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COMPARATIVE STUDIES OF Yersinia pestis OUTER MEMBRANE ISOLATION TECHNIQUES AND THEIR POTENTIAL USE IN PLAGUE EPIDEMIOLOGY

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SUMMARY

In the present study three techniques for obtaining outer membrane enriched fractions from **Yersinia pestis** were evaluated. The techniques analysed were: differential solubilization of the cytoplasmic membrane with Sarkosyl or Triton X-100, and centrifugation in sucrose density gradients. The sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane isolated by the different methods resulted in similar protein patterns. The measurement of NADH-dehydrogenase and succinate dehydrogenase (inner membrane enzymes) indicated that the outer membrane preparations obtained by the three methods were pure enough for analytical studies. In addition, preliminary evidences on the potential use of outer membrane proteins for the identification of geographic variants of **Y. pestis** wild isolates are presented.

KEY WORDS: Yersinia pestis, outer membrane proteins, membrane isolation techniques.

INTRODUCTION

Most of the outer membrane proteins of **Escherichia coli** and **Salmonella typhimurium** have been extensively studied[6, 8, 15]. In contrast, the knowledge of outer membrane proteins of other Gram-negative bacteria, as **Yersinia pestis**, the causative agent of bubonic plague, is still deficient.

In addition to the important role of outer membrane proteins in host-parasite relationship, including virulence and eliciting of host immune response[6, 20], the outer membrane has been useful in the characterization of pathogenic variants of **Enterobacteriacea** by means of electrophoretic analysis[1, 8, 12, 15]. In the **Yersinia genus**, some outer membrane proteins are induced by growth in Ca^{++} deficient medium at 37°C and partially secreted in the growth medium[3, 21]. These yersinia outer membrane proteins (YOPs) are directly involved in the pathogenesis of these organisms[2, 3, 4, 19, 20, 21].

In the present study some techniques for obtaining outer membrane enriched fractions were evaluated in **Y. pestis.** Additionaly, we showed preliminary evidence that electrophoretic profiles of outer membrane proteins might be useful for the identification of geographic variants of **Y. pestis.**

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MATERIAL AND METHODS

Strains and growth conditions

The wild **Y. pestis** strains isolated from Northeast Brazil are listed on Table I. The **Y. pestis** reference laboratory strains A1122 and KIM were supplied by Dr. R. R. Brubaker (Michigan State University, USA) and were previously described[5, 11]. The cells were grown to stationary phase at 28°C in YT medium (1% tryptone-Difco, 0.5% yeast extract-Difco and 0.5% sodium chloride-Merck) for 48 hours with agitation.

TABLE 1
Yersinia pestis strains isolated from Northeast of Brazil.

Strain	Origin	Region	Year
P.BA 2	Flea	Eastern Plateau	1984
P.BA 3	Human	Eastern Plateau	1984
P.EXU 800	Rodent	Triunfo Mountain Range	1979
P.EXU 841	Human	Triunfo Mountain Range	1980
P.EXU 310	Flea	Araripe Plateau	1969
P.EXU 424	Rodent	Araripe Plateau	1974
P.EXU 801	Human	Ibiapaba Mountain Range	1978
P.EXU 834	Rodent	Ibiapaba Mountain Range	1979
P.EXU 805	Human	Baturité Mountain Range	1978
P.EXU 811	Rodent	Borborema Plateau	1979
P.EXU 843	Rodent	Borborema Plateau	1980
P.PB 873	Rodent	Borborema Plateau	1986
P.PB 874	Rodent	Borborema Plateau	1986

Isolation of outer membranes by differential solubilization with Sarkosyl

Fractionation of **Y. pestis** cells was carried out as described by BOLIN, NORLANDER & WOLF-WATZ[2]. Briefly, cells were harvested in phosphate buffered saline (PBS, pH 7.0) and centrifuged at 10.000 x g for 10 min. The bacterial cell pellet was suspended in 10 mM tris-hidrochloride (pH 7.8), 5 mM EDTA and 1 mM 2-mercaptoethanol and disrupted in a Kubota refrigerated ultrasonic disintegrator (model 200 M) at full power for 10 min. Cell debris were removed by low speed centrifugation and the envelope was pelleted from the supernatant fraction by centrifugation at 100.000 x g for 1 h. The membrane pellet was then, suspended in a solution containing 0.5% Sarkosyl and 1 mM 2-mercaptoethanol, incubated overnight at 4°C and centrifuged at 100.000 x g for 1 h to obtain the outer membrane containing pellet.

