Epidem. Inf. (1989), **103**, 595–602
Printed in Great Britain

Electrophoretic characterisation of the outer membrane proteins of *Yersinia pestis* isolated in north-east Brazil

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(*Accepted 9 June 1989*)

SUMMARY

The outer membrane proteins of 38 *Yersinia pestis* isolates from all known plague foci of north-east Brazil were analysed by SDS-PAGE. Approximately 20 bands were consistently found in all strains analysed and 11 were selected for comparative studies. Although qualitative differences among the electrophoretic profiles of outer membrane proteins of wild *Y. pestis* isolates were not observed, quantitative alterations were clearly noted for most of these proteins. No particular quantitative alteration of the electrophoretic profile of outer membrane proteins could be associated with the period of isolation and geographic origin of the isolates. The 64 kDa outer membrane protein was significantly expressed in higher amounts among *Y. pestis* strains isolated from a recent plague outbreak. The possible use of electrophoretic profiles of outer membrane proteins of wild *Y. pestis* isolates as a tool for epidemiological studies and for the analysis of virulence determinants is discussed.

INTRODUCTION

The genus *Yersinia* is composed of three pathogenic species: *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, which are easily distinguished by biochemical and physiological characteristics (1). Whereas *Y. enterocolitica* shows considerable variation in serogroups and biochemical reactions, *Y. pestis* and *Y. pseudotuberculosis* have a limited number of variants. In the case of *Y. pestis*, the causative agent of plague, three major distinct biotypes were established based on the fermentation of glycerol (G) and reduction of nitrate (N): Orientalis (G−, N+), Antiqua (G+, N−) and Mediaevalis (G+, N+) (2). However, the isolation of strains which do not fit in such a classification demonstrated that biotyping can not be applied alone in epidemiology and systematic studies of *Y. pestis* (3–5).

Hudson and collaborators (3, 6, 7) introduced electrophoretic techniques of whole cell extracts in the study of geographic variants and epidemiology of *Y. pestis* strains isolated from all over the world. In these studies, the authors

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analysed 17 *Y. pestis* strains from Brazil (Pernambuco) and six electropherotypes could be established. However, the low number of isolates examined precluded any final conclusion with regard to the validity of the electrophoretic analysis of geographic distribution of various electropherotypes within specific localities. Moreover, the electrophoresis of whole cell extracts might lead to ineffective visualisation of important protein bands due to the complex band profiles of the gels (6, 7). This problem could be overcome with the isolation and investigation of a cellular fraction such as the outer membrane which regulates most of the interactions with the environment. Several virulence-associated factors of *Y. pestis* are localized on the outer membrane (8, 9).

In this study we have analysed outer membranes of *Y. pestis* samples isolated from all known plague foci in north-east Brazil obtained in different periods, hosts, and biological conditions. The usefulness of electrophoretic profiles of outer membrane protein on the identification of geographic variants and on epidemiological studies of *Y. pestis* is discussed.

MATERIALS AND METHODS

In this study 38 *Y. pestis* wild strains derived from different plague foci in north-east Brazil were used and are listed in Table 1. *Y. pestis* EV76 and KIM-10 were obtained from Dr R. R. Brubaker, Michigan State University and the A1122 was obtained from the Center for Disease Control (Fort Collins, USA).

The bacteria were grown in liquid YT medium (1% tryptone-Difco, 0·5% yeast extract-Difco and 0·5% sodium chloride-Merck) at 28 °C for 48 h. Bacterial stocks were kept in agar stabs at 4 °C.

Fractionation of *Y. pestis* cells was carried out by the method described by Bolin and co-workers (9). Briefly, cells were harvested in phosphate buffered saline and centrifuged at 10000 g for 10 min. The bacterial cell pellet was suspended in 10 mM Tris-hydrochloride (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 100000 g for 1 h. The membrane pellet was suspended in a solution containing 0·5% Sarkosil and 1 mM 2-mercaptoethanol, incubated overnight at 4 °C and then centrifuged at 100000 g for 1 h to obtain the outer membrane containing pellet.

