used at 500 nm with the suspending medium as a blank for zero extinction. Suspensions were prepared from the sedimented cells at a concentration giving OD_{500} of 0.875 to 0.900. Within 15 min, 4-ml portions were rapidly mixed in cuvettes containing equal volumes of the plasmolyzing substances dissolved in the medium used for the cell suspension at twice the desired final concentration. OD measurements were taken at time zero (15 to 20 s after mixing) and at intervals over periods of 1 to 2.5 h. All Ec values ranged from 0.48 to 0.54 and showed ±5% variation over the experimental period. The Es values never exceeded 0.85. Values of E' were either positive or negative and ranged from −35% to 60%. All the manipulations were carried out at 22 to 24 C (room temperature).

The extent of plasmolysis was determined from the proportion of plasmolyzed cells identifiable by phase microscopy. It was calculated from counts in 20 to 30 fields, each with 50 to 100 cells and graded from "++++" to "−," denoting the presence of 80 to 100%, 50 to 80%, 25 to 50%, 10 to 25% and no plasmolyzed cells.

Penetration was studied in parasite-host mixtures always containing 10^9 to 5×10^9 hosts/ml and 1.5 to 2.5 parasites per host cell. Volumes of 6 to 8 ml of the mixtures in 250-ml flasks were incubated at 30 C with shaking. To study the effect of plasmolysis on penetration, 3 or 4 ml of host cells in water, 0.02 M KCl, or TY/5 broth (2×10^9 to 10^{10} cells/ml) was mixed with equal volumes of the suspending medium containing the plasmolyzing agent at twice the desired final concentration. After 30 to 45 min at 22 to 24 C, when the proportion of plasmolyzed cells became constant (see Results), 0.1 to 0.2 ml of parasites suspended in water was added and the mixtures were incubated. Changes in the concentrations of the plasmolyzing agents or the suspending media due to the parasite inoculum were disregarded. Samples of 0.2 ml drawn at intervals over a period of 2.5 h were examined by phase microscopy to determine the percentage of penetrated host cells, averaged from cell counts as described above for plasmolysis. Parasite-host mixtures without the plasmolyzing agents and suspensions of hosts alone served as controls to determine maximum penetration and extent of plasmolysis, respectively.

Parasite-host mixtures for microscope observations of infection under optimal conditions were always prepared in TY/5 broth. Under these conditions 70 to 80% of the hosts were penetrated within 30 to 40 min after mixing the partners. During this period the mixtures were sampled at intervals of 5 to 10 min and afterwards at longer intervals. To study the effect of EDTA on parasite-host interaction in these mixtures, 0.5-ml samples, drawn at 2-min intervals during 30 min after mixing the partners, were added to 0.1 ml of aqueous EDTA to give a final concentration of 4×10^{-3} M. After standing for 5 to 10 min at room temperature, they were examined by phase microscopy or negatively stained for electron microscopy. Parallel controls without EDTA were kept at 2 C until examined by phase microscopy.

Negatively stained and shadow-cast specimens were prepared as previously described (1). Aqueous solutions of 0.5% ammonium molybdate and 0.5% potassium borotungstate at pH 7.0 were used for staining, and carbon-platinum was used for shadowing.

For thin sectioning, samples were drawn every 5 to 10 min for 30 min only from mixtures of *B. bacteriovorus* 109 and *E. coli* B. The cells were fixed with OsO_4 or with glutaraldehyde (GA) followed by OsO_4. The Ryter-Kellenberger buffer (15; R-K buffer) used throughout the fixation procedures was diluted twofold to balance it osmotically with the diluted suspending broth (TY/5). The calcium concentration in the diluted buffer, however, was maintained at 0.01 M. The procedure for OsO_4 fixation was essentially that of Ryter-Kellenberger (15), including prefixation. For fixation with GA, 9 volumes of cell suspension were mixed with 1 volume of 10 or 20% aqueous GA (final pH 6.0), and after 2 to 3 h at 2 C the cells were

centrifuged and suspended in the R-K OsO_4 fixative. The last step was repeated after 10 to 20 min, and the cells were left in the fresh fixative for 2 to 16 h. Fixed specimens were directly dehydrated in graded series of ethanol, or prior to dehydration they were washed once with the R-K buffer and postfixed for 2 h with 0.5% (wt/vol) uranyl acetate (UA) prepared in the same buffer. In later experiments postfixation with UA was omitted since it only effected a fibrillar appearance of the nuclear material which, without UA fixation, appeared loosely aggregated. Specimens were infiltrated for 12 to 16 h and embedded in Epon 812, which was polymerized at 70 C. Thin sections were contrasted first with 4% UA (wt/vol) in 50% ethanol for 15 to 30 min and then with lead citrate (25) for 2 min or with lead citrate only.

Specimens were examined in an AEI 6B electron microscope at accelerating voltage of 80 kV. Electron micrographs were recorded at instrumental magnification of 7,500 to 40,000. Micrographs illustrating shadow-cast material were printed as negatives from contact intermediates.

A Zeiss Universal microscope with phase-contrast optics was used. Micrographs were recorded at magnifications of 500 or 640 by using a microflash device. They were photographically enlarged to ×4,000 for measurements of intact and infected cells of *S. serpens* NCIB 9150. Fifty measurements were averaged, to compare the cell volumes. For volume calculations it was assumed that intact cells are cylinders, that swollen ellipsoidal penetrated cells or protoplasts are prolate spheroids, and that rounded cells and protoplasts are spheres.

RESULTS

The structural features of the successive stages in *Bdellovibrio* infection, through the enclosure of the parasite in the host periplasm, are shown in Fig. 1 to 3, 6 to 10, and 12 to 29. The finer structural details that relate to the penetration process are clearly revealed in thin sections of fixed specimens. Parasites at first are associated at their penetration poles with the periphery of host cells (Fig. 1 to 3, 6, 7, 12 to 15). During the initial surface contact, the cell wall of the parasite is flattened against the outer membrane of the host cell wall (Fig. 1, 2, 13). The interspace (5 to 15 nm wide) of these two apposed intact "unit membranes" often contains electron-opaque material (Fig. 13) that occasionally appears to extend from structured material present in, and characteristic of, the parasite periplasm at the penetration pole. Free, attached, and penetrating parasites often reveal this material as several (3 to 7) well-organized bands, 7 to 12 nm wide, which extend from the protoplast to the cell wall (Fig. 1 to 5b, 6 to 8, 15). Parasites occasionally appear to have been pulled away from adjacent hosts, probably by mechanical forces during the preparative procedure (Fig. 4a, b). In these cases the host wall shows a discontinuity, while membranous material that appears to have originated in this lesion adheres to the parasite penetration pole (Fig. 4b). Such views indicate a firm association between the walls of the partners and the weakening of the host cell at the contact site, already early in the infection. At this stage of the infection, the host cell (Fig. 4a) is almost indistinguishable from uninfected cells (Fig. 4); its nuclear material is fibrillar or loosely aggregated (depending on the fixation), and its cell wall always shows an outer membrane and an inner peptidoglycan R-layer (12), which often is discontinuous at the contact site (Fig. 1, 2). Later in the infection, a passage pore forms at the attachment site in the host wall, and the penetration pole becomes flattened against the host cytoplasmic membrane (Fig. 3, 6, 7, 14, 15). This contact site is a complex structure consisting of two apposed intact "unit membranes," 5 to 20 nm apart, and strands of electron-dense material (described above), clearly extending from the parasite cytoplasmic membrane, through the parasite wall and the interspace, to the host cytoplasmic membrane (Fig. 6). Although almost the entire body of the parasite is still outside the host (Fig. 6, 14, 15), a generalized effect on the structural organization of the host cell can be recognized. In addition to the dispersed appearance of the nuclear material previously described (20), infected hosts reveal irregular boundaries with bulges especially pronounced in GA-fixed specimens (Fig. 14, 15). The R-layer of the cell wall is still preserved except at the penetration site (Fig. 6); it completely disappears only at later stages of the infection (Fig. 8). Penetrated hosts, however, in which the wall is completely devoid of R-layer, show smooth boundaries irrespective of the fixation procedure (Fig. 16).

