Identification of surface-exposed *Yersinia pestis* proteins by radio-iodination and biotinylation

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**Summary.** When whole cells (stationary phase) of *Yersinia pestis* strain EV76 were radiolabelled with Iodogen and $^{131}$I, 16 major and 10 minor surface-exposed outer membrane proteins (OMPs) were identified. Labelling with N-hydroxysuccinimidy 6-biotinylamino-hexanoate (biotin X-NHS) resulted in a complex protein profile detectable after blotting and developing with peroxidase-conjugated avidin. *Y. pestis* cell fractionation revealed that biotin X-NHS labelled not only OMPs but also proteins of inner cell compartments. Therefore, radiolabelling was the more reliable technique for identifying the OMPs of *Y. pestis*.

**Introduction**

The outer membrane of gram-negative bacteria is usually the outermost cell envelope layer. Some surface-exposed outer membrane proteins (OMPs) play a role in invasion, adherence and ability to evade humoral and cellular defence mechanisms.1 *Yersinia pestis*, the aetiological agent of plague,2 is a gram-negative organism whose plasmid-coded OMPs are associated with survival and growth in vivo.3-4

The surface proteins of several bacterial species have been identified by iodination with membrane-impermeable enzymes5-8 or the water insoluble reagent 1,3,4,5,6-tetrachloro-3,6-diphenylglycouril (Iodogen).9-11 Labelling with Iodogen has proved reliable for viruses, bacteria and eukaryotic cells,12-14 but the handling of radioactive material and the short half-life of $^{125}$I or $^{131}$I are serious drawbacks.

The avidin-biotin system is a useful alternative for cell-labelling and affinity isolation techniques.15-17 Biotin derivatives conjugated with N-hydroxysuccinimide were synthesised for the specific labelling of proteins and peptides under mild conditions.18 Recently, a new biotin derivative, N-hydroxysuccinimidyld 6-biotinylamido-hexanoate (biotin X-NHS), was used for the specific labelling of cell-surface proteins of embryonic neural chick cells.19 It is an ideal reagent for the electrophoretic analysis of cell surface components because it binds to molecules under mild conditions and does not penetrate cell membranes or interfere with one- or two-dimensional gel electrophoresis. However, the use of this technique for labelling the cell surface proteins of gram-negative bacteria has not been evaluated.

This study compared the effectiveness of Iodogen-catalysed iodination and biotinylation with biotin X-NHS for the specific labelling of *Y. pestis* OMPs.

**Materials and methods**

**Bacteria and growth conditions**

*Y. pestis* strain EV76, kindly donated by Dr R. R. Brubaker, was used. Cells were grown in YT medium (tryptone 1%, yeast extract 0.5%, NaCl 0.5%) at 28°C up to the stationary phase.

**Radio-iodination of intact cells**

The *Y. pestis* cells were radio-iodinated as previously described.9 Stationary phase cells were washed twice in phosphate-buffered saline (PBS) and suspended to a final concentration of $10^9$ cells/ml. The cell suspension (0.2 ml) was allowed to react with 300 µCi of carrier-free $^{125}$I (IPEN, São Paulo, Brazil) (10 mCi/ml) in reaction vials containing 45 µg of Iodogen (Pierce Chemical Co., Rockford, USA) for 90, 45 or 30 s at room temperature. Radio-iodination was interrupted by transferring the samples to another tube containing 3 ml of 10 mM NaI, followed by three washes with the same solution and one wash with 0.5 M Tris-HCl (pH 6.8). Labelled cells were solubilised in electrophoresis sample buffer and subjected to electrophoresis and autoradiography.