The proteins were analysed by SDS-PAGE essentially as described by Laemmli (10). Outer membrane samples were solubilized in the Laemmli sample buffer after 10 min incubation at 95 °C. Improved separation and detection of *Y. pestis* proteins were obtained in gradient gels (15 to 10% acrylamide, 0·4 to 0·27% bis-acrylamide), followed by Coomassie Blue or silver staining as described by Morrisey (11).

The densitometric studies of Coomassie Blue stained gels were performed with a computerized Bio-rad videodensitometer (Model 620). The quantification of the optical density of each peak was calculated automatically by the densitometer using integral values of each curve in relation to the complete electrophoretic profile. Relative integral values for each of the 11 selected protein bands were calculated in order to permit comparisons.

Protein contents were measured by the method of Lowry and co-workers (12).

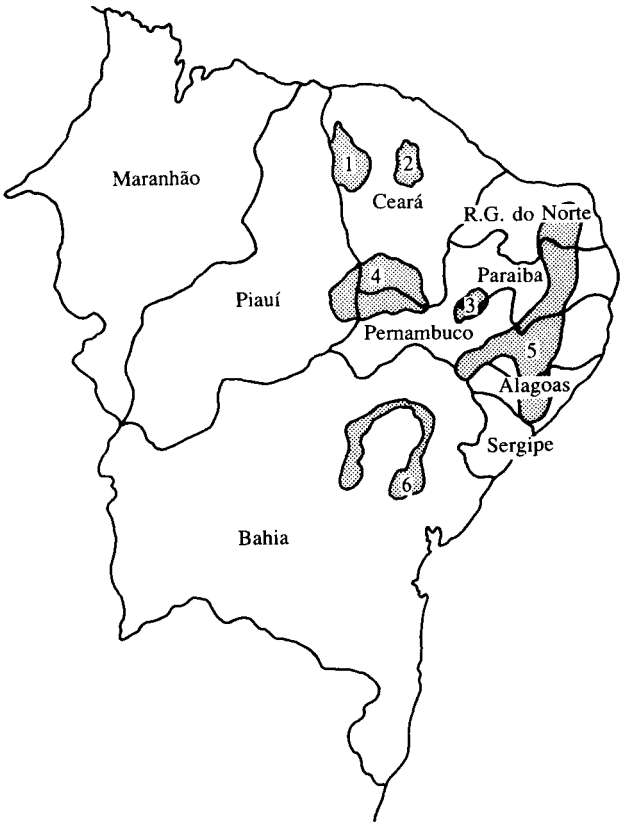

Fig. 1. Geographic distribution of the different plague foci localized in north-eastern Brazil. The shaded areas represent the regions where *Y. pestis* was isolated under endemic or epidemic conditions. 1. Ibiapaba Mountains, 2. Baturité Mountains, 3. Triunfo Mountains, 4. Araripe Plateau, 5. Borborema Plateau, 6. Eastern Plateau.

RESULTS

Six well established *Y. pestis* foci are localized in the Brazilian north-east region (Fig. 1). All these sites share similar geographic and climatic conditions with elevated altitudes, mild temperatures and rather regular and high rain falls when compared to the surrounding dry and hot plains (13). *Y. pestis* samples were collected in such places from different host and biological conditions during the last 23 years and kept at the Aggeu Magalhães Research Institute, Recife (14). Thirty-eight representative isolates of the six plague foci in this region were chosen arbitrarily (Table 1) including 20 samples recovered from a recent plague outbreak at the Borborema Plateau region which resulted in four deaths (15).