The simultaneous use of two fixation procedures provided complementary information. Intact *E. coli* (Fig. 11) and *Bdellovibrio* (Fig. 5b) fixed with GA reveal undulated envelopes and have a narrower periplasm and a more compact appearance than OsO_4-fixed cells (Fig. 4, 5a, 5c). Infected hosts reveal early changes in the integrity of their wall more clearly after GA fixation (Fig. 14, 15), whereas the fine details of parasite-host association are better seen after OsO_4 fixation (Fig. 1 to 3, 6 to 8).

In both negatively stained and shadowed preparations, attached parasites often adhere along their bodies to the host surface. This relationship between the cells is a drying effect, since phase microscopy reveals the parasites to be attached only at one pole to the host surface.

Observations on later stages in the infection

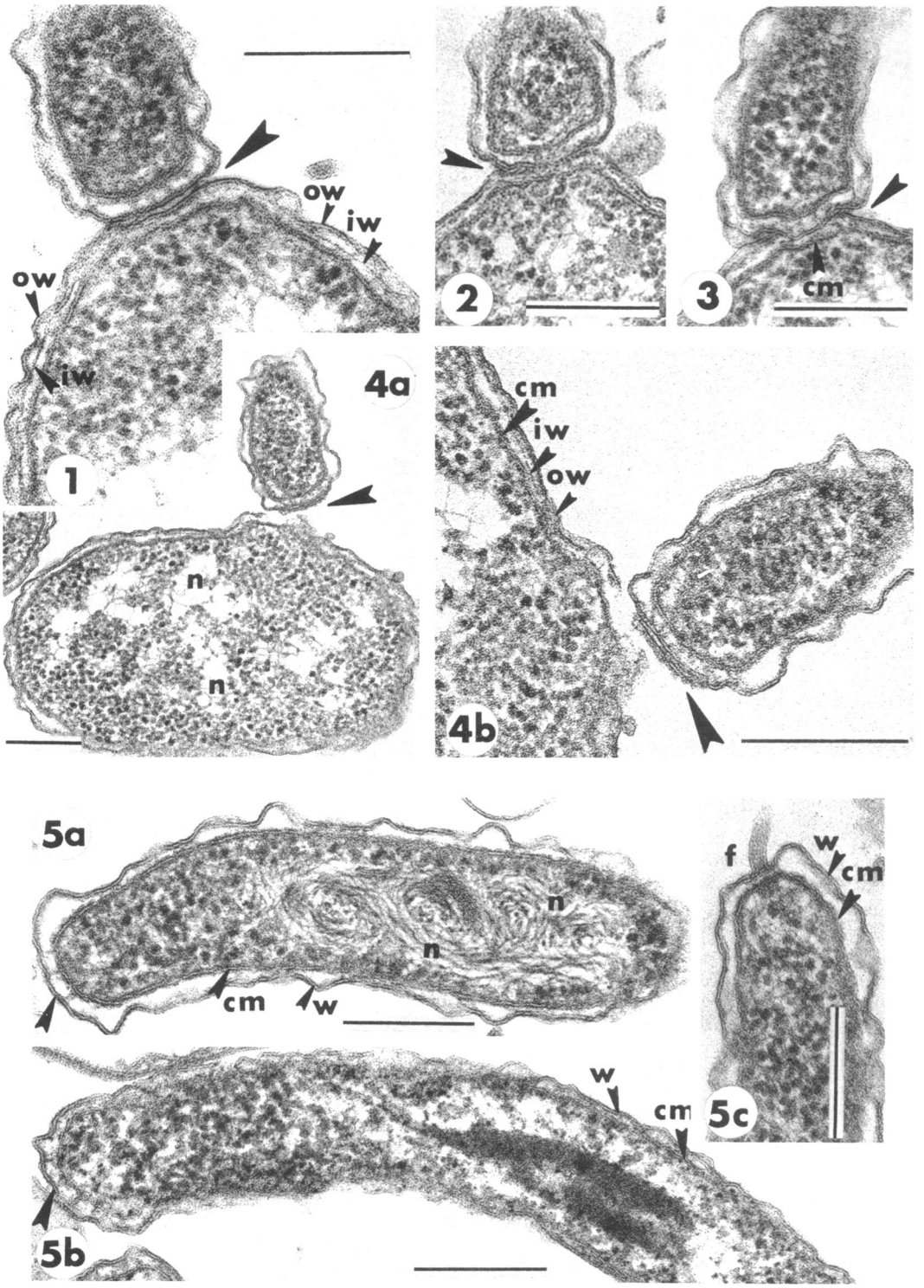

FIG. 1. *B. bacteriovorus 109 attached to the surface of E. coli. The differentiated penetration pole of the parasite reveals, in the periplasm, strands of electron-dense material extending from the protoplast to the wall (arrow). At the contact site, the parasite wall is flattened against the outer membrane of the host wall (ow), and the inner R-layer (iw) of the host wall is discontinuous. Specimen obtained from parasite-host mixture incubated for 10 to 15 min. OsO_4 fixation without UA postfixation. Bar represents, in this and in all subsequent electron micrographs, 0.2 μm, unless otherwise stated.*

FIG. 2, 3. *Early parasite-host interaction during the opening of the passage pore in the host cell wall (arrows). In Fig. 3 a portion of the parasite penetration pole is already in contact with the host cytoplasmic membrane (cm). Specimens were obtained and processed as in Fig. 1.*

relate to the following points. (i) The attachment of the parasite at its penetration pole to the host protoplast persists throughout the penetration and after the completion of this process (Fig. 3, 6 to 10, 14 to 29). In negatively stained preparations, the parasites often also adhere along their entire body or part of it to the host protoplast (Fig. 18 to 20). This appearance most probably is an artifact of this preparative procedure, similar to that noted above for parasites attached to the host surface. (ii) As the penetration process progresses, the infected host always swells (increases in volume) and sometimes completely rounds up, whereas the periplasm around the passage pore expands (Fig. 6, 8, 9, 15, 17 to 19). Ultimately the parasite is located in this space (Fig. 10, 16, 20, 22 to 24, 28, 29). The expanded periplasm usually is asymmetric with respect to the penetration pore (Fig. 8, 9, 17 to 19), and, at later stages in the infection, often the parasite penetration pole is displaced by 40 to 180° from the entry pore (Fig. 9, 19). (iii) The parasite body always appears constricted at this pore (Fig. 6 to 9, 14, 15, 17 to 19), as previously described (3, 20). The constriction usually is more pronounced after OsO_4 (Fig. 6 to 9) than after GA fixation (Fig. 15), and, in negatively stained preparations, only a slight constriction is revealed (Fig. 17 to 19). In the course of penetration, the constriction advances along the parasite without disturbing the continuity of its cell wall, which always appears as a three-layered structure, as previously noted (20). The host cell wall at the passage pore appears to be invaginated (Fig. 6 to 9, 15). Hosts that enclose one-third or more of the parasite body never exceed 0.2% of the host population, both in fixed and in negatively stained specimens. Neither the formation of the penetration pore nor partially penetrated hosts could ever be recognized with certainty by phase microscopy. (iv) During penetration and after its completion, the host protoplast is a membrane-bound domain which always remains associated with the cell wall along a portion of its surface (Fig. 9, 10, 16, 18 to 20). (v) Penetrated host cells always appear swollen. *E. coli* and *P. fluorescens* cells often completely round up, whereas cells of *S. serpens* usually become ellipsoidal. The volumes of penetrated *S. serpens* cells, and of the protoplasts within them, were found to exceed the volume of uninfected cells by factors of 1.4 to 1.6 and 1.3 to 1.4, respectively. (vi) Parasites can clearly be seen by phase microscopy in the expanded periplasm. Observations on infected *S. serpens* by phase microscopy are particularly revealing. In the expanded periplasm which by far exceeds the size of the enclosed parasite, the latter can clearly be seen attached only at one pole to the protoplast (Fig. 28). This polar association is confirmed also by a pendular movement of the enclosed parasites which rarely are seen to move freely.

The observations of infected *E. coli* by phase microscopy, and by electron microscopy of thin sections and negatively-stained and shadow-cast preparations, are in good agreement. These observations, except for thin sections which were done only for *E. coli*, hold true for all other parasite strains and host species used in this study.

