Studies on Outer Membrane Proteins of *Moraxella nonliquefaciens*

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Outer membrane fractions of *Moraxella nonliquefaciens* 7784 strains SC-c and N-b, isolated by extraction with lithium acetate, were analysed by SDS-PAGE. Three main proteins were found, of which one, with an apparent molecular weight of 19 500, was undetectable in membranes isolated by lysis of lysozyme/EDTA spheroplasts. All three major proteins were heat modifiable.

INTRODUCTION

The protein pattern seen after SDS-PAGE of outer membrane fractions seems to be dependent upon the isolation method used. We have previously shown that the outer membrane fraction of *Moraxella nonliquefaciens* 7784 SC-c obtained by lysis of lysozyme/EDTA spheroplasts according to Osborn (1972) contained partly degraded peptidoglycan (Andersen *et al.*, 1980). In order to obtain a peptidoglycan-free outer membrane fraction, we used extraction of whole cells with lithium acetate (Heckels, 1977). The major protein band obtained by this method was undetectable in the outer membrane of lysed lysozyme/EDTA spheroplasts. Variation of the solubilization conditions in SDS before SDS-PAGE revealed differences in the protein pattern, suggesting that *Moraxella nonliquefaciens* contains several different heat-modifiable outer membrane proteins.

METHODS

Bacteria and media. Organisms and growth conditions were as previously described (Andersen *et al.*, 1980).

Preparation of the membrane fraction. The outer membrane fraction was extracted from the bacteria essentially as described by Heckels (1977). The cells were harvested in the late-exponential phase of growth ($A_{650} = 1.5-2.0$; 1 cm light path; Beckman DB spectrophotometer) by centrifuging at 3900 *g* for 10 min (all centrifugations were done at 4 °C). The sediment was suspended in 0.01 M-Tris/HCl, pH 8.0, and recentrifuged at 10000 *g* for 30 min. Washed cell pellets were suspended in 0.2 M-lithium acetate, pH 6.0, at 5 ml per g of wet cells and extracted for 2.5 h at 100 r.p.m. in a gyratory water bath shaker (Baird and Tatlock) at 45 °C. The outer membrane preparations were stored at -20 °C for further studies.

SDS-PAGE. Outer membrane fractions were analysed on an 18% (w/v) polyacrylamide gel with 0.5% (w/v) *N,N'*-methylenebisacrylamide (BioRad) with the Laemmli buffer system (Frøholm & Sletten, 1977). The following standard proteins were used for molecular weight determinations: human albumin (molecular weight 68000, Behringwerke), ovalbumin (43000, Miles Labs), myoglobin (17200, Koch-Light) and lysozyme (14300, Sigma). The staining conditions were those described by Fairbanks *et al.* (1971) using Coomassie brilliant blue R 250 (BioRad). For heat modification studies, samples of outer membrane preparations were treated for 1, 5, 10 and 20 min at 56 °C, and for 10 and 20 s, and 1, 5, 7 and 10 min at 100 °C. Two samples were used as controls: one was immediately applied to the gel after adding Laemmli buffer and the other after 30 min at 20 °C.

Trypsin treatment. The membrane suspension was divided into two samples before trypsin treatment (Nurminen, 1978). One was boiled for 5 min at 100 °C, the other not. To both samples 1.5 µg trypsin (Koch-Light, twice-crystallized) in 15 µl 0.01 M-EDTA/0.2% (v/v) Triton X-100 (Merck)/0.2% (w/v) SDS (BioRad) was added to 20 µg protein in 25 µl extraction buffer. Incubation was overnight at 37 °C.

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Fig. 1. SDS-PAGE patterns of outer membrane proteins from *M. nonliquefaciens* 7784 strain N-b obtained with 0.2 M-lithium acetate, pH 6.0, at 45 °C. (a) After heating in SDS under the following conditions: (1) 20 °C, directly applied to the gel; (2) 20 °C, 30 min; (3) 56 °C, 1 min; (4) 56 °C, 5 min; (5) 56 °C, 10 min; (6) 56 °C, 20 min; (7) 100 °C, 10 s; (8) 100 °C, 20 s; (9) 100 °C, 1 min; (10) 100 °C, 5 min; (11) 100 °C, 10 min; (12) 100 °C, 20 min. (b) After the following treatments with trypsin: (1, 6, 12) untreated controls; (2-5) not preboiled before trypsin added; (7-11) preboiled before trypsin added. Heating in SDS was under the following conditions: (1, 6, 12) 100 °C, 5 min; (2, 7) 56 °C, 5 min; (3, 8) 56 °C, 10 min; (4, 9) 100 °C, 1 min; (5, 10, 11) 100 °C, 5 min. Molecular weight standards used: human albumin (68000 daltons), ovalbumin (43000), myoglobin (17200) and lysozyme (14600).

RESULTS

Outer membrane fractions of *Moraxella nonliquefaciens* 7784 strains SC-c or N-b were extracted from washed cells in 0.2 M-lithium acetate, pH 6.0, according to Heckels (1977). The outer membrane vesicles thus obtained showed, on SDS-PAGE under Laemmli conditions, the presence of three major protein bands with apparent molecular weights of 19 500, 41 000 and 53 000 (proteins A, B and C, Fig. 1*a*, lane 10). The 53 000 band is clearly triple in this lane, but double in lanes 9 and 12 of Fig. 1*a*). Washing and slight homogenizing of the cells before extraction complicated the protein pattern and reduced the bands in the regions of B and C (not shown). Protein A remained distinct.

