Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *85* (1): 107-111, jan./mar. 1990

STRAIN SPECIFIC VARIATION OF OUTER MEMBRANE PROTEINS OF WILD *YERSINIA PESTIS* STRAINS SUBJECTED TO DIFFERENT GROWTH TEMPERATURES

FREDERICO GUILHERME COUTINHO ABATH; ALZIRA MARIA PAIVA DE ALMEIDA & LUIS CARLOS DE SOUZA FERREIRA*/+

Centro de Pesquisas Aggeu Magalhães – FIOCRUZ, Caixa Postal 7472, 50730 Recife, PE, Brasil
*Departamento de Biofísica e Radiobiologia e Laboratório de Imunopatologia Keizo Asami, Cidade Universitária, 50739 Recife, PE, Brasil

Three Yersinia pestis *strains isolated from humans and one laboratory strain (EV76) were grown in rich media at 28 °C and 37 °C and their outer membrane protein composition compared by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Several proteins with molecular weights ranging from 34 kDa to 71 kDa were observed to change in relative abundance in samples grown at different temperatures. At least seven* Y. pestis *outer membrane proteins showed a temperature-dependent and strain-specific behaviour. Some differences between the outer membrane proteins of full-pathogenic wild isolates and the EV76 strain could also be detected and the relevance of this finding on the use of laboratory strains as a reference to the study of* Y. pestis *biological properties is discussed.*

Key words: *Yersinia pestis* – outer membrane proteins

The cell envelope of *Yersinia pestis*, the causative agent of plague, is composed by three layers: the inner cytoplasmic membrane, an intermediary peptidoglycan layer and the outer membrane (Lugtemberg & Alphen, 1983). The outer membrane, the most outward structure of the cell, is an essential element in host-bacterium relationship including virulence mechanisms, mediated by some *Yersinia* outer membrane proteins (Yops) (Straley & Brubaker, 1982; Bolin et al., 1988). Previous reports have indicated that alterations of incubation temperature and composition of the growth medium could induce drastic changes on the protein composition of the *Y. pestis* outer membrane (Darveau et al., 1980, 1983; Staley & Brubaker, 1981, 1982). However, these observations were based on few widespread used *Y. pestis* laboratory reference strains. The present report deals with the effects of incubation temperature on the outer membrane protein composition of wild *Y. pestis* strains isolated from northeast Brazil in comparison with a reference laboratory strains, the EV76 strain.

MATERIALS AND METHODS

Three *Y. pestis* strains were isolated from humans cases at the Borborema Plateau (Paraíba State) during a plague outbreak in 1986 as previously described (Almeida et al., 1989) (Table I). The *Y. pestis* EV76 was kindly donated by Dr R. R. Brubaker, Michigan State University. The virulence-associated factors (synthesis of fraction 1, V and W antigens, ability to adsorb exogenous pigments and production of fibrinolysin-coagulase) were carried out as previously described (Almeida et al., 1989). Quantification of distinct proteins bands were carried out in a computorized video densitometer with stained gels (Bio-Rad, Model 620, USA). Unless otherwise stated, bacteria were grown in solid YT medium (0.5% yeast extract-Difco; 1% tryptone-Difco and 0.5% sodium chloride) at 28 °C or at 37 °C for 48 h. After growth, cells were harvested with TE buffer (10 mM Tris-HCl; 5 mM EDTA, pH 7.8) and a Pasteur pipette. Some experiments were carried out with 4% blood agar base plates (BAB medium) supplemented with 5% defibrinated sheep blood under 5% CO_2 atmosphere.

Research supported by the Japanese International Cooperation Agency and CNPq.
+ Corresponding author.

Received November 14, 1989.
Accepted January 11, 1990.

Cells were washed twice in TE buffer, and submitted to sonication (Kubota, Model 200 M, Japan) in TE buffer containing 1 mM 2-mercaptoethanol. Outer membranes were obtained after solubilization of the inner membrane with Sarkosil and differential centrifugation (Bolin et al., 1988). Proteins were analyzed by SDS-PAGE essentially as described by Laemmli (1970). Improved separation of outer membrane proteins were obtained in gradient gels (15 to 10% acrylamide, 0.4 to 0.27% bis-acrylamide), followed by silver staining (Morrissey, 1981). Proteins contents were measured by the method of Lowry et al. (1951).