Phase microscopy of parasite-host mixtures exposed to EDTA reveals that most of the attached parasites are either released by the chelator or remain attached to phase-transparent ghosted cells. At all times, only a few parasites remain attached to cells as phase-dense uninfected hosts; free hosts rarely appear phase-transparent, whereas penetrated ones always do. The proportion of infected transparent host cells increases as the infection progresses. Initially (5 to 10 min after mixing) they are mostly elongated, and later they become swollen or spherical. Infected hosts exposed to EDTA and negatively stained reveal collapsed protoplasts or large membranous fragments, to which *Bdellovibrio* clearly is attached at one pole (Fig. 21).

Plasmolysis in suspensions of *E. coli*. *P. fluorescens*, and *S. serpens* exposed to osmotic up-shocks was followed by phase microscopy and turbidity changes, simultaneously (Fig. 30 to 32). The decrease in the number of plasmolyzed cells after an initial maximum always paralleled the decrease in the *E'* values. De-

FIG. 4. *Parasite apparently torn away from adjacent host which shows a zone of discontinuity of the wall layers corresponding to the membrane fragment that coats the parasite penetration pole (arrow). The general appearance of the host (4a) is essentially that of uninfected cells. Its nuclear material (n) is loosely aggregated, and its envelope consists of the wall outer membrane (ow), the wall inner R-layer (iw), and the cytoplasmic membrane (cm). Specimen was obtained and processed as in Fig. 1.*

FIG. 5. *Free B. bacteriovorus 109 from a fresh lysate fixed with OsO_4 followed by UA postfixation (5a, c) and with glutaraldehyde followed by OsO_4 (5b). At the penetration pole (arrows), delicate strands extend from the protoplast to the cell wall (5a, b). They are absent at the flagellated (f) cell pole in Fig. 5c. Both the cell wall (w) and the cytoplasmic membrane (cm) are three-layered structures. Note the narrow periplasm in Fig. 5b, and the fibrillar nuclear material (n) in Fig. 5a.*

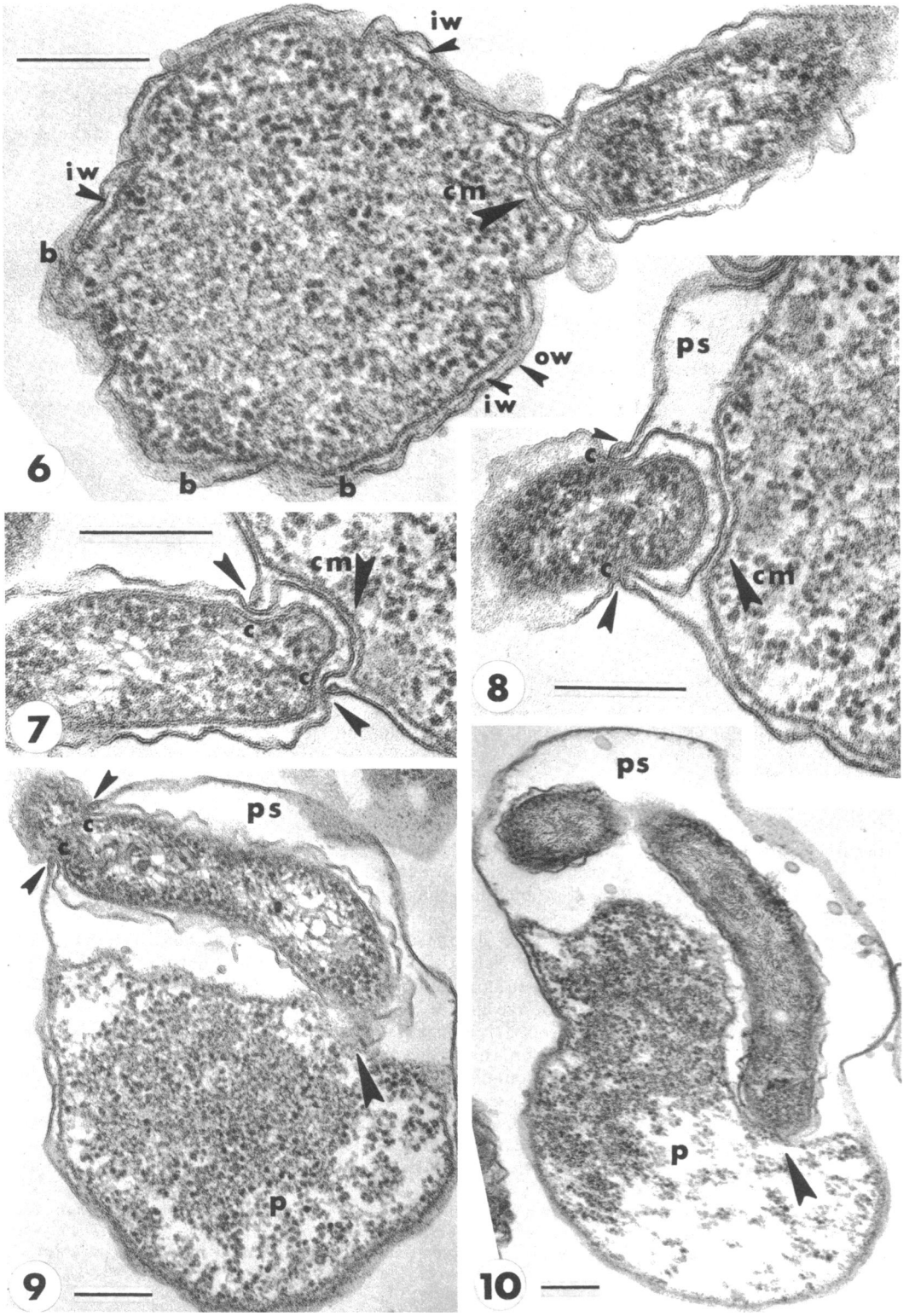

FIG. 6, 7. *After the formation of the passage pore, the parasite cell wall at its penetration pole is flattened against the host cytoplasmic membrane (cm). Note in Fig. 6 at the contact site fine strands that extend from the parasite protoplast, through its cell wall and the interspace, to the host cytoplasmic membrane. The nuclear material of the host cell is dispersed, the cell boundaries are irregular with slight bulges (b), and the R-layer (iw) is still preserved. At the edges of the passage pore the host cell wall invaginates (arrows) and the parasites show a constriction (c). Specimens were obtained and processed as in Fig. 1.*

pending upon the osmotic environment, some or all of the cells undergo deplasmolysis. After the optical response levels off, however, the number of plasmolyzed cells (whenever present) remains constant for at least 4 to 5 h. A fraction of the population, therefore, always is more refractory to changes in external osmotic pressure, as previously shown (8). The profiles of the optical responses with time are characteristic of the species and depend on the environmental conditions (Fig. 31). Extent of plasmolysis for *E. coli* and *P. fluorescens* suspended in water and osmotically shocked with NaCl, KCl, or sucrose, or suspended in TY/5 broth and 0.02 M KCl and shocked with NaCl was routinely estimated turbidimetrically. In these cases, final (leveled off) E' values agree well with visible plasmolysis. For *E. coli* and *P. fluorescens* suspended in TY/5 broth or 0.02 M KCl and osmotically shocked with sucrose, and for *S. serpens* under all the conditions tested, extent of plasmolysis could be determined only by microscopy because the final E' values were low or negative (Fig. 32a, b, c). In these cases, however, the susceptibility of the cells to plasmolysis always could be roughly estimated from the initial (zero time) E' values.

Results of preliminary experiments, using washed and unwashed exponential- and stationary-phase cells (Fig. 30b), showed that washing increases the susceptibility of exponential-phase cells to plasmolysis but decreases that of stationary-phase cells. Data similar to those shown in Fig. 30b for *E. coli* suspended in water and plasmolyzed by NaCl were obtained also with KCl or sucrose and for *P. fluorescens* and *S. serpens* with all three plasmolyzing agents. In most subsequent experiments, therefore, washed exponential cells were used (Fig. 32). The three host organisms used in this study were found to differ with respect to their susceptibility to undergo plasmolysis. Under all conditions tested, the most sensitive is *S. serpens*, followed by *E. coli*, and lastly by *P. fluorescens*. In water suspensions and for equimolar solutions, these three organisms plasmolyze more in NaCl than in KCl or sucrose. Cells suspended in TY/5 broth and 0.02 M KCl, which increase the osmolarity of the medium (in ca. 20 mosM), always are more resistant to plasmolysis than cells suspended in water.

