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Cell Surface Proteins of the Infectious Form of the Symbiotic Bacterium *Holospora obtusa*

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Abstract

In order to identify surface proteins of the infectious form of *Holospora obtusa*, a bacterium infecting the macronucleus of the ciliate *Paramecium caudatum*, the bacteria were labelled with the biotin derivative biotinyl-aminocaproic acid N-hydroxysuccinimide ester (BNHS). The BNHS-labelled proteins were then detected on Western blots by means of the streptavidin-peroxidase technique. Approximately 20 different polypeptides of 25–100 kDa could be identified. Ultrastructural cytochemical investigations gave strong evidence that the proteins which were labelled are in fact surface proteins of the bacteria.

Keywords: *Paramecium*, infection, *Holospora*, endonucleobiosis, nuclei, bacteria, ciliates, recognition, proteins, biotinylation

1. Introduction

The bacterium *Holospora obtusa* infects the macronucleus of the ciliate *Paramecium caudatum* (Ossipov et al., 1975). The infection of a host cell by a bacterium affords a cascade of recognition events and interactions between the host cell and the invading microbe. Bacteria invading a host cell are therefore expected to have certain proteins on their surfaces which function in the recognition process. Whereas it has been possible with the help of monoclonal

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antibodies to localize a number of proteins in the periplasm and cytoplasm of *H. obtusa* (Wiemann and Görtz, 1991), those polypeptides that are exposed at the bacterial surface have not been identified until now. In order to identify the surface polypeptides the infectious form of *Holospora* was incubated in the presence of biotinyl-aminocaproic acid N-hydroxysuccinimide ester (BNHS). This biotin derivative is assumed not to penetrate the cell membrane. The labelling of polypeptides then was followed with biochemical and cytochemical methods.

2. Materials and Methods

P. caudatum infected with the bacterium *H. obtusa* (strain ATCC #50009) was cultured at 26°C in a decoction of dehydrated cereal leaves inoculated with the bacterium *Enterobacter aerogenes*. Harvesting of *H. obtusa* cells was done using a discontinuous Percoll gradient as described (Görtz et al., 1990). For biotinylation freshly isolated bacteria (4×10^8) were incubated in 1 ml PBS (3.3 mM KH_2PO_4, 6.7 mM K_2HPO_4, 18 mM NaCl) with 50 μg BNHS (Sigma) for 5 min at room temperature. To remove unbound BNHS the cells were sedimented twice for 5 min at 14,000 g and the resulting sediment was taken up in 1 ml PBS.

Cells were lysed by the addition of 0.5 volumes of 1 N NaOH to a packed cell sediment, and then taken up in 8 volumes of lysis buffer containing 3% Na-dodecylsulfate (SDS), 5% mercaptoethanol, 10% glycerol and 0.063 M Tris-HCl, pH 6.8 (Schmidt et al., 1987). SDS-PAGE of the total protein mixtures was performed essentially according to Laemmli (1970) using a 5% stacking gel and a 10–20% polyacrylamide gradient in the separation gel.

Proteins were either stained using Coomassie Brilliant Blue R-250 (CBB, Merck, Darmstadt, FRG) or blotted onto a Fluorotans membrane (Pall, Dreieich, FRG). Blots were blocked for 60 min in 3% bovine serum albumine (BSA, Serva, Heidelberg, FRG) in PBS, washed 2×5 min in 0.05% Tween 20 in PBS (TPBS) and then 60 min in 1% BSA in PBS + 20 μg streptavidin-horseradish peroxidase (Boehringer, Mannheim, FRG). To remove the unbound streptavidin the blot was incubated 2×5 min in PBS and subsequently developed for 15 min in staining solution (25 mg 4-chloro-1-naphtol dissolved in 7.5 ml cold methanol, then addition of 45 ml PBS + 30 μl cold H_2O_2). The reaction was stopped by washing 2×5 min in dH_2O. To detect any endogenous biotin, freshly isolated bacteria which were not incubated with BNHS were lysed and treated as described above.

For electron microscopy freshly isolated infectious forms of *H. obtusa* were biotinylated as described above and fixed with 2.5% glutaraldehyde in 0.01 M

PIPES (piperazin-N,N′-bis-(2-ethane sulfonic acid)) buffer, pH 7.2 for 30 min, washed once with 0.1% Triton X-100 in PBS pH 7.2 and subsequently 3 times in PBS. Then cells were incubated in 3% BSA in PBS for 1 hr, thereafter for 1 hr in 1% PBS + 20 μg streptavidin-horseradish peroxidase. Unbound streptavidin was removed by washing with Tris-HCl, pH 7.6. Then 0.05% (w/v) 3,3′-diaminobenzidine in the same buffer was added for 5 min and 6 μl H_2O_2 were added for a further incubation of 5 min. Cells were washed twice in Tris-HCl, treated with 0.5% OsO_4 for 30 min and embedded in Epon. Sections were investigated unstained.

