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HOMOLOGY AMONG EXTRA-CRYPTIC DNA BANDS AND THE TYPICAL PLASMIDS IN BRAZILIAN *YERSINIA PESTIS* STRAINS

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ABSTRACT

Yersinia pestis, the etiologic agent of plague, harbors three well-characterized plasmids: pFra (90-110kb), pYV (70 kb) and pPst (9.5 kb). Furthermore, some extra-cryptic DNA bands have been observed in a number of wild strains from several foci of the world. Additional bands have also been reported in Brazilian strains. Looking for any relationship among these cryptic DNA bands and the three-prototypical plasmids, we analyzed twelve strains displaying different plasmid content. The DNA bands were hybridized by southern blot with probes directed at the genes *caf1*, *lcrV* and *pla* located respectively on the plasmids pFra, pYV and pPst. The probes were constructed by PCR amplification and labeled with digoxigenin. The Pla probe hybridized with its target (pPst) and with bands of about 35 kb suggesting some homology among them. The Caf1 probe hybridized with the target (pFra) as well as with higher bands. The LcrV also hybridized with the target (pYV) and both with the bands higher than pFra and the bands between pFra and pYV. These results suggest that the large-cryptic bands could represent some rearrangement, open circular or linearized forms of the pFra and pYV plasmids.

Key words: *Y. pestis*, plasmids, probes, virulence genes

INTRODUCTION

In Brazil, there are several independent *Yersinia pestis* foci in rural areas, mainly in the Northeast region (21). During the period of 1966 to 1997, we have isolated 882 strains of *Y. pestis* from different hosts and distinct geographic foci (1, 2). A preliminary evaluation of the plasmid content of 26 of these strains freshly isolated during a plague outbreak (13) has revealed a homogeneous pattern composed of the three well-characterized *Y. pestis* plasmids: pFra (90-110 kb), pYV (70 kb) and pPst (9.5 kb), plus an extra-cryptic DNA band of about 23 kb (13). Another work conducted on more than 250 *Y. pestis* strains that were stored in our bacterial culture collection for several years, confirmed that most of the strains displayed the typical plasmid profile composed of the three classical plasmids (14). However, a number of the tested strains missed at least one of these plasmids, whereas a few strains carried extra-cryptic DNA bands (14). Based on the molecular weight, they could be grouped into three classes: one composed of bands greater than the pFra band (>110 kb), another group with band sizes ranging between those corresponding to pFra and pYV (<110 kb, >70 kb), and one group with bands smaller than the pYV but larger than the pPst bands (<70 kb, >9.5 kb) (14).

In this work, using probes directed at the typical *Y. pestis* plasmids, we show a homology among them and the cryptic-extra DNA bands found in strains of *Y. pestis* from our collection.

MATERIALS AND METHODS

The *Y. pestis* strains studied were obtained from the bacterial collection of the "Centro de Pesquisas Aggeu Magalhães" (1, 2, 13, 14). Since isolation, they have been stored in stabs of Blood Agar Base (BAB, Difco), at +4°C. Subculturing some of these isolates, for different purposes, at different periods, originated some spontaneous variants. Twelve isolates and four variants, displaying different plasmid content, were selected for

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this study. Table 1 shows the origin of these strains. One strain of *Y. pseudotuberculosis*, (YP 2952, from the Institut Pasteur, Paris collection) one of *Y. enterocolitica* (Ye 37, from IOC/FIOCRUZ collection) and the Girard & Robic vaccine-strain *Y. pestis* EV76 were employed as controls. Before plasmid examination, the strains were cultivated at 28ºC in Brain Heart Infusion Broth (BHI, Difco) during 24 up to 72 h, plated on BAB, to ensure purity, and grown for 24 h in BHI for plasmid extraction.

In order to know if passages “in vivo” altered the plasmid content of *Y. pestis* strains, five cultures displaying different plasmid content (Table 1), have been submitted to the natural cycle of infection (rodent/flea/rodent). With this purpose, five pools of fleas (*Xenopsylla cheopis*) were infected by feeding on rodents (*Calomys callosus*) inoculated with the strains. Infection was transmitted to healthy *C. callomys* through infected fleas bites. Dead *C. callomys* were autopsied, and their spleens were cultured for *Y. pestis* isolation and analysis of the plasmid content of the cultures recuperated. Rodents and fleas used in this study, obtained from the animal facilities of the CPqAM, were handled as recommended by Bahmanyar and Cavanaugh (4).

Table 1: Origin of the *Yersinia pestis* strains used

Identification of the strains	Source	Foci	Year of obtention
P.EXU 208	Man	Chapada do Araripe	1968
P. EXU 248	Man	Triunfo	1968
P. EXU 324	Man	Triunfo	1969
P. EXU 281	Man	Chapada do Araripe	1968
P. EXU 554	Man	Ibiapaba	1971
P. EXU 674	Man	Chapada do Araripe	1974
P. EXU 767	Man	Chapada do Araripe	1975
P. EXU 778	Man	Chapada do Araripe	1975
P. EXU 807	Man	Ibiapaba	1978
EV76	Man	Madagascar	1929
P. EXU 216	Rodent	Chapada do Araripe	1968
YP 216/80	Derived from P.EXU 216		1980
YP 216/89	Derived from P.EXU 216		1989
P. EXU 228	Rodent	Chapada Araripe	1968
YP 228/89*	Derived from P.EXU 228		1989
YP 228/93*	Derived from P.EXU 228		1993
P. PB 881*	Man	Planalto da Borborema	1986
P. PB 543*	Man	Ibiapaba	1971
P. EXU 547*	Man	Ibiapaba	1971

*= strains used in passage “in vivo”

The phenotypic plasmid markers: calcium-dependent growth at 37°C encoded by pYV, synthesis of coagulase and the bacteriocin pesticin encoded by pPst, and synthesis of the F1 antigen encoded by pFra (17) were studied as described previously (4).

Plasmid extraction was performed by a small scale alkaline lysis technique based on the procedure described by Birnboim and Doly (6), followed by electrophoresis in 0.6% agarose gels, in Tris-Borate buffer (TBE), under constant voltage of 100 V, staining with ethidium bromide, and photographed under ultraviolet light (UV). The gels were then treated with 0.2 N HCl at room temperature for 15 minutes, under gentle agitation for depurination, washed in distilled water and denatured with 1.5 M NaCl, 0.5 M NaOH, for 30 minutes, under gentle agitation. The DNA bands were then transferred from the gels to a nylon membrane by slow diffusion, overnight, in alkaline buffer (0.25 M NaOH, 1.5 M NaCl) as described (15). After transfer, the membranes were washed for 2 min in 0.3 M NaCl, 0.03 M Trissodic citrate, pH 7.0 (2 x SSC), air dried, and exposed to UV for 10 min to fix the DNA on the membrane.

The probes were constructed by PCR amplification with primers directed at published sequences