Data provided by the above experiments were used to obtain cell suspensions plasmolyzed to various known degrees for penetration experiments (Table 1).

Suspensions of *Bdellovibrio* of both fresh and old lysates exposed to increased external osmotic pressure, under similar conditions used to osmotically shock the host organisms, do not show an appreciable optical response (Fig. 32d). Lack of plasmolysis in *Bdellovibrio* could not be confirmed by phase microscopy due to the size of the cells. It has been assumed that parasites of fresh lysates do not plasmolyze since they remained motile under all the conditions used, whereas other motile organisms are paralyzed upon plasmolysis (13). On the other hand, under similar conditions, parasites of old lysates were partially or completely paralyzed by 0.1 M NaCl or KCl (sucrose was not tested), and, therefore, they were not used in the following penetration experiments. The responses of the four parasite strains (109, ATCC 15143, D, and 6-5-S) used in this study were found to be similar.

Table 1 summarizes some of the penetration experiments with *B. bacteriovorus* 109 and *E. coli* B designed to examine the relationships between extent of plasmolysis and the proportion of penetrable cells in host populations under various osmotic conditions. In controls with washed and unwashed exponential cells suspended in TY/5 broth or in 0.02 M KCl, almost all (80 to 100%) of the cells are infected within 1 h after mixing the partners. In similar controls with stationary-phase cells and in the experimental mixtures, only a portion of the host population is affected, and the number of penetrated cells gradually increases for 1 to 2 h after mixing the partners.

The percent of penetrated hosts, therefore, was determined approximately half an hour after maximum infection had occurred and not later than 2.5 h after mixing the partners, when progeny are not yet released from the early infected cells. Considering the values of the controls (Table 1), it follows that, for populations of exponential hosts under all conditions

FIG. 8 to 10. *B. bacteriovorus 109 attached at the penetration pole (arrows) to the protoplasts of partly (Fig. 8, 9) and completely (Fig. 10) penetrated E. coli. The constriction (c) in the parasite body at the passage pore advances during penetration toward the cell distal end (Fig. 9; see also Fig. 18, 19). The host cell wall at the edges of the passage pore is invaginated (Fig. 8, 9). The expanded periplasm (ps) is asymmetric with respect to the passage pore (Fig. 8), and the parasite penetration pole is dislocated relative to it (Fig. 9; see also Fig. 18, 19). The infected host protoplast (p) is an intact membrane-bound domain (Fig. 9, 10) partly associated with the cell wall. Specimens from parasite-host mixtures incubated for 20 to 30 min and fixed with OsO_4 without (Fig. 8, 9) and with (Fig. 10) UA postfixation.*

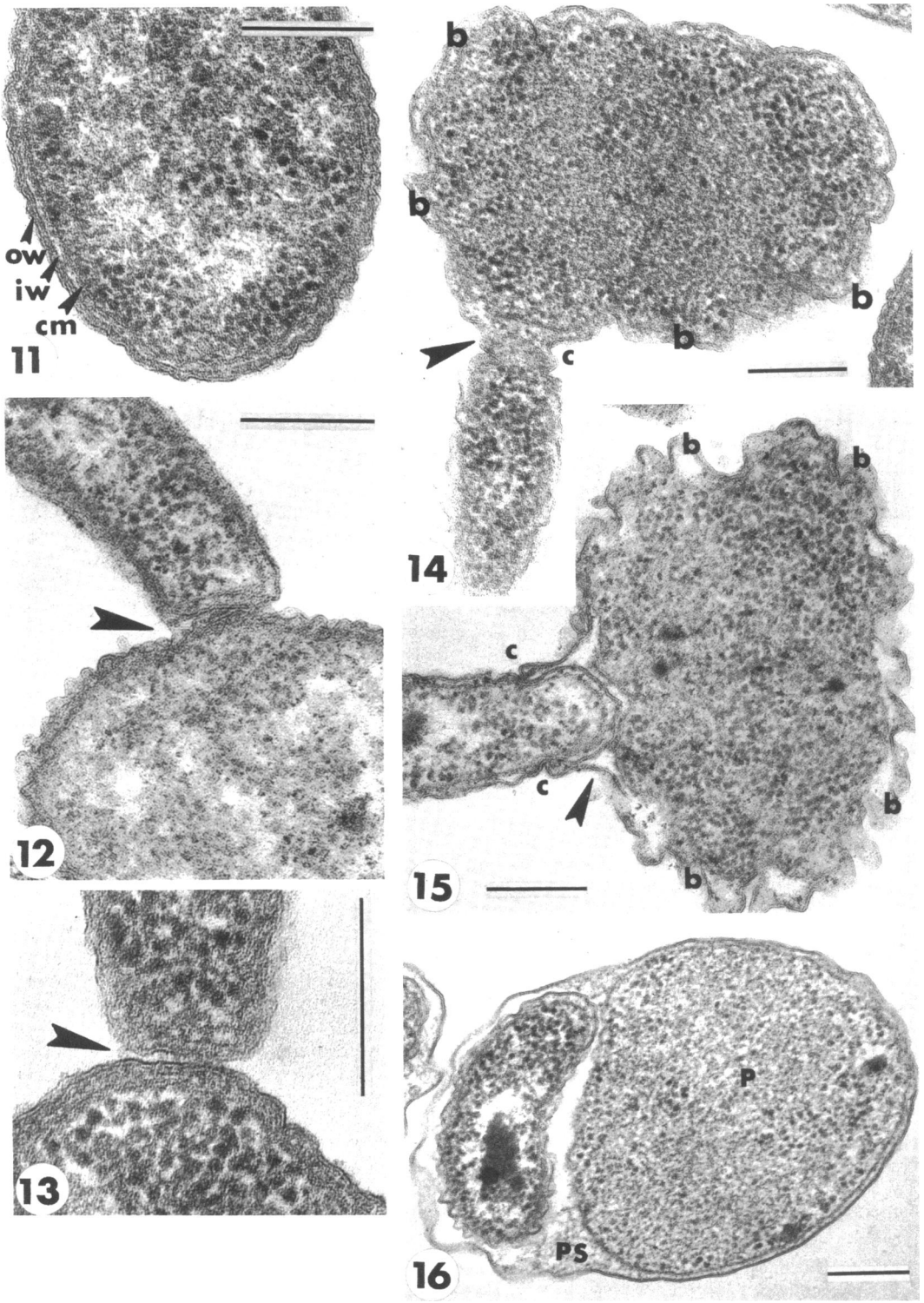

FIG. 11 to 16. *Specimens, fixed with glutaraldehyde followed by OsO_4, from mixtures of B. bacteriovorus 109 and E. coli, incubated for 15 min (Fig. 12 to 15) and 30 min (Fig. 16). Fig. 11, an uninfected host. Fig. 12, note the lack of details at the contact site (arrow) between the undulated host surface and the parasite penetration pole. Fig. 13, the interspace between the apposed parasite and host walls contains dense material (arrow).*

FIG. 14, 15. *Infected hosts with parasites attached to their periphery (arrows) show irregular boundaries with pronounced bulges (b). Fig. 16, a penetrated host with smooth boundaries. Periplasm (ps); protoplast (p).*

tested, an inverse relation exists between the proportions of penetrable and plasmolyzed cells. This holds for the three host species with different osmotic sensitivities and for the conditions conferring increased resistance to plasmolysis (TY/5 broth or 0.02 M KCl). Under conditions of high osmolarity, the impenetrable hosts comprise plasmolyzed and morphologically unaffected cells. The proportion of the former is similar to that of plasmolyzed cells in control suspensions of uninfected hosts. *Bdellovibrio* can be seen to attach to the two types of impenetrable host.