The outer membrane protein of the *Y. pestis* isolates were analysed by the SDS-PAGE technique. The electrophoretic profile of the outer membrane proteins of all strains investigated comprized from 20–25 visible bands. Silver staining resulted in deficient staining of some protein bands. Based on this observation all densitometric analyses had to be carried out with Coomassie Blue stained gels. Among the outer membrane protein bands, 11 were chosen for further comparison

https://doi.org/10.1017/S0950268800030995 Published online by Cambridge University Press

Table 1. Yersinia pestis *strains isolated from north-east Brazil*

Strain	Origin	Region	Year
P.BA 2	Flea	Eastern Plateau	1984
P.BA 3	Human	Eastern Plateau	1984
P.EXU 196	Human	Triunfo Mountain Range	1967
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 559	Flea	Ibiapaba Mountain Range	1972
P.EXU 801	Human	Ibiapaba Mountain Range	1978
P.EXU 834	Rodent	Ibiapaba Mountain Range	1979
P.EXU 795	Human	Baturité Mountain Range	1978
P.EXU 796	Human	Baturité Mountain Range	1978
P.EXU 805	Human	Baturité Mountain Range	1978
P.EXU 810–811/ 817/819–820	Rodent	Borborema Plateau	1979
P.EXU 843	Rodent	Borborema Plateau	1980
P.EXU 851	Rodent	Borborema Plateau	1980
P.PB 862–863	Human	Borborema Plateau	1986
P.PB 864–877/ 879	Rodent	Borborema Plateau	1986
P.PB 881	Human	Borborema Plateau	1986

due to their clear densitometric recording and reduced migration artifacts associated with the heat treatment of the membrane samples before loading into the gels. These protein bands were indicated by letters as follows: A (71 kDa), B (67 kDa), C (64 kDa), D (49 kDa), E (44·5 kDa), F (42 kDa), G (38 kDa), H (34 kDa), I (33·5 kDa), J (32·5 kDa), and K (16 kDa). Fig. 2 shows the densitometric recording of the outer membrane protein profile of some selected strains. The general electrophoretic pattern is represented by the profile of the strain P.PB 874 (Fig. 2, chart 1). This set of peptides was observed in most strains, including the reference laboratory strains A1122, KIM-10 and EV76 (data not shown). Even though qualitative differences in the expression of outer membrane proteins of *Y. pestis* wild isolates could not be seen, quantitative alterations were clearly noted specially for those proteins corresponding to bands B, C, D, E, and F. Protein G was usually barely detectable, with a remarkable exception of the strain P.BA 2. where protein G is one of the most prominent protein bands of the outer membrane profile (Fig. 2, chart 3). Considerable variation could also be observed in the relative amounts of protein bands H, I, and J, but the similar electrophoretic mobilities made them difficult to resolve as individual peaks by densitometer. Some outer membrane proteins with molecular weight ranging form 30 kDa to 16·5 kDa showed a marked heat-modifiable behaviour in all samples analysed. Based on the lack of reproducibility of these protein bands in our electropherograms they were not considered for further comparisons. The protein corresponding to peak K showed also a heat-modifiable behaviour in some samples and was carefully considered when comparing the electrophoretic profiles.

The gel densitometric profiles of outer membrane proteins of 38 representative *Y. pestis* isolates of all six plague foci in north-eastern Brazil are presented in Table 2. The quantification of the different bands was possible by the direct measurement