RESULTS

The three *Y. pestis* strains collected from infected persons were full virulent in the guinea pig model and proficient for all virulence-associated factors (synthesis of fraction 1, V and W antigens, production of fibrinolysin-coagulase and ability to adsorb Congo red) analyzed (Table I and data not shown). On the other hand, the EV76 stain was non-virulent and deficient in the ability to adsorb Congo red (Table I and data not shown).

TABLE I

Yersinia pestis strains used in this work

Strain	Isolation (host)	Virulance factors[a]	Reference
P.PB 862	Borborema Plateau, Paraíba State (man)	$F1^+$, Vwa^+, Pfc^+, Pgm^+	Abath et al., 1989
P.PB 863	Borborema Plateau, Paraíba State (man)	$F1^+$, Vwa^+, Pfc^+, Pgm^+	Abath et al., 1989
P.PB 881	Borborema Plateau Paraíba State (man)	$F1^+$, Vwa^+, Pfc^+, Pgm^+	Abath et al., 1989
EV-76	–	$F1^+$, Vwa^+, Pfc^+, Pgm^-	Darveau et al., 1980

a: F1 – production of F1 antigen; Vwa – production of V and W antigens; Pfc – production of pesticin, fibrinolysin and coagulares; Pgm – ability to adsorb Congo red.