Washed stationary-phase cells are less susceptible to plasmolysis than washed exponential cells under homologous osmotic conditions (Fig. 30b). Suspensions of the former, however, contain a lower proportion of penetrable cells, and most of the impenetrable hosts are not visibly plasmolyzed. Suspensions of washed and unwashed stationary-phase cells in TY/5 broth or in water and without plasmolyzing agents contain less penetrable cells than homologous exponential populations. Thus, when similarly treated stationary and exponential cells are compared, the relation between penetrable and plasmolyzed cells is not inverse. Here it should also be mentioned that populations of stationary-phase *E. coli* and *P. fluorescens* (harvested 10 h into the stationary phase) were found to contain 20 to 40% nonviable cells.

In some experiments *Bdellovibrio* strains 109 and D or 109 and ATCC 15143 have been used simultaneously, for comparative purposes (see footnote to Table 1). In all cases the results obtained for strain 109 also hold for strain D and for strain ATCC 15143.

DISCUSSION

During its entry into the host periplasm, *Bdellovibrio* is firmly attached to the host protoplast. This contact is apparent at a very early stage of the infection and persists after the parasite entry is completed. It involves the differentiated portion of the penetration pole previously described for negatively stained free *Bdellovibrio* (1). The organized strands seen at this pole in thin sections are believed to represent the fibers shown before to emerge from it (1). These linearly oriented thin structures probably mediate the association between the parasite and the host protoplast across the host cell wall, even before a passage pore is opened. The appearance of a bulge in the host envelope at the attachment site before the passage pore forms, as described schematically by Burnham et al. (3), could not be confirmed. Instead, as the passage pore opens and the parasite attaches to the host protoplast, the host cell wall appears to cuff the parasite penetration tip. Sections through such a cuff may give, depending upon their orientation, the impression of a bulge probably analogous to that previously described (3). At early stages of the penetration, when the parasite is still associated with the periphery of the host cell, irregularities in the cell boundaries are observed as distinct bulges, but they are not restricted to the areas of parasite attachment. At the passage pore, the parasite body always shows a transient constriction (3, 20) that advances along the parasite during its entry into the host, suggesting that the *Bdellovibrio* envelope is excessively plastic. In fact, other unique properties of the *Bdellovibrio* wall have already been reported (1). In addition, in this study it is shown that the *Bdellovibrio* wall appears devoid of the R-layer after fixation procedures that always reveal this layer in the host wall. Although components characteristic of the R-layer have been identified in *Bdellovibrio* (23), their amount (1% of cell weight) may be at the limit for the existence of a closed sacculus, or even of peptidoglycan patches thick enough to be identified in thin sections by electron microscopy (6, 26). *Bdellovibrio* also are readily lysed by Triton X-100 and sodium dodecyl sulfate and are converted to spheroplasts by lysozyme alone under conditions whereby most gram-negative organisms are unaffected (unpublished data). All these facts are likely to be related to the plastic nature of the *Bdellovibrio* envelope, which might be essential for the parasite to pass through a pore clearly narrower than its body.

Our results show that *Bdellovibrio* do not penetrate visibly plasmolyzed host, but that penetration does occur in media of high osmolarity containing substances (TY/5 broth or 0.02 M KCl) that render the hosts resistant to plasmolysis by osmotic up-shocks. With osmotically shocked hosts, an inverse relation always exists between penetrable and plasmolyzed cells, and when cells of various species are under similar osmotic conditions, the less susceptible the species is to undergo plasmolysis, the higher is the proportion of penetrable cells. These results exclude the possibility that the primary effect of the plasmolyzing agents is on the capacity of the parasites to penetrate. Electron and phase microscopy show that a tight contact exists between the parasite penetration tip and the host cytoplasmic membrane. This association apparently is required for the penetration process, and it cannot be established in plasmo-

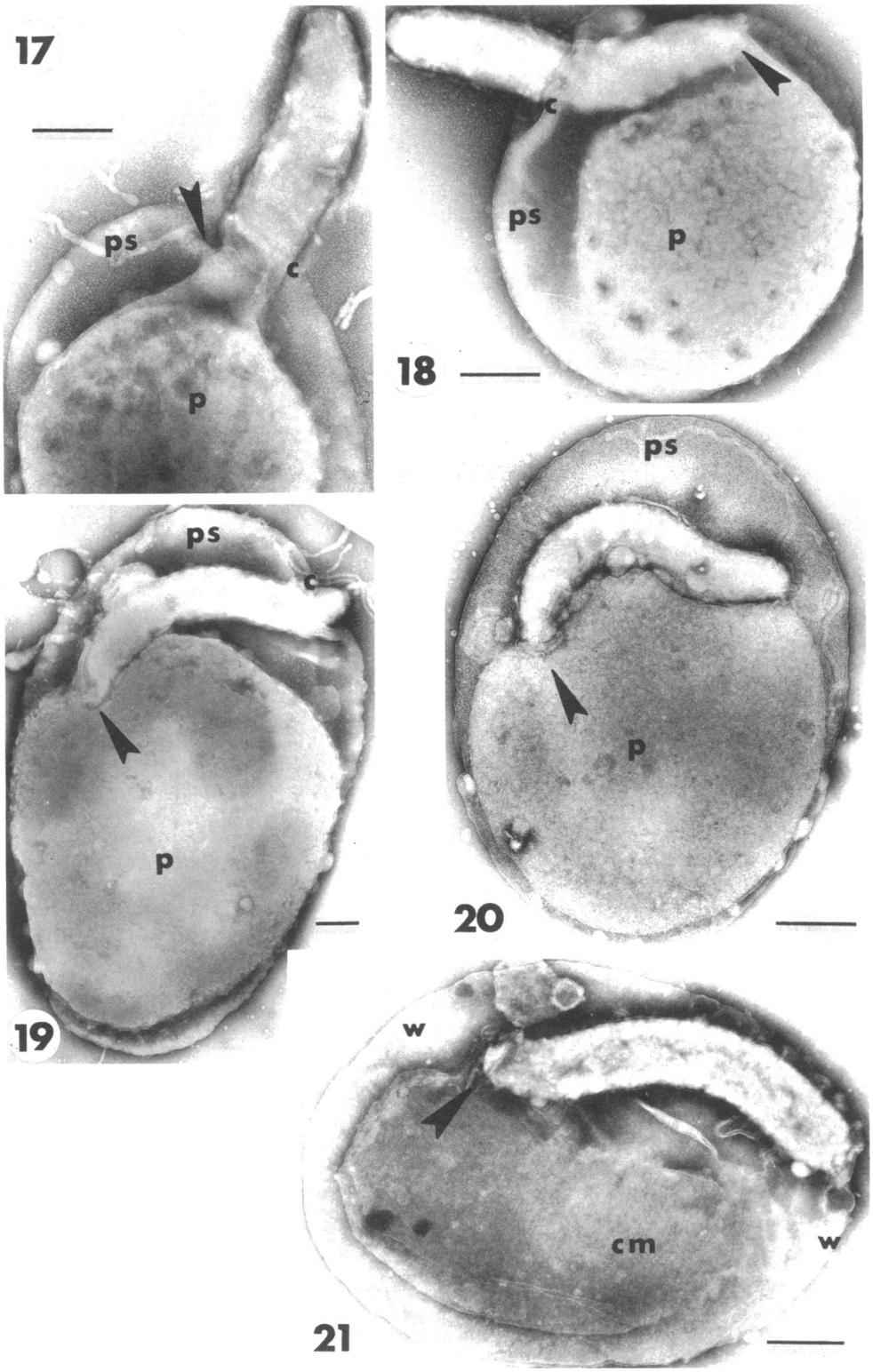

FIG. 17 to 19. *B. bacteriovorus 109 penetrated partway into E. coli (Fig. 17, 18) and P. fluorescens (Fig. 19). The parasites are attached at one pole (arrows) to the host protoplast (p), and adhere to it along a portion of their body (Fig. 18, 19). Note expanded periplasm (ps) and slight constriction (c) in the parasite body at the passage pore. Specimens from parasite-host mixtures were incubated for 20 min and stained negatively with ammonium molybdate.*

lyzed cells with protoplasts retracted from the wall and, therefore, not within reach of the parasite.