**Biotinylation of intact cells**

The *Y. pestis* cells were biotinylated by a previously published procedure.9 Cells corresponding to 0.2 ml...
of a stationary phase culture were washed twice with labelling buffer (NaCl 8 g, KCl 0.4 g, glucose 1 g, MgSO₄·7H₂O 0.2 g, CaCl₂·2H₂O 0.2 g, and NaHCO₃ 0.5 g/L, pH 7.4) and then transferred to an Eppendorf tube containing different amounts of biotin X-NHS (Calbiochem Boehringer Diagnostics, La Jolla, CA, USA) solubilised in dimethyl formamide (Fluka, Buchs, Switzerland) for 10 min at room temperature. After labelling, cells were washed twice with M9 medium containing casamino acids (Na₂HPO₄ 0.6%, KH₂PO₄ 0.3%, NaCl 0.05%, NH₄Cl 0.01%, casamino acids 0.02%; Difco). The cells were solubilised in sample buffer and subjected to SDS-PAGE and blot transfer.

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**Fig. 1. Autoradiogram of Iodogen-catalysed iodination of stationary phase _Y. pestis_ cells grown at 28°C.** Cells were labelled with iodogen and 131I for 90 (lanes 1, 4, 7), 45 (2, 5, 8), and 30 (3, 6, 9) s. Different amounts of protein were applied in each run: 64 µg (1, 2, 3), 32 µg (4, 5, 6) and 16 µg (7, 8, 9). After separation on SDS-PAGE, gels were exposed to X-ray film for 24 h at −80°C. The amount of radioactivity in each sample ranged from 8000 to 90000 cpm.
Fig. 2. *Y. pestis* protein labelled with different amounts of biotin X-NHS. Each sample contained intact *Y. pestis* cells, grown at 28°C, corresponding to 0.2 ml of stationary phase cells (4.5 mg wet weight). The amount of biotin X-NHS used in each reaction was 1 mg (lane 1), 500 µg (2), 250 µg (3), 125 µg (4), 62.5 µg (5), 31.25 µg (6), 15.625 µg (7), 7.5 µg (8). Each sample contain 25 µg of protein. The labelling period was 10 min.

Fig. 3. Labelling of *Y. pestis* cells with biotin X-NHS for different incubation times. Stationary phase cells grown at 28°C were incubated with biotin X-NHS (1 mg) for 30 s (lane 1), 1 min (2), 3 min (3), 5 min (4) and 10 min (5). Samples containing 25 µg of protein were applied on the gel.

Results

Radio-iodination of intact *Y. pestis* cells

Sixteen protein bands were regularly detected in *Y. pestis* whole cells labelled with Iodogen and ^131^I. A further set of 10 minor OMPs could be seen also, but their optimal resolution depended on sample preparation, electrophoresis and exposure time. The most prominent OMPs detected in autoradiograms had mol. wts of 65, 39, 32–35 and 12 kDa. A heavily labelled broad band migrating to a position of 32–35 kDa was formed by a triplet not clearly distinguished on autoradiograms but easily resolved on silver-stained SDS-PAGE gels. These proteins were represented by the protein E and probably two other porin-like proteins also exposed on the cell surface (data not shown). An estimation of the optimal labelling period and ideal protein load was performed to obtain a better resolution on autoradiograms after exposure for 24 h (fig. 1). Best results were obtained with a labelling period of 45 s and 32 µg of total protein/well.

Biotinylation of intact *Y. pestis* cells

The labelling of *Y. pestis* whole cells with biotin X-NHS resulted in a complex protein profile composed of at least 30 major bands of different staining intensities on blots. The most prominent bands revealed by this technique were clearly distinct from the *Y. pestis* OMPs detected in silver-stained gels or after Iodogen-catalysed iodination of surface-exposed proteins. To establish the best labelling conditions for biotin X-NHS, different ratios of biotin to bacterial...
cells were used. Decreasing amounts of biotin X-NHS were used to label a fixed amount of cells, corresponding to 0.2 ml of stationary phase Y. pestis culture (4.5 mg wet weight; fig. 2). With a labelling period of 10 min, 1 mg of biotin/reaction gave the best results. The influence of the labelling period on the profile of biotinylated surface proteins was also evaluated. Biotinylation of the most prominent proteins was observed after labelling periods as short as 30 s (fig. 3). However, several additional bands were detected after prolonged labelling periods.