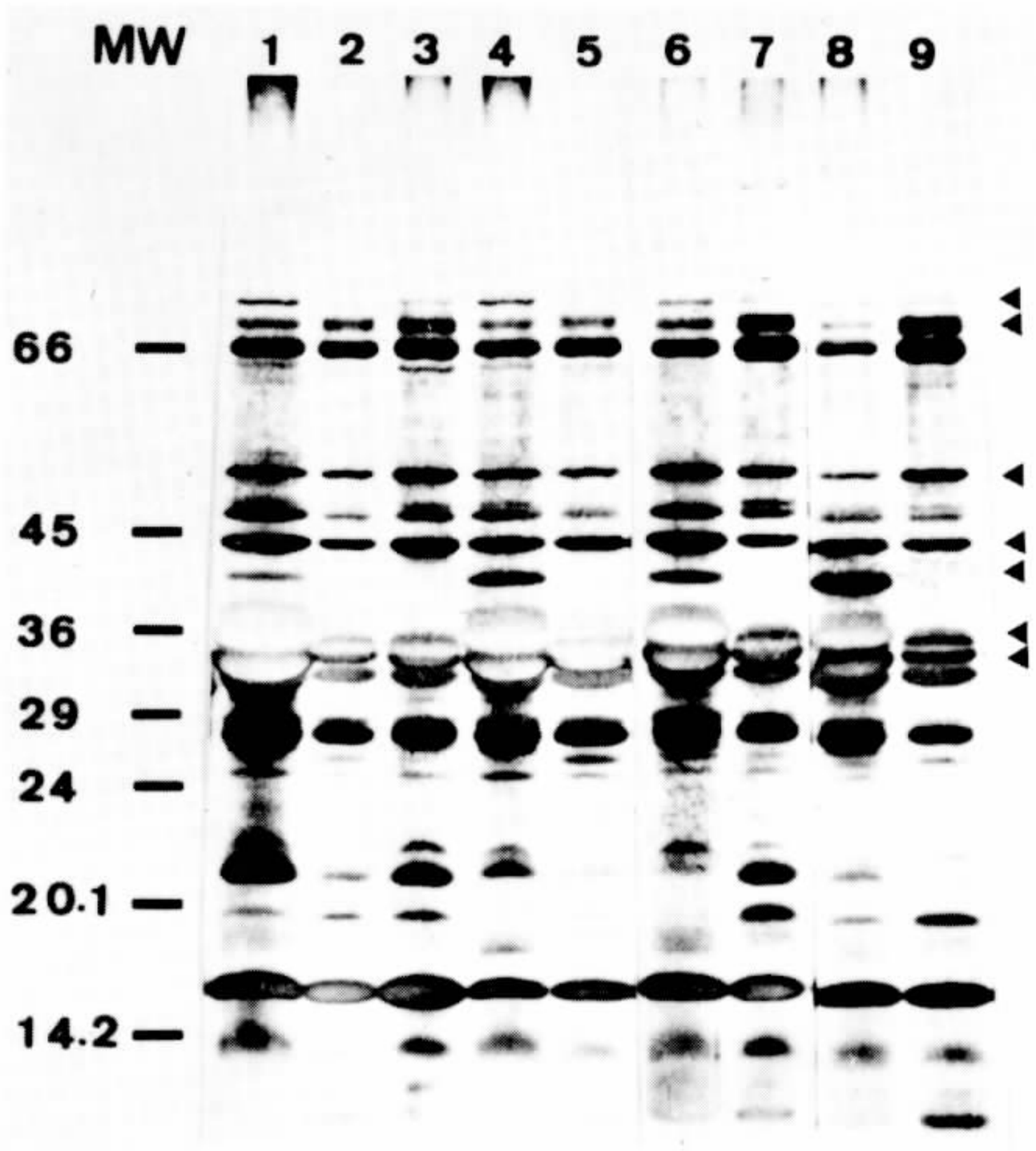
The *Y. pestis* strains were grown at 28 °C and at 37 °C and their outer membrane proteins analyzed in SDS-PAGE. Approximately 20 major protein bands could be identified in the outer membranes of the samples. Our data confirmed the marked influence of the growth temperature on the expression of several outer membrane proteins of *Y. pestis* (Fig.). For comparative purposes 7 major proteins with molecular weights ranging from 34 to 71 kDa were selected from the outer membrane protein profile based on their stable behavior on SDS-PAGE. Other proteins with lower molecular weight were heat modifiable proteins, changing their electrophoretic mobility upon heat exposure of the samples before gel loading, and were not taken into consideration. Relative amounts of some major outer membrane proteins, based on densitometric data, are presented on Table II. It can be clearly seen that some proteins are expressed in higher amounts in all strains after incubation at 28 °C, as the porin-like proteins (36 and 34 kDa). However, the relative abundance of the 71 kDa, 67 kDa, 49 kDa and 44.5 kDa proteins were not uniform among the strains analyzed (Fig., Table II). In some cases, the results observed with the EV76 strain was in clear contrast with the results found in the virulent strains, as the 49 kDa protein. This protein was present in increased amounts in the outer membrane of the EV76 strain grown at 28 °C but in reduced levels in the outer membrane of P.PB 881; when compared to cells grown at 37 °C (Table II). Even proteins with similar behavior as the 36 kDa protein, expressed in higher levels after growth at 28 °C, displayed a strain-specific induction pattern as in the EV76 strain which express the 42 kDa protein in higher amounts than the others strains (Fig.).

TABLE II

Effect of growth temperatures on the relative amounts of some outer membrane proteins of *Yersinia pestis*

Bands (kDa)	Strains			
	P.PB 862	P.PB 881	P.PB 863	EV76
(71)	+	+	+	NA
(67)	NA	–	NA	–
(49)	NA	NA	NA	–
(44.5)	+	+	+	NA
(42)	NA	+	+	+
(36)	+	+	+	+
(34)	+	+	+	+

(–): absent or diminished in 28°C grown cells.
(+): increased or only present in 28°C grown cells.
NA: not altered.



SDS-Page of outer membrane proteins of *Yersinia pestis*. The strains P.PB 863 (1, 2, 3), P.PB 862 (4, 5), P.PB 881 (6, 7) and EV76 (8, 9) were grown in YT medium at 28 °C (1, 4, 6, 8) or at 37 °C (2, 5, 7, 9) and in BAB medium at 37 °C (3). MW, molecular weight markers. Positions of proteins refered in the text are indicated on the right side of the figure. Each sample contained 15 μg of protein.