Whenever a portion of the host population is impenetrable, either in the presence of plasmolyzing agents or in their absence (e.g., when washed stationary-phase cells are used as hosts), the time required for all penetrable hosts to be infected is longer than in normal infection. Here, it is not possible to exclude a temporary effect of the plasmolyzing agent on the parasites; however, a more plausible explanation will be presented later. The arrest of *Bdellovibrio* motility and the resultant loss of its infectivity by 0.9% (0.155 M) NaCl (24) could be reproduced in our study only with parasites from old lysates. Structural and metabolic changes that occur in *Bdellovibrio* from old lysates and upon starvation (1; Abram, unpublished data; and Rittenberg, private communication) may account for the paralyzing effect of 0.9% NaCl. Penetration by *Bdellovibrio* from fresh lysates, however, is partially prevented by NaCl even at concentrations lower than 0.9% if the host cells undergo plasmolysis, substantiating a primary effect of the salt on the host rather than on the parasites.

The lower degree of closeness between the host cytoplasmic membrane and cell wall may explain the smaller number of penetrable cells in populations of washed host cells than in unwashed ones. This is most striking when the infected washed hosts are in the stationary phase. It is known that washing cells in water, i.e., an osmotic down-shock, reduces their internal solutes concentration (5) and that, in stationary-phase *E. coli*, the internal solutes are at a concentration several times lower than that of rapidly growing cells (8, 17). In washed cells and even more so when they are at the stationary phase, the membrane probably is not compressed against the cell wall due to a low protoplast turgor pressure and it is not, therefore, within reach of the parasite. Whenever a longer time is required for the infection of penetrable cells, it is likely to be due to an intermediate state of turgor pressure that reduces the membrane area that is apposed to the wall and consequently decreases the number of sites appropriate for infection. The low proportion of penetrable cells in populations of washed stationary cells is probably also related to a permanent damage caused by osmotic down-shock, which renders these cells incapable of regaining the original internal solute concentration.

The resistance to osmotic stresses is known to vary for different species (8, 10), and some species lose most of their intracellular K^+ when washed with distilled water (2, 9). The decreased proportion of penetrable, washed, exponential *P. fluorescens* cells is restored to normal in 0.02 M KCl, probably due to a rapid accumulation of K^+ ions. These cells, unlike washed stationary-phase cells, retain the capacity to restore a level of turgor pressure compatible with the penetration process discussed below.

Penetrated hosts always increase in volume and in some cases completely round up, yet they maintain their integrity. They are at osmotic equilibrium with the surrounding milieu, probably as a consequence of influx of water and efflux of small-molecular-weight substances. These fluxes occur after changes in the selective permeability of the host membrane which are known to be among the early effects of *Bdellovibrio* infection (14). The permeability changes may be due to direct action of the parasite on the host membrane (as suggested by the structural evidence) or they can result, indirectly, from a preceding lesion to the cell wall. Changes in the wall of infected hosts already at an early stage of infection, perhaps as soon as the passage pore opens, are substantiated by the susceptibility of the protoplasts to EDTA and the irregular appearance of the cell boundaries. The expansion of the periplasm in the infected host is always restricted to an area surrounding the passage pore, indicating that the cell wall at this zone is affected earlier and more extensively than at zones distal to it.

The morphological and structural changes displayed by the infected host, the firm contact between the parasite and the host protoplast during penetration, the increased permeability, and the osmotic stability of penetrated hosts lend credibility to the following penetration mechanism, by which the rapid entry is a passive act of the parasite. When *Bdellovibrio* is attached to the host protoplast at the periphery of the cell, the integrity of host wall and the

FIG. 20. *B. bacteriovorus 109 enclosed in the expanded periplasm (ps) of penetrated E. coli. The parasite is associated at its penetration pole (arrow) with the host intact protoplast (p) and adheres along its body to it. Specimens from parasite-host mixtures were incubated for 30 min and stained negatively with ammonium molybdate.*

FIG. 21. *Similar to Fig. 20, but after exposure to EDTA and stained negatively with potassium borotungstate. The parasite, enclosed by the intact host cell wall (w), is attached at one pole (arrow) to a large membranous remnant (cm) of the host-disrupted protoplast.*

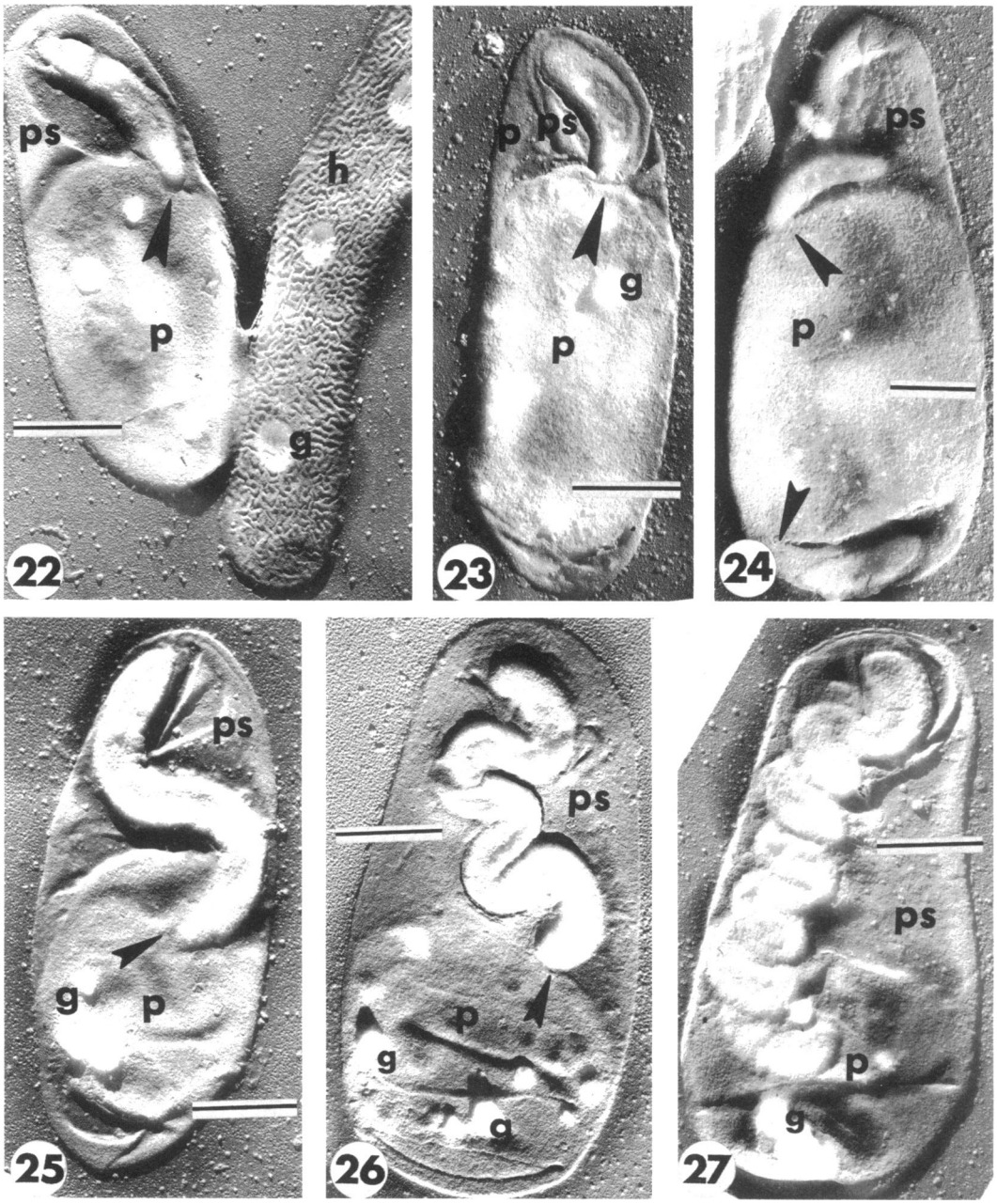

FIG. 22 to 27. *Shadowed preparations of S. serpens infected with B. bacteriovorus 109. Fig. 22 to 24, specimens from mixtures incubated for 30 min. The swollen ellipsoidal penetrated hosts (compare to slender uninfected host cell [h] in Fig. 22) enclose parasites attached at one pole (arrows) to intact protoplasts (p). The expanded periplasm (ps) is restricted to one pole, except in the host penetrated by two parasites (Fig. 24).*