Isolation of outer membrane by differential solubilization with Triton X-100

The technique was performed according to FERREIRA[7]. Cells grown in 50 ml of YT medium washed in 30 mM tris-HCl (pH 8.1), suspended in 0.5 ml 20% sucrose in 30 mM tris-HCl (pH 8.1). Then, 50 μl lysozyme (10 mg/ml) was added. The mixture was kept on ice for 30 min. After adjusting the volume to 5 ml with 3 mM EDTA (pH 7.3) the cells were disrupted by sonication (200 watts, 10 min, Kubota model 200 M), under refrigeration. Cell debris were removed by slow speed centrifugation and the supernatant centrifuged at 20.000 x g for 1 h at 4°C. The pellet was dissolved in 3 ml 30 mM tris-HCl (pH 8.1) containing 0.2% Triton X-100 and 2 mM $MgCl_2$ and kept on ice for at least 2 hours. Finally, the preparation was centrifuged at 25.000 x g for 1 h at 4°C to obtain the outer membrane enriched fraction.

Isolation of the outer membrane by centrifugation in sucrose gradient

The method was described by ITO and colleagues was followed[10]. Briefly, **Y. pestis** cells were washed with 0.03 M tris-HCl (pH 8.1), suspended in 0.2 ml of 20% sucrose containing 0.03 M tris-HCl (pH 8.1) and converted to spheroplasts by incubating on ice bath for 30 min with 50μl of lysozyme freshly dissolved in 0.1 M EDTA (pH 7.3). All subsequents steps were carried out at 4°C. The preparation was diluted to 5 ml with 3 mM EDTA (pH 7.3) and the cells sonically disrupted (200 watts, 10 min, Kubota model 200 M) under refrigeration. After removing of cell debris, the supernatant was centrifuged at 35.000 xg for 1 h at 4°C. The pellet was suspended in 1 ml 3 mM EDTA (pH 7.3) and layered onto 9 ml of 53% (w/v) sucrose-3 mM EDTA with 70% sucrose-3 mM EDTA (1 ml) at the bottom and centrifuged at 152.000 xg for 18 h at 4°C. Outer membrane was recovered as a band above the 70% layer and the inner membrane as a second band in the middle of the tube.

Isolation of cell envelopes

Cell envelopes of **Y. pestis** strains were obtained essentially by the procedure cited above for outer membrane described by BOLIN et al.[2], except by the Sarkosil treatment which was ommited.

SDS - polyacrylamide gel electrophoresis

The electrophoresis was performed according to LAEMMLI[13]. Improved protein separation was achieved in gradient gels (15 to 10% acrylamide, 0.4 to 0.27% bis-acrylamide). Samples solubilized in LAEMMLI[13] sample buffer were heated at 95°C for 10 min and subjected to electrophoresis at a constant current of 20 mA. The gels were silver stained as described by MORRISSEY[17]. Protein contents were determined according to LOWRY et al.[14].

Enzyme assay

Succinate-dehydrogenase (EC 1.3.99.1) and NADH-dehydrogenase (EC 1.6.99.3) activities were determined according to OSBORN et al.[18] and MACKLER[16], respectively.

RESULTS

Electrophoretic analysis of outer membrane fractions prepared by different methods

Figure 1 shows the electrophoretic protein profile of outer membranes of the **Y. pestis** A1122 strain isolated by centrifugation in sucrose density gradient, differential solubilization with Sarkosyl or Triton X-100. Some differences were noted, mainly a 70 kDa peptide absent in outer membrane isolated by sucrose density gradient method. Other minor differences among the outer membrane protein profiles could be seen, as the increased amount of a 45 kDa protein and the reduction of a 22 kDa protein in samples subjected to the sarkosyl extraction method.

Enzymatic analysis

In order to evaluate a possible contamination with inner membrane proteins, the outer membrane fractions were subjected to enzymatic analysis to demonstrate the specific activities of NADH-dehydrogenase and succinate-dehydrogenase, two cytoplasmic membrane enzymes. The results indicated that the preparations were rather pure since the amount of enzymatic activity detected in the outer membrane samples were only a minor fraction of activity present in inner membrane samples (Table 2). The sarkosyl insoluble fraction showed less than 10% of the enzymatic activity detected in the cell envelope sample, whereas the Triton X-100 solubilization and the sucrose density gradient methods gave even lower values for both enzymes, indicating a good membrane separation efficiency.