Growth of the strains in a different growth medium (BAB) under 5% CO_2 atmosphere did not altere significantly the outer membrane protein profile observed for a specific incubation temperature. The Fig. shows the outer membrane protein of strains P.PB 863 grown in the BAB medium at 37 °C. Similar results were obtained with the other strains (data not shown). Preliminary results indicated that no significant difference could be observed in outer membrane proteins of *Y. pestis* grown at the same temperature at solid or liquid medium (data not shown).

DISCUSSION

The bacterial envelope changes its composition in response to variation on enviromental conditions (Braun & Hantke, 1981). This is particularly relevant in *Y. pestis*, which alternates from a mammalian host to the flea vector in its infective cycle. The two hosts represent significantly different environments in regard to growth temperature and nutrition.

It has been previously shown that the *Y. pestis* KIM and EV76, two widespread used laboratory strains, change their outer membrane protein composition *in vitro* in response to the incubation temperature (Darveau et al., 1980, 1983; Straley & Brubaker, 1981, 1982; Bolin et al., 1985). Some of these alterations might be related to an *in vivo* adaptative process induced by the changing environment. Indeed, some outer membrane proteins induced at 37 °C, in low calcium containing medium, were also expressed *in vivo* and can be recognized by specific antibodies produced in plague recovering patients (Bolin et al., 1988).

A temperature-controlled multigenic system was previously described in *Escherichia coli* and *Salmonella typhimurium* (Craig, 1985). The usually called heat shock response involves at least seventeen distinct genes which are activated when these bacteria are incubated at temperatures above 42 °C (Neidhardt & vanBogelen, 1987). More interesting is the fact that the heat shock response is quite well conserved in several procaryotic and eucaryotic cells and is probably associated to a general stress-induced cellular response (Neidhardt & vanBogelen, 1987).

The temperature-controlled behavior of some outer membrane proteins of *Y. pestis*, *Shigella* and probably other mammalian pathogens represents another multigenic adaptative system employed by such pathogens. In these cases, induction of proteins at 37 °C are clearly related to a pathogen strategy by which the bacteria had to evolve a system to regulate the expression of virulence-associated genes only when they are in contact with the mammalian host (Maurelli, 1988).

In the present results, we showed that some *Y. pestis* outer membrane proteins are repressed by incubation at 37 °C. The possible involvement of these proteins on the adaptation of *Y. pestis* to a low temperature environment, as the flea vector, is still speculative and deserve a better understanding. Although our results corroborate previous reports regarding the behavior of EV76 strain subjected to different growth temperatures (Darveau et al., 1980, 1983), the common view that variation of the outer membrane proteins induced by the incubation temperature is a similar process for all *Y. pestis* strains does not hold true based on our findings. Indeed, some outer membrane proteins displayed a clearly distinct induction pattern in wild *Y. pestis* strains in relation to the laboratory strain EV76, as the 71 kDa and the 44.5 kDa proteins.

No difference in outer membrane protein composition could be observed among wild and laboratory *Y. pestis* strains subjected to different growth media. The inclusion of sheep red cells and a CO_2 enriched atmosphere represented a rough simulation of the extracellular condition found by the pathogen in the blood stream. Previous works showed also that *Y. pestis* cells did not change their protein composition in a minimal medium similar to mammalian intracellular environment in respect to salt composition (Straley & Brubaker, 1981). Except for some *Yersinia* outer membrane proteins (Yops) and the VW antigens, regulated by temperature and the Ca^{++} level in the growth medium, no relevant alteration of *Y. pestis* outer membrane protein composition seems to take place during growth in high content Ca^{++} containing medium as the one we had used.

Yersinia pestis populations are remarkably homogeneous in respect to several diagnostic aspects, as phagotypes, serotypes, plasmid content, metabolic reactions and outer membrane protein composition, when samples of different geographic foci, but grown under similar conditions, are compared (Abath et al., 1989; Hudson et al., 1976). The observed strain-specific response of outer membrane proteins subjected to different incubation temperatures demonstrate that the assumed homogeniety of some *Y. pestis* characteristics should be carefully considered as a general characteristic in this organism.