FIG. 25 to 27. *Specimens from mixtures incubated for 2 h. The hosts enclose developing elongated parasites, still attached at one pole to the host protoplast (arrows). Note the polymetaphosphate granules (g). In Fig. 27 they are located at one pole of a cell in which almost all the protoplast has been exhausted during the development of the parasite. Bar, 1 μm.*

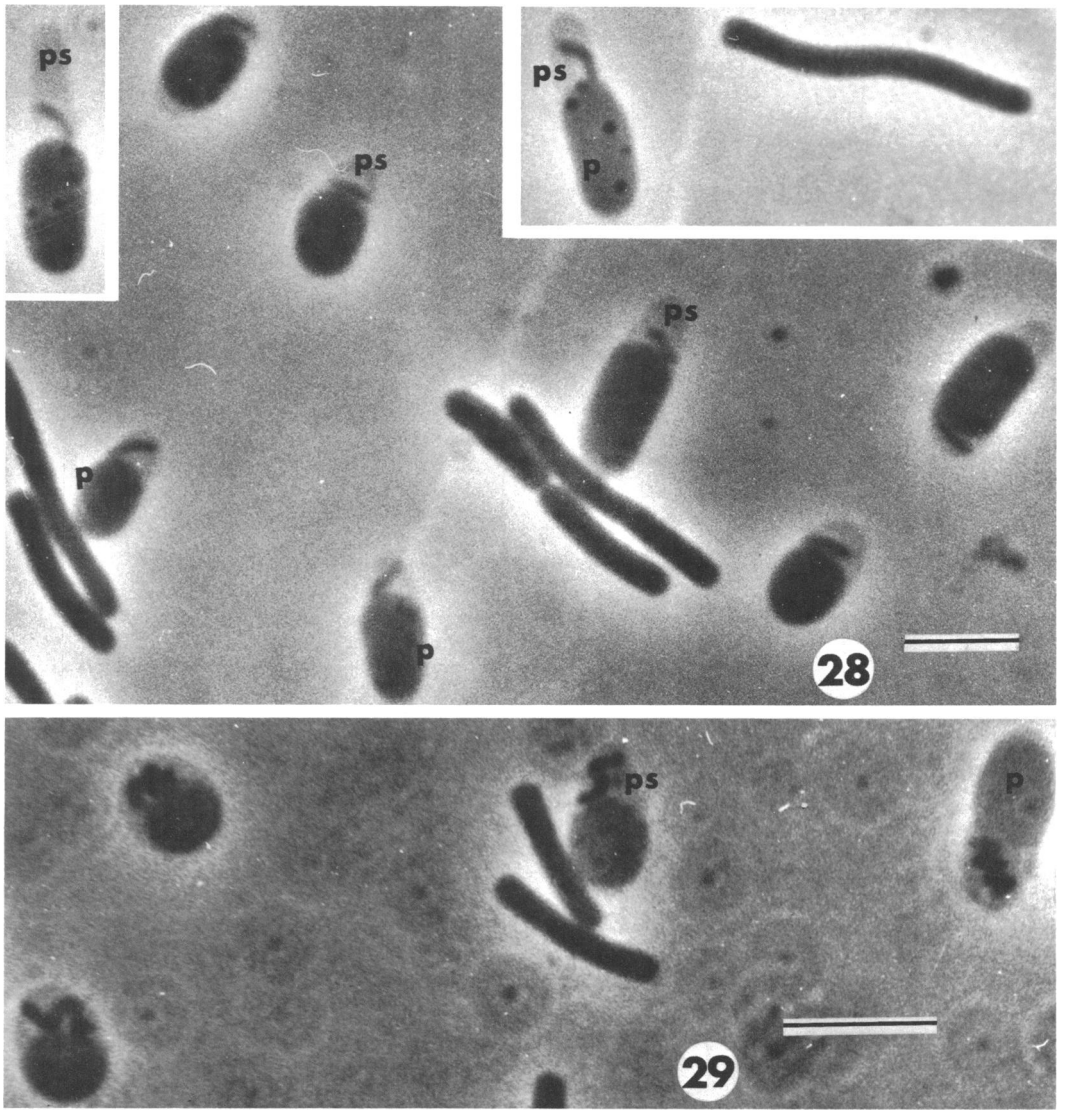

FIG. 28, 29. *Phase micrographs of S. serpens NCIB 9510 and LVH (Fig. 29) in mixtures with Bdellovibrio 109 and 6-5-S, respectively. Note the dense slender uninfected hosts and the swollen ellipsoidal infected ones showing dense protoplasts (p) and transparent expanded periplasms (ps) in which the parasites are enclosed (Fig. 28) and develop (Fig. 29) while associated with the protoplast. Specimens obtained from mixtures incubated for 35 min (Fig. 38) and 2 h (Fig. 29).*

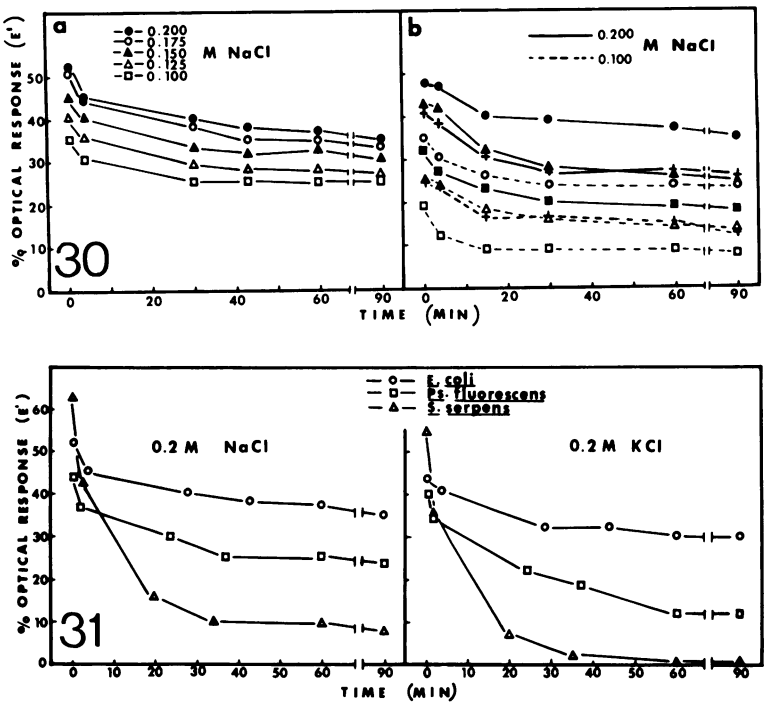

FIG. 30. *(a) Turbidity changes with time in water suspensions of washed exponential E. coli exposed to increasing concentration of NaCl. (b) Effect of the cell age and washing of the cells on turbidity changes in water suspensions of E. coli exposed to osmotic upshock. Symbols:* ●——●, ○-----○, *exponential washed;* ▲——▲, △-----△, *exponential unwashed;* +——+, +-----+, *stationary washed;* ■——■, □-----□, *stationary unwashed.*

FIG. 31. *Turbidity changes with time characteristic for different host species. Suspensions in water of washed exponential cells were exposed to osmotic upshock by 0.2 M NaCl and 0.2 M KCl.*

selective permeability of its membranes are affected. At a critical time, fluxes of solutes and water generate forces sufficient to cause the cell wall of the cytoplasmic membrane to expand. At this time, the weakened zone in the wall around the passage pore expands rapidly and to a greater extent than the protoplast, resulting in an enlarged pariplasmic space adjacent to the penetration site. As the wall expands, the passage pore slides along the body of the parasite, which is anchored to the host protoplast. Consequently, the parasite ends up enclosed in the expanded host periplasm. The penetration process, therefore, would be independent of the parasite's active motion after its attachment; attached parasites, in fact, appear, by phase microscopy, nonmotile before penetration occurs. It is reasoned that, for a host to be penetrable, a certain level of turgor pressure is required. first to establish a contact between the parasite and the host protoplast, and then, upon an increase in the permeability of the protoplast, to generate a force sufficient for parasite entry.