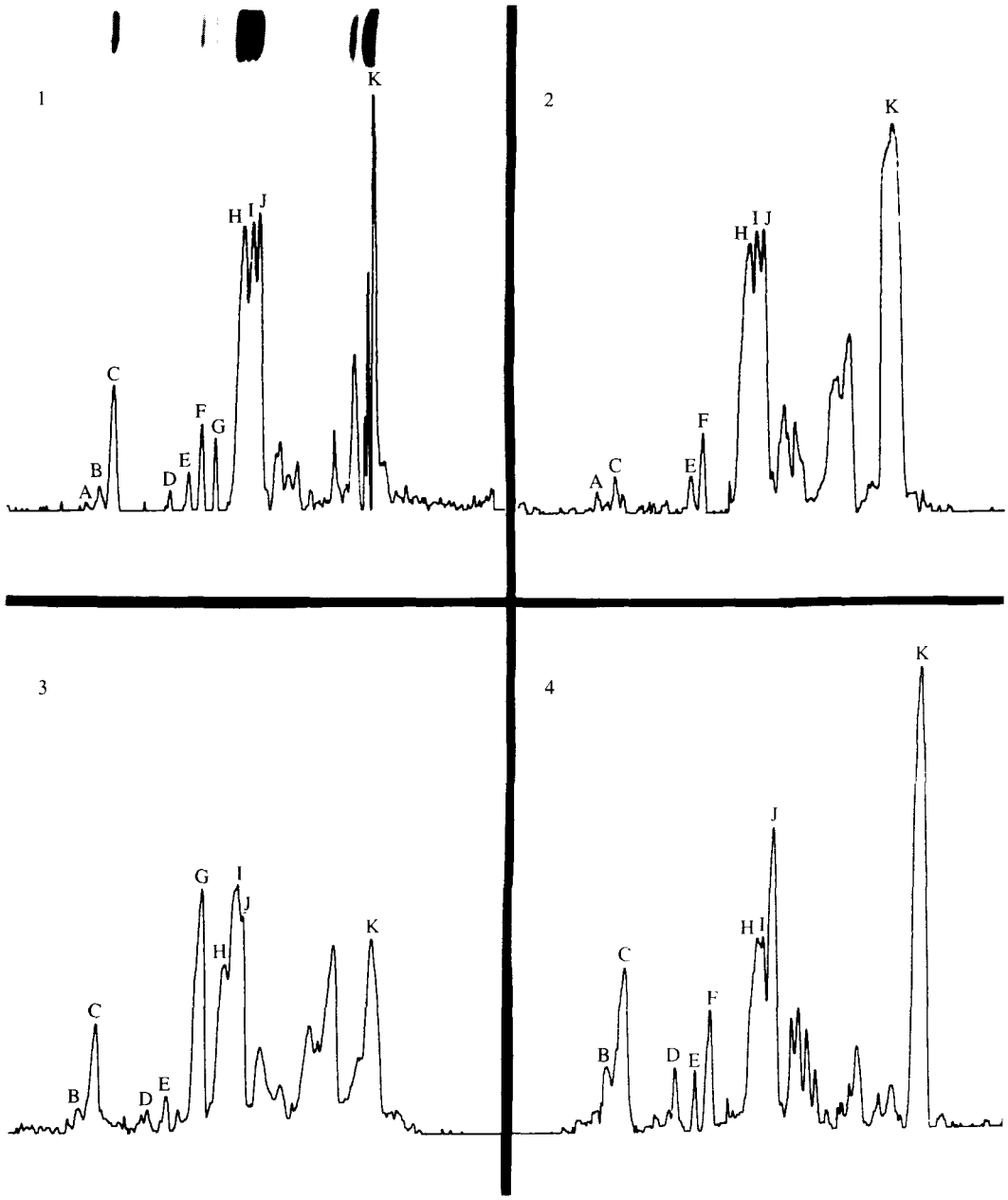

Fig. 2. Densitometric tracings of some representative SDS-PAGE of *Y. pestis* outer membrane proteins. 1. P.PB 874, 2. P.PB 817, 3. P.BA 2 and 4. P.BA 3. The letters refer to the protein bands described in the text. The most frequently found outer membrane protein profile is illustrated by the Coomassie Blue stained gel (top left).

https://doi.org/10.1017/S0950268800030995 Published online by Cambridge University Press

Table 2. Yersina pestis *strains isolated from north-east Brazil: densitometric analysis*