**Fractionation of biotinylated Y. pestis**

The increasing number of peptides identified in cells labelled for prolonged periods with biotin may indicate a permeation of biotin X-NHS to inner compartments of the Y. pestis cell and consequent labelling of intracellular proteins. Fractionation of biotinylated Y. pestis into soluble components (periplasm and cytoplasm), cytoplasmic membrane and outer membrane indicated that biotinylation of whole cells resulted in the labelling of soluble and cytoplasmic membrane proteins (fig. 4).

**Discussion**

Surface proteins of *Vibrio cholerae*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Treponema pallidum* and *Aeromonas salmonicida* have been identified by the Iodogen-catalysed iodination technique. The results of this study strongly suggest that the proteins detected by the Iodogen procedure are indeed surface-located. The protein profile obtained in autoradiograms after radio-iodination of intact Y. pestis cells was remarkably similar to the band pattern of isolated outer membranes after SDS-PAGE. Sixteen major proteins and 10 other minor bands were detected in stationary phase Y. pestis cells grown at 28°C. This number represents almost all the OMPs of this organism detected in silver-stained SDS-PAGE and approximately half of the OMPs detected in twodimensional gels. Increased amounts of cells and longer reaction periods did not change the number of proteins specifically labelled by this procedure (fig. 1). Moreover, only traces of radioactive contamination, represented by OMPs, could be found in cytoplasmic membrane after fractionation of radiolabelled cells (and unpublished observations). Additional evidence of the surface-exposed nature of proteins identified by Iodogen-catalysed iodination would be their recognition by antibodies produced against intact Y. pestis cells. The profile of proteins recognised by specific anti-Y. pestis sera on immunoblots shares several features with the set of proteins labelled by Iodogen. At least eight major Iodogen-labelled OMPs were recognised by the sera of guinea-pigs immunised with *Y. pestis* or by sera from convalescent plague patients. These observations further emphasise that the Iodogen-catalysed iodination of *Y. pestis* cells is restricted to surface-located OMPs.

Apart from lectins, protein A and antibodies, the avidin-biotin complex represents an excellent binding system for characterising chemical and biological materials. Properties of avidin and biotin relevant to their use as technical tools include high affinity (KD < 10^-15 m) and multivalent binding for free and conjugated biotin, great stability, relatively small size, homogeneity, ready availability, the polar nature of biotin, and easy attachment of biotin to other macromolecules.

Attempts to identify surface-exposed proteins with the avidin-biotin method have been restricted mainly to eukaryotic cells, e.g., leucocytes, trypanosomes and nematodes. In particular, biotin X-NHS was used for selective labelling, isolation and electrophoretic analysis of surface proteins of embryonic chick retina cells. However, the labelling of *Y. pestis* cells with biotin X-NHS showed that this compound can permeate the cell envelope.

The failure to label specifically the surface proteins of *Y. pestis* with biotin X-NHS may have been due to its low mol. wt (454-3) which is well below the exclusion limit of the outer-membrane hydrophilic pores. Moreover, gram-negative bacteria are endowed with a specific biotin transport system, including outer-membrane receptors, responsible for the intracellular accumulation of this vitamin. Attempts to block the penetration of biotin X-NHS into the cells by changing the labelling conditions, e.g., by altering the composition of the labelling buffer, adding osmoprotectants such as proline, or incubating at different temperatures, did not change the results (data not shown).

Some *Y. pestis* OMPs could be detected readily by the biotin X-NHS reagent but others could not be
labelled even in reactions with isolated outer membranes (fig. 4, and unpublished results). One possible explanation is the varied amino-acid composition of the proteins and the different specificities of Iodogen and biotin X-NHS, which mainly catalyse the binding of surface-exposed tyrosine and lysine residues respectively.

The present study has shown that Iodogen-catalysed iodination is more specific than biotin X-NHS for labelling Y. pestis surface-exposed proteins. However, because of its simplicity, safety and successful application to eukaryotic cells, biotin-mediated labelling of surface-exposed proteins of gram-negative bacteria should be studied further with larger biotin derivatives or improved labelling conditions.

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References