The displacement of the parasite penetration pole relative to the passage pore is a result of the periplasm expansion which is asymmetric with respect to the passage pore. The geometry of the expanded periplasm is related to the normal heterogeneity of the cell wall in rod-shaped cells, known from studies on the wall chemical structure (18) and on the degree of association between wall and cytoplasmic membrane (4). What determines the final orientation of the penetrating parasite is, therefore, the location of the passage pore, whether it is at or close to the poles of the cell or at its lateral portion.

The suggested penetration process is likely to be very rapid. A rapid entry is in agreement with documented observations by cinematography showing that, whereas the parasite is attached to the host surface for 10 to 20 min, the actual entry of the parasite is almost instantaneous (H. Stolp, Film C-972. Institut für den wissenschaflichen Film, Göttingen, West Germany, 1967). A rapid entry also would account for the low proportion of parasites seen during the course of this process.

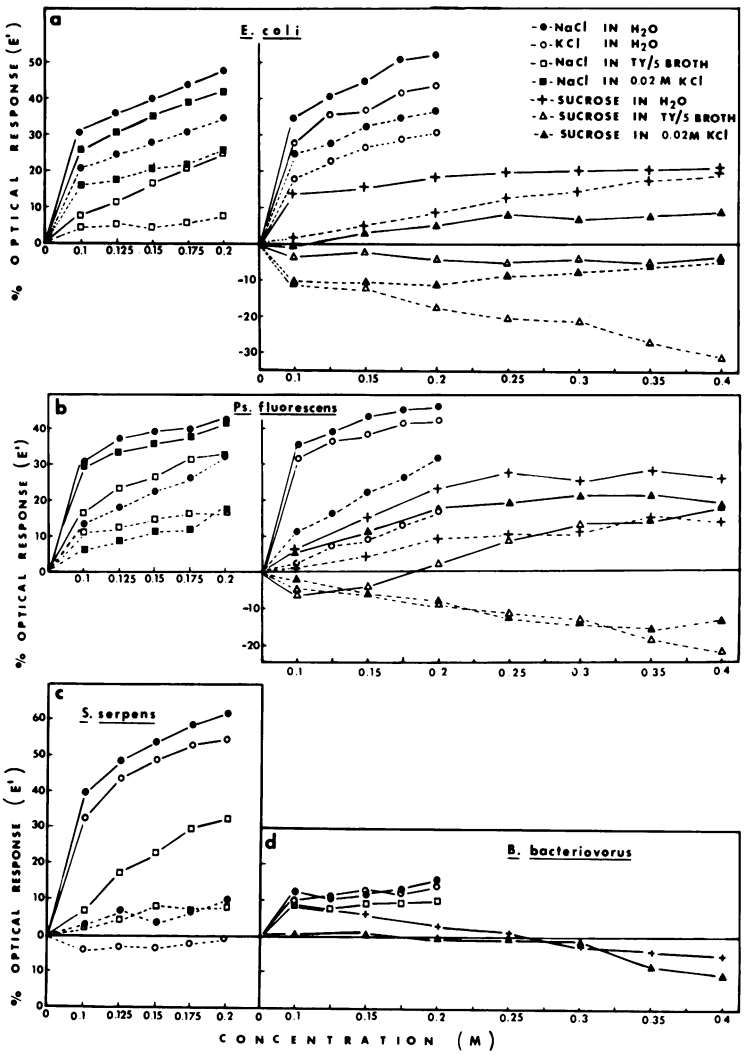

FIG. 32. *Effect of the solute concentration and the composition of the suspending medium on the optical response of suspensions of washed exponential cells of the three host organisms (a, b, c) and of B. bacteriovorus 109 from fresh lysates (d). Solid and broken lines depict initial (zero time) and final (leveled off, 60 min after the osmotic shock) E' values, respectively.*

TABLE 1. *Penetration of B. bacteriovorus 109 into host cells under various osmotic conditions*[a]

Organism; growth phase	Suspending medium washing	Plasmolyzing agent (M)	Penetrated cells (%)	Extent plasmolysis
E. coli; exponential	UW, broth	—	100	–
	UW, water	—	100	–
	W, broth	—	100	–
	W, water	—	80-90	–
E. coli; stationary	UW, broth	—	50-60	
	UW, water	—	30-40	–
	W, broth	—	20-30	–
	W, water	—	5-10	–
E. coli; exponential	W,[b] water	NaCl, 0.10-0.125	50-60	–
		0.15	20-30	++
		0.175	5	+++
		0.20	0	++++
E. coli; exponential	W,[b] broth	NaCl, 0.10-0.125	100	–
		0.15	60-70	–
		0.175	40-50	+
		0.2	20-30	++
E. coli; exponential	W,[b] 0.02 M KCl	NaCl, 0.10-0.125	80-90	–
		—	70-80	–
		0.15	50-60	–
		0.175	20-30	++
		0.20	5-10	+++
E. coli; exponential	W, water	KCl, 0.10-0.125	60-80	–
		0.15	40-50	+
		0.175	20-30	++
		0.20	5-10	+++
E. coli; exponential	W,[b] water	Sucrose, 0.10-0.15	60-80	–
		0.20	40-50	+
		0.25	5-10	++
		0.3-0.4	0	++++
E. coli; exponential	W, broth	Sucrose, 0.10-0.30	100	–
		0.35-0.4	50-60	+
E. coli; exponential	W, 0.02 M KCl	Sucrose, 0.10-0.25	100	–
		0.30-0.35	50-60	–
		0.4	20-30	+
E. coli; stationary	UW, water	NaCl, 0.10-0.15	20-30	–
	UW, water	0.175-0.20	0	+
	UW, broth	0.10-0.15	30-40	–
	UW, broth	0.175-0.20	0	+
	W, water	0.10-0.20	0-10	+
	W, broth	0.10-0.15	10-20	+
	W, broth	0.175-0.20	0	+
P. fluorescens; exponential	W, water	—	50-60	–
	W, broth	—	100	–
	W, 0 02 M KCl	—	100	–

TABLE 1—*Continued*

Organism; growth phase	Suspending medium washing	Plasmolyzing agent (M)	Penetrated cells (%)	Extent plasmolysis
P. fluorescens; exponential	W,[b] water	NaCl, 0.10–0.15	30–50	+
		0.175	20–30	++
		0.20	5–10	+++
P. fluorescens; exponential	W,[b] broth	NaCl, 0.10–0.15	80–100	–
		0.175	40–50	+
		0.20	20–30	++
P. fluorescens; exponential	W,[b] 0.02 M KCl	NaCl, 0.10–0.15	80–100	–
		0.175	30–40	+
		0.20	20–30	++
P. fluorescens; exponential	W, water	KCl, 0.10–0.15	60–70	–
		0.175	30–40	++
		0.20	10–20	+++
S. serpens; exponential	W, water	—	80–90	–
	W, broth	—	100	–
	W, 0.02 M KCl	—	80–90	–
S. serpens; exponential	W, water	NaCl, 0.10–0.125	30–50	++
		0.15	10–20	+++
		0.175	0	++++
S. serpens; exponential	W, broth	NaCl, 0.10–0.125	50–60	+
		0.15–0.175	20–30	++
		0.2	10–20	+++
S. serpens; exponential	W, 0.02 M KCl	NaCl, 0.10–0.15	40–50	+
		0.175	10–20	++
		0.20	5–10	+++

[a] The data represent reproducible results of two to five separate experiments for each variable. UW, unwashed host cells; W, washed host cells; broth, TY/5.

[b] Conditions under which *Bdellovibrio* strains 109 and D or 109 and 15243 have been used simultaneously.

ACKNOWLEDGMENTS

We are grateful to Josephine Yao for capable technical assistance.

This investigation was supported by National Science Foundation grant GB-8155, and Public Health Service grant AI-08715 from the National Institute of Allergy and Infectious Diseases. J.C.M. is a recipient of a fellowship from the Fundação Calouste Gulbenkian and D.C. was supported by the University of Pittsburgh Medical Alumni Association fund.

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