Strain		Integral values (%) A	B	C	D	E	F	G	H	I	J	K
P.BA	2	0·8	0·8	8·9	0·8	0·8	0·8	18·6	16·5	21·6	6·3	24·0
P.BA	3	0·7	6·3	10·4	2·1	3·1	6·3	0	13·6	4·1	17·8	35·6
P.EXU	196	0·9	0·9	7·0	0·9	0·9	4·3	0	12·7	9·7	16·8	46·0
P.EXU	800	0·7	0·7	7·1	0·7	2·0	3·2	0·7	20·5	16·4	8·2	40·0
P.EXU	841	0·9	6·8	13·5	0·9	0·9	4·0	0·9	17·5	8·2	17·8	28·6
P.EXU	310	0·9	7·0	15·5	4·3	2·7	4·3	0·9	15·3	2·9	6·5	39·5
P.EXU	424	0·8	0·8	3·9	0·8	1·8	2·5	0·8	17·9	15·4	26·9	28·2
P.EXU	559	0·6	3·9	11·0	4·0	0·6	5·0	0	11·0	7·0	14·9	41·9
P.EXU	801	0·7	0·7	4·4	0·7	0·7	0·7	0	37·7	21·4	16·3	16·7
P.EXU	834	0·7	0·7	0·7	0	3·3	0·7	0·7	38·8	9·9	12·2	32·2
P.EXU	795	1·0	4·5	10·9	6·1	6·3	6·1	0		(24·8)		40·2
P.EXU	796	0·8	7·7	18·1	2·6	2·5	5·3	0		(28·3)	12·8	21·9
P.EXU	805	0·8	5·9	17·8	2·3	0·8	5·8	0		(23·8)	15·4	27·4
P.EXU	810	1·4	1·4	1·4	0	4·5	1·4	0	21·9	9·0	8·7	50·3
P.EXU	811	0·9	2·9	4·1	0	0·9	4·3	0	15·4	16·7	9·9	44·9
P.EXU	817	0·9	0·9	2·8	0	0·9	4·5	0	16·0	17·3	10·2	46·5
P.EXU	819	0·6	0·6	5·4	4·0	2·1	2·1	6·0	20·4	26·3	6·1	26·3
P.EXU	820	1·0	1·0	1·0	0	1·0	8·2	0	9·8	17·9	9·6	50·4
P.EXU	843	0·6	0·6	2·0	0·6	0·6	2·8	0	18·3	14·5	5·8	54·1
P.EXU	851	0·9	3·0	4·4	4·4	5·9	8·9	0	7·4	13·3	13·4	38·4
P.PB	862	0·7	0·7	4·4	0·7	0·7	8·8	0·7		(26·2)	8·8	48·2
P.PB	863	0·9	0·9	10·2	0·9	3·0	7·2	0		(18·9)	14·5	43·4
P.PB	864	0·8	3·7	6·3	2·4	3·9	3·7	0·8	8·9	11·3	10·0	48·1
P.PB	865	0·8	2·3	11·1	2·3	0·8	0·8	0	14·7	(15·9)		51·3
P.PB	866	0·8	0·8	7·0	0·8	0·8	2·3	0	16·6	11·8	13·0	46·1
P.PB	867	0·9	0·9	11·0	0·9	3·9	0·9	0·9	26·5	30·6	2·7	19·9
P.PB	868	0·8	0·8	11·8	4·0	5·2	6·5	0·8	9·2	10·3	14·3	36·3
P.PB	869	0·9	0·9	11·0	0·9	0·9	4·0	0	13·8	12·5	13·8	41·3
P.PB	870	0	1·0	8·1	1·0	1·0	6·4	1·0		(40·8)		40·6
P.PB	871	2·6	2·6	13·8	5·0	2·4	8·8	0·8		(27·7)	11·2	25·1
P.PB	872	0·8	2·6	11·4	3·7	2·6	6·3	0		(28·0)	15·3	29·3
P.PB	873	0·8	2·3	15·5	2·3	2·4	5·9	0·8	12·9	13·0	14·3	29·8
P.PB	874	0·8	0·8	7·7	0·8	2·6	3·8	2·6	20·5	13·0	15·3	32·0
P.PB	875	0·8	0·8	2·5	3·7	6·2	4·9	2·5	15·0	8·6	15·0	40·0
P.PB	876	0·8	0·8	4·8	0·8	0·8	3·5	0·8	20·2	11·8	9·5	46·2
P.PB	877	0·8	2·5	15·0	2·5	2·5	6·2	0·8	8·8	15·0	16·1	29·8
P.PB	879	0·8	0·8	8·0	0·8	5·3	10·5	0·8	18·5	11·9	13·2	29·3
P.PB	881	0·8	0·8	9·0	0·8	2·6	3·8	0		(28·3)	17·9	35·9

of the integral value of the total area subtended by each curve. All outer membrane protein bands showed a considerable variation in their expression but no clear geographic correlation could be established. Indeed, some samples isolated at the same plague focus showed striking quantitative differences in electrophoretic profile, as exemplified by the P.BA 2 and P.BA 3 isolates (Table 2). No significant quantitative difference in the expression of outer membrane proteins was noted among *Y. pestis* strains isolated from different hosts.