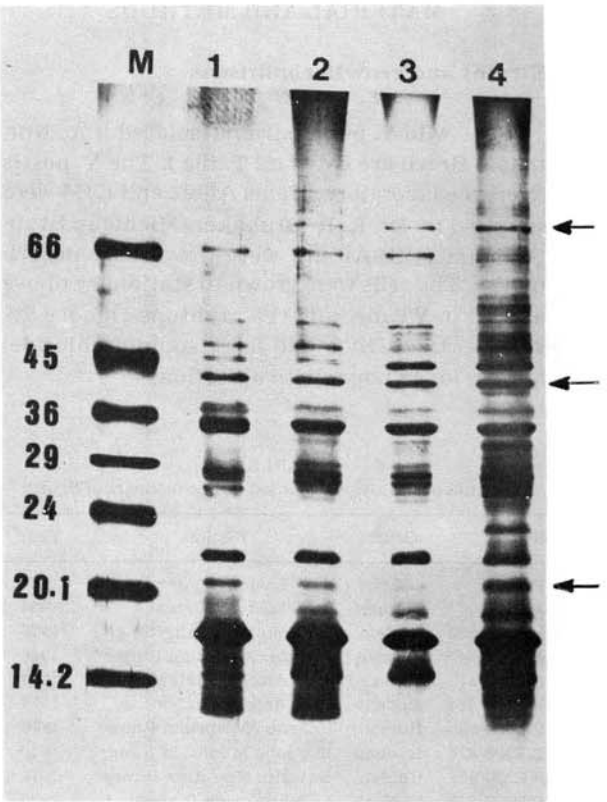

Fig. 1 — Comparison of silver stained SDS-PAGE of **Y. pestis** A1122 strain outer membrane fractions obtained by different methods. 1. Sucrose gradient, 2. differential solubilization with Triton X-100, 3. differential solubilization with Sarkosyl. Sample 4 represents the cell envelope fraction. MW, molecular weight markers. The arrowheads indicate position of proteins with Mr of 70, 45 and 22 kDa. Each well was loaded with 10 μg of protein.

TABLE 2
Specific acitivity of inner membrane enzymes in different outer membrane preparations (μmol.min^{-1}.mg^{-1})

Method	NADH deshidrogenase	Succinato deshidrogenase
Sucrose gradient	0.010	< 0.005
Solubilization with Sarkosyl	0.017	0.009
Solubilization with Triton X	0.005	0.000
Cell envelope	0.189	0.175
Inner membrane[a]	0.580	0.520

(a) obtained by separation in sucrose gradient.

Electrophoretic analysis of outer membrane proteins from different *Y. pestis* strains

Based on the previous use of the sarkosyl solubilization method for the isolation of **Y. pestis** outer membranes and the similar performance of the three outer membrane extraction methods used, we decided to apply this procedure to 13 wild **Y. pestis** isolates.

In general, the outer membrane protein profiles were similar with approximately 20 distinct protein bands revealed by silver staining. Nevertheless, some differences can be noted among the outer membrane protein profile of some strains as: a 70 kDa protein found in higher amounts in the A1122, P.BA-2, and P.EXU 424 strains; a 60 kDa protein present in strains P. EXU-805, P.BA-3 and KIM; and a 38 kDa protein in the P.BA-2, A1122, P.EXU-800 and P.EXU-424 strains. Some additional differences related to proteins with Mr lower than 25 kDa could also be seen in the outer membrane profiles studied However, these proteins proved to be heat-modifiable proteins subjected to great variations on their electrophoretic mobilities depending on the heat treatment of the samples prior the running and were not taken into consideration for comparative purposes (data not shown).

DISCUSSION

Three methods for obtaining outer membrane enriched fractions of **Y. pestis** were evaluated and proved to be suitable. However, two proeminent cell envelope proteins with Mr of 70 kDa and 42 kDa were preferentially extracted by the sarkosyl solubilization method. It is conceivable that the two proteins represent minor contaminations with cytoplasmic membrane since their relative concentrations in outer membrane and envelope fraction were similar. The analysis of cytoplasmic membrane enzymes further demonstrated that the sarkosyl solubilization method was significantly more contaminated with inner membrane enzymes than the other two methods.

In spite of the apparent lower efficiency of the sarkozyl solubilization method we chose this procedure for further analyses of **Y. pestis** strains based on its general use for the isolation of outer membrane in this organism[2, 3, 4, 19, 21].

An electrophoretic approach, using whole cell extracts, has been used by HUDSON et al., 1973[9] in the identification of **Y. pestis** geographic variants in a worldwide basis. The use of outer membrane proteins in the epidemiological characterization of **Y. pestis** seems to be more advantageous than whole cell extracts since the number of bands is reduced and comparisons are easier. Moreover, outer membrane proteins are essential elements in any exchange between the bacterial cell and the surrounding environment, including host-parasite relationships. It is conceivable that different environments could reflect in different outer membrane protein composition.

The initial attempt to identify geographic variants among 13 **Y. pestis** samples isolated at the six known plague foci localized at the Northeast region of Brazil, although not conclusive, was promising. In some cases, as in the strains P.PB-873 and P.PB-874 similarities between their outer membrane protein profiles are evident. On the other hand, strains from the same area as the P.BA-2 and P.BA-3 showed marked differences as the expression of the 38 kDa protein (Figure 2).

Recent publication demonstrated that outer membrane proteins of **Y. pestis** are essential

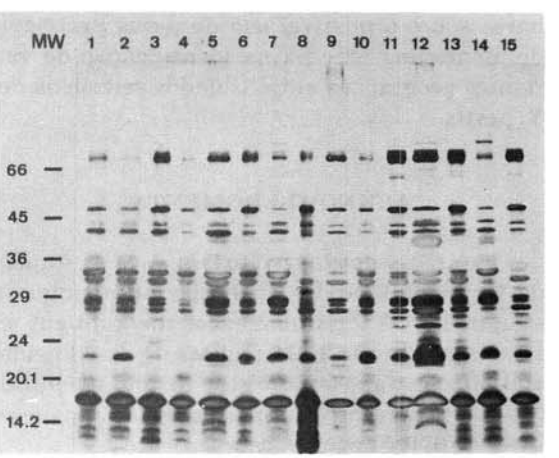

Fig. 2 — Silver stained SDS-PAGE of outer membrane fraction (differential solubilization with Sarkosyl) obtained from **Y. pestis** strains isolated from different plague foci. 1. P.EXU 811, 2. P.EXU 843, 3. P.PB 873, 4. P.PB 874, 5. P.EXU 800, 6. P.EXU 841, 7. P.EXU 424, 8. P.EXU 310, 9. P.EXU 801, 10. P.EXU 834, 11. P.EXU 805, 12. P.BA 2, 13. P.BA 3, 14. A1122, 15. KIM. Each well was loaded with approximately 10μg of protein. MW, molecular weight markers.

elements to the comprehension of the pathogenic properties of this organism[2, 3, 4, 19, 21]. It is possible that the analysis of a greater number of **Y. pestis** isolates can give important contributions to epidemiological studies of plague. Experiments are in progress in our laboratory to further evaluate the potential use of outer membrane protein pattern as a epidemiological markers for **Y. pestis** studies.

RESUMO

Estudo comparativo de técnicas de isolamento de membrana externa de Yersinia pestis **e seu uso na epidemiologia da peste.**

No presente estudo três técnicas para isolamento de frações enriquecidas em membrana externa de **Y. pestis** foram avaliadas. As técnicas utilizadas foram: centrifugação em gradiente de densidade em sacarose e solubilização diferencial com Sarkosyl ou Triton X-100. A análise por eletroforese em gel de poliacrilamida na presença de dodecil sulfato de sódio (SDS-PAGE) das membranas externas extraídas pelos diferentes métodos evidenciou perfis protéicos semelhantes. A determinação das atividades de NADH-desidrogenase e succinato-desidrogenase (enzimas de membrana interna) indicou que todas as preparações estudadas eram adequadas a estudos analíticos. Obteve-se evidências preliminares sobre o possível uso de perfis protéicos de membrana externa na identificação de variantes geográficos entre isolados selvagens de **Y. pestis.**

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