3. Results and Discussion

Approximately 20 different polypeptides of 25–100 kDa were labelled, when infectious forms of *H. obtusa* were incubated in the presence of the biotin derivative BNHS (Fig. 1, lane 2). When cells were incubated in the absence of

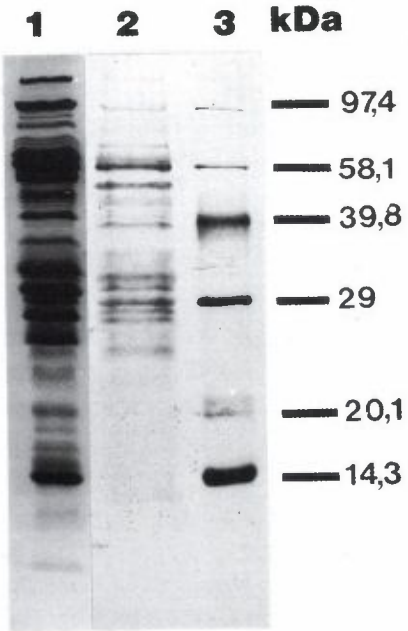

Figure 1. SDS-PAGE of total protein derived from ≈ 10^7 cells of *Holospora obtusa* (infectious form)
lane 1: polypeptide pattern after staining with CBB
lane 2: biotinylated polypeptides
lane 3: biotinylated marker polypeptides

BNHS no labelling could be detected (data not shown). This indicated that the labelling was exclusively caused by the added BNHS and not due to endogenous biotin. It has been shown for eukaryotic cells that BNHS does not pass membranes in considerable amounts (Freiburg, unpublished). This observation and the fact that only a small number of polypeptides were biotinylated indicate a selective labelling presumably of those polypeptides that are at least in part located at the cell surface.

To get further evidence that only surface proteins of the bacteria were labelled by BNHS, infectious forms were incubated with BNHS, fixed with glutaraldehyde and then treated with streptavidin-horseradish peroxidase and processed for the electron microscope. The ultrastructural analysis clearly indicated that indeed exclusively surface structures were labelled by BNHS. As the result of the enzyme reaction the bacteria were strongly labelled exclusively on their surfaces (Fig. 2a). A thin basic layer of label was found all over the cells. In addition the label formed small aggregates which were irregularly distributed over the surface (Fig. 3). In controls, where the bacteria were not incubated with BNHS, no label was detected (Fig. 2b).

The infectious form of *H. obtusa* reveals a very typical polypeptide pattern (Görtz et al., 1988) that is characterized by the fact that some bands are much broader and more heavily stained than others (Fig. 1, lane 1). This was taken as an evidence that some proteins are much more abundant than others. In contrast, the bands generated by biotinylated polypeptides are similar to each other in size and staining intensity. This may indicate that the very abundant polypeptides do not belong to that fraction that is accessible to the added BNHS but are rather located within the cell.

The sensitivity of the detection of biotinylated polypeptides is much higher than staining by CBB (von Boxberg et al., 1990). One may therefore assume that the number of cell surface polypeptides is in fact as low as found on the blots (Fig. 1). This assumption is supported by the finding that incubation of a cell lysate (derived from the same cell number as in experiments with intact cells) with BNHS leads to a general labelling without any selectivity (data not shown). The results show that the labelling with BNHS is a convenient method to identify surface proteins of *H. obtusa*. The observation that only a few polypeptides are biotinylated corresponds to the low number of intramembrane particles in the outer membrane of the infectious form of *H. obtusa* found in freeze-fracture preparations (Görtz et al., 1989).

The polypeptides exposed at the cell surface of the infectious form of *H. obtusa* may have a function in the recognition process in the first stages of infection. In order to verify such a function it is now necessary to compare the surface proteins of the infectious form with the surface of the bacteria at later