To get more information on the outer membrane proteins, heat modification experiments were done. The outer membrane fractions were heated for different periods of time at 56 °C and 100 °C. After 1 min at 56 °C (Fig. 1*a*, lane 3) no marked changes could be observed from the untreated control (Fig. 1*a*, lane 1), although a different intensity distribution between the bands labelled B was noticed.

With increasing time at 56 °C a graded transition in molecular weight was seen with the protein (double band) with an apparent molecular weight of 46 000, called C*. After 5 min at 56 °C another band with a higher molecular weight (53 000, called C) was observed (Fig. 1*a*, lane 4). The amount of the latter increased with time and temperature. The C* band was initially double and the lower one in Fig. 1*a*) had disappeared after 5 min at 100 °C (Fig. 1*a*, lane 10) whereas the upper one gradually became fainter after 100 °C treatment with a concomitant appearance of an extra band at the position of C.

The B band also was double in the untreated samples and after treatment at 56 °C for 1 min. After 5 min at 56 °C the lower-running component had disappeared and the upper one was distinctly darker stained. No further change took place on prolonged treatment or at 100 °C. (These bands are not labelled B* and B, but may be analogous.)

On close inspection one can also see changes in some of the minor bands in the regions between A and B. In the region close to B a change is evident between 1 and 5 min at 56 °C. Closer to A a new band seems to appear after 1 min at 100 °C.

No change in molecular weight of protein A* (22 000) was observed at 56 °C. After 20 s at 100 °C part of this protein migrated with a lower molecular weight, A (19 500), (Fig. 1*a*, lane 8) and after 5 min at 100 °C (Fig. 1*a*, lane 10) the change was almost complete.

Similar results were obtained with the SC-c strain (not shown).

Proteolytic treatment with trypsin (Fig. 1*b*) changed the protein pattern of the outer membrane distinctly. The most pronounced difference was the appearance of split products with apparent molecular weights of 16 000 and about 9000. These bands probably derive from protein A which has disappeared from both unboiled and preboiled samples. It looks as if trypsin-treated A* has unchanged mobility and heat stability. The split products appear when A* disappears. This disappearance of the band in the region of A* was complete after 5 min at 100 °C (Fig. 1*b*, lane 5). The same observations were made with the preboiled samples (Fig. 1*b*, lanes 10 and 11). Another marked difference was the disappearance of the bands in the regions of bands B and C, together with the emergence of bands in the apparent molecular weight region about 35 000 (Fig. 1*b*, lanes 2–5). This leads us to suggest that the latter bands may be split products of either protein B or C, or both. No heat modifications were observed with the split products in the region of about 35 000. Heating the outer membrane fractions before protease treatments made this band almost disappear (Fig. 1*b*, lanes 7–11).

DISCUSSION

The major protein of the outer membrane fraction of *Moraxella nonliquefaciens* 7784 strains SC-c and N-b isolated by extraction with lithium acetate according to Heckels (1977), has been shown to have an apparent molecular weight of 19 500. Previously (Andersen *et al.*, 1980), we did not detect this protein in the outer membrane fraction obtained after lysis of lysozyme/EDTA spheroplasts. This demonstrates how dependent the outer membrane protein patterns in SDS-PAGE are upon the method used. This observation has previously been made by several

investigators, including Dassa *et al.* (1978), who compared the protein distribution obtained in outer membrane fractions of *Escherichia coli* K12, isolated according to the methods of Osborn *et al.* (1972), Wolf-Watz *et al.* (1973), Mizushima & Yamada (1975) and Koplow & Goldfine (1974). A possible explanation for this difference according to the method used might be found in the results of the trypsin treatments. The present results indicate that protein A is degraded into split products with apparent molecular weights of 16000 and 9000. It is well known that during formation of EDTA/lysozyme spheroplasts, periplasmic enzymes are released (Neu & Heppel, 1965) which can degrade outer membrane proteins into lower molecular weight polypeptides. Accordingly, the protein pattern obtained after lysis of lysozyme/EDTA spheroplasts revealed a distinct band with an apparent molecular weight of 16000. This band may well be a degradation product of protein A.

Another explanation for the lack of protein A might be that this protein is very weakly bound and hence solubilizes easily in the buffers used. The several manipulations of the Osborn technique make it possible for any solubilized protein to escape during the process of membrane recovery.

No major differences in protein pattern could be detected between the strains SC-c and N-b. This close resemblance is in line with the fact that SC-c is a fimbriation variant derived from N-b (Frøholm & Bøvre, 1978) and does not contradict strain-specific variations in major membrane proteins as found among neisseriae (Frasch & Mocca, 1978).

Moraxella nonliquefaciens apparently contains at least three types of heat-modifiable proteins. The fact that protein A was degradable by trypsin may seem to exclude it as a possible porin protein (Nurminen, 1978). But no other trypsin-resistant major protein was detected and heat treatment experiments also showed that protein A* (molecular weight 22000) shifted down to apparent molecular weight 19500 only after boiling at 100 °C for 5 min in SDS, indicating that this protein does not rapidly dissociate in SDS.

The finding that some of the protein bands were double or triple in the best electrophoretic separations indicates either that several individual proteins are involved, or that some protein may show several different mobilities depending on the conditions. Thus, experiments with isolated proteins are required to explain in detail what changes are taking place. Finally, these experiments also showed how important it is to boil outer membrane samples in SDS for a minimum time of 5 min before application to the gel, in order to avoid protein patterns with bands which are in transition between two positions (Frasch & Mocca, 1978).

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