ACKNOWLEDGMENTS

To Dr R. R. Brubaker for the kind gift of *Y. pestis* EV76 strain and Mr S. H. Almeida and Mrs Y. Nakasawa for their technical assistance.

REFERENCES

ABATH, F. G. C.; ALMEIDA, A. M. P. & FERREIRA, L. C. S., 1989. Electrophoratic characterization of the outer membrane proteins of *Yersinia pestis* isolated in Northeast Brazil. *Epidem. Infect., 103:* 592-602.

ALMEIDA, A. M. P.; BRASIL, D. P.; MELO, M. E. B.; LEAL, N. C.; RÊGO, R. V. B. & ALMEIDA, C. R., 1989. Estudos bacteriológicos e sorológicos de um surto de peste no Estado da Paraíba, Brasil. *Mem. Inst. Oswaldo Cruz, 84:* 249-256.

BOLIN, I.; FORSBERG, A.; NORLANDER, L.; SKURNIK, M. & WOLF-WATZ, H., 1988. Identification and mapping of the temperature-inducible, plasmid-encoded proteins of *Yersinia* spp. *Infect. Immun., 56:* 343-348, 1988.

BOLIN, I.; PORTNOY, D. A. & WOLF-WATZ, H., 1985. Expression of temperature-inducible outer membrane proteins of *Yersinia. Infect. Immun., 48:* 234-240.

BRAUN, V. & HANTKE, K., 1981. Bacterial cell surface receptors. p. 1-73. In B. K. Ghosh, *Organization of prokaryotic cell membranes.* Florida. CRC Press, Inc., vol. 2.

CRAIG, E. A., 1985. The heat shock response. *Crit. Rev. Biochem., 18:* 239-280.

DARVEAU, R. P.; CHARNETZKY, W. T. & HURLBERT, R. E., 1980. Outer membrane protein composition of *Yersinia pestis* at different growth stages and incubation temperatures. *J. Bacteriol., 143:* 942-959.

DARVEAU, R. P.; CHARNETZKY, W. T.; HURLBERT, R. E. & HANCOCK R. E. W., 1983. Effects of growth temperature, 47-megadalton plasmid, and calcium deficiency on the outer membrane protein porin and lipopolysaccharide composition of *Yersinia pestis* EV 76. *Infect. Immun., 42:* 1092-1101.

HUDSON, B. W.; QUAN, T. J. & BAILEY, R., 1976. Electrophoretic studies of the geographic distribution of *Yersinia pestis. Int. J. Syst. Bacteriol., 26:* 1-16.

LAEMMLI, K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227:* 680-685.

LAWRY, A. H.; ROBERBROUGH, N. J.; FARR, A. L. & RANDAL, R. J., Protein measurement with the Folin phenol rreagent. *J. Biol. Chem., 193:* 269-275.

LUGTEMBERG, B. & ALPHEN, L. V., 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. *Biochim. Biophys. Acta, 737:* 51-115.

MAURELLI, A. T., 1988. Temperature regulation of virulence genes in pathogenic bacteria: a general strategy for human pathogens? *Microb. Pathog., 7:* 1-10.

MORRISSEY, J. M., 1981. Silver stain for proteins in polyacrilamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem., 117:* 307-310.

NEIDHARDT, F. C. & vanBOGELEN, R. A., 1987. Heat shock response. p. 1334-1345. In F. C. NEIDHARDT, *Escherichia coli and* Salmonella typhimurium *cellular response and molecular biology*, vol. 2. Washington, American Society for Microbiology.

STRALEY, S. C. & BRUBAKER, R. R., 1981. Cytoplasmic and membrane proteins of *Yersiniae* cultivated under conditions simulating mammalian intracellular environment. *Proc. Natl. Acad. Sci., USA, 78:* 1224-1228.

STRALEY, S. C. & BRUBAKER, R. R., 1982. Localization in *Yersinia pestis* of peptides associated with virulence. *Infect. Immun., 36:* 129-135.