Several strains of *Y. pestis* analysed in this study were isolated during a plague outbreak at the Borborema Plateau in 1986 (P.PB 862–P.PB 881) and were compared to eight independent isolates obtained previously in the same area but

https://doi.org/10.1017/S0950268800030995 Published online by Cambridge University Press

under endemic condition from wild rodents (Table 1). In spite of the low number of isolates, protein band C was significantly increased ($P < 0{\cdot}001$, Student t test) in samples derived from the epidemic outbreak (Table 2). Based on the available data it is not possible to decide if this observation could indicate the selection of a mutant variant raised in the local *Y. pestis* population.

DISCUSSION

Y. pestis isolates are characterized by a remarkable molecular and biochemical homogeneity. The lack of markers has considerably inhibited the development of epidemiological studies regarding this organism. Preliminary studies of Brazilian *Y. pestis* isolates (4, 5, 16), have demonstrated that most of the strains pertain to the Orientalis variety (G−, N+) with some variants which were G−, N−, indicating the *Y. pestis* population of Brazil was not completely homogeneous.

The studies of Hudson and co-workers (6, 7) demonstrated that electrophoretic techniques can be used to differentiate geographic variants of *Y. pestis*. The 17 Brazilian isolates analysed formed 6 distinct groups, based on the variability of 9 selected proteins in addition to biochemical and physiological characteristics (3). It is noteworthy that the samples analysed by Hudson, Quan & Bailey (3) were representative of only 2 of the 6 established plague foci in north-east Brazil (Triunfo Mountain Range and Araripe Plateau), and were collected during a short period between 1966 and 1967. Even with a limited representation of the Brazilian plague foci, those authors were able to demonstrate a clear heterogeneity in the *Y. pestis* population from Brazil based on the electrophoretic profile of whole cell extracts.

The results presented here extend the initial observations made by Hudson and colleagues (3, 6, 7) by including a representative set of isolates from all plague foci in north-east Brazil, as well as samples collected during more than 20 years and in different host and biological conditions, i.e., epidemic or endemic. These results suggest that *Y. pestis* isolates in north-east Brazil display an overall similarity regarding the outer membrane electrophoretic profile and no clear geographic variants can be established based on this parameter. It should be noted that the classification of the Brazilian *Y. pestis* geographic variants carried out by Hudson and his group took into consideration the expression of the murine toxin, a cytoplasmic protein (3, 6, 17).

The finding that one outer membrane protein was expressed in significantly higher levels in strains isolated in an epidemic condition suggests a possible involvement of this peptide on the virulence of *Y. pestis*. However, on the basis of difference in virulence this hypothesis is not supported as all strains studied were highly virulent for animals (unpublished observations). It should be noted that protein C was detected in outer membranes of some other strains isolated under endemic conditions. The identification of any biological role of protein C (64 kDa) on the enhanced virulence of Brazilian *Y. pestis* isolates should await further physiological and gene cloning experiments.

The overall similarity of the outer membrane electrophoretic profiles of the 38 representative *Y. pestis* isolates indicated that these organisms are still quite conserved and uniform, despite the geographic isolation for at least 80 years after

its introduction into the continent. In contrast, the presence of clear individual clones with altered expression of some outer membrane proteins indicate that a possible evolutionary process is taking place. It is possible that in the future one of these variants will be established as a clear geographic variant. Well maintained bacterial collections are essential.

ACKNOWLEDGMENTS

We thank Dr R. R. Brubaker for the kind gift of *Y. pestis* strains EV76 and Kim-10, as well as Mr S. H. Almeida and Mrs Y. Nakasawa for their excellent technical assistance.

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