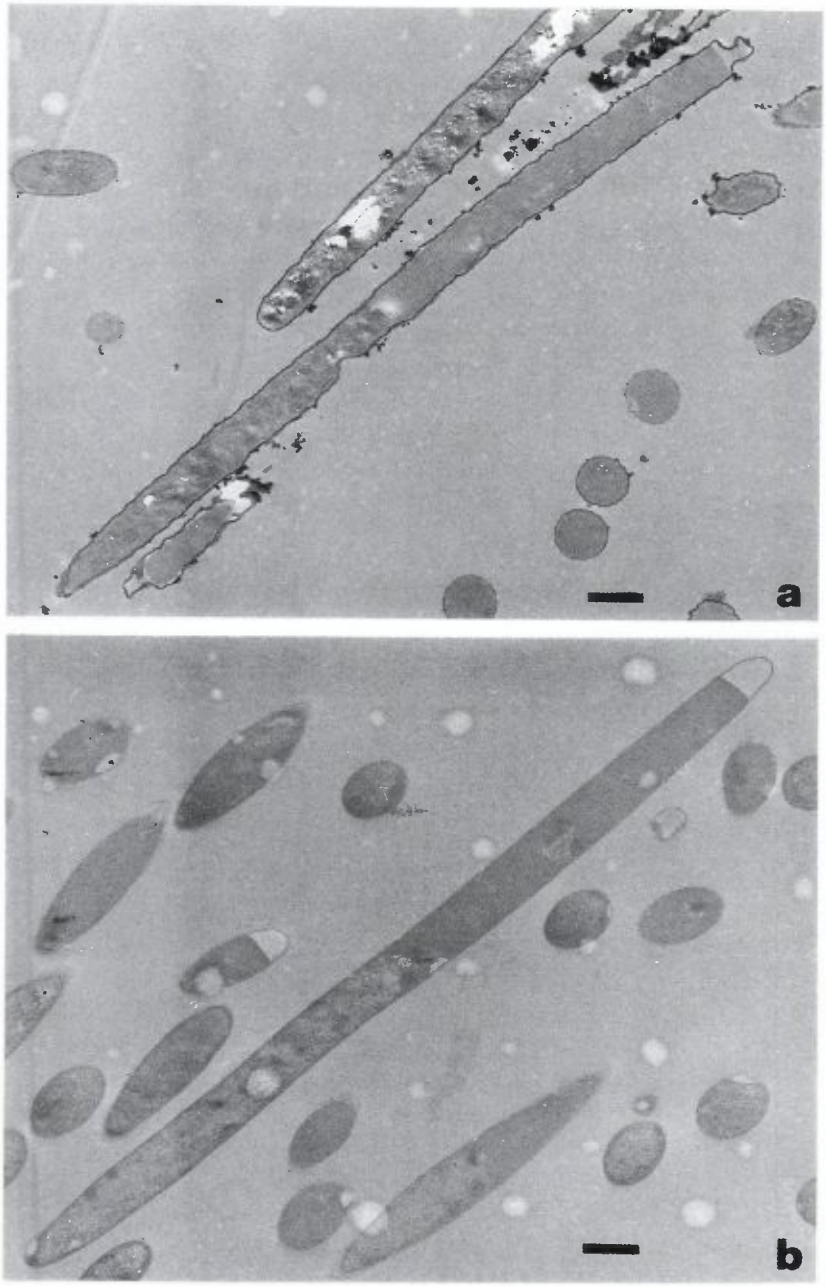

Figure 2. Electron micrographs of biotinylated (a) and non-biotinylated (b) infectious forms of *Holospora obtusa*. Unstained, bar 1 μm.

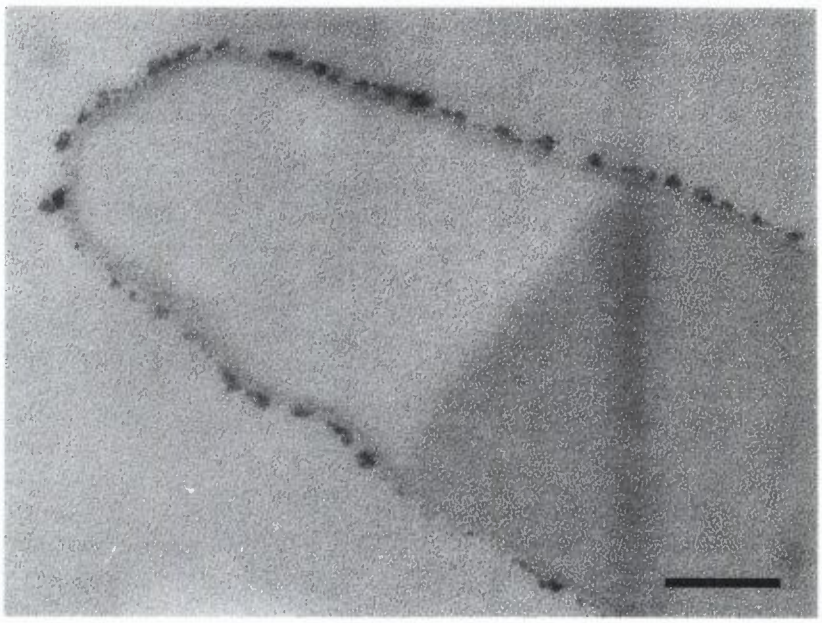

Figure 3. Electron micrograph of a biotinylated infectious form of *H. obtusa* at higher magnification. Unstained, bar 0.25 μm.

stages of the infection process. Whereas the infectious form has to interact with the phagosomal membrane (Görtz and Wiemann, 1989), later stages may have to interact with the cytoskeleton and the nuclear envelope of the host cell. The different steps of the invasion process could afford different proteins on the bacterial surface. The identification of these proteins is one of the prerequisites to study the dynamics of the bacterial cell surface during the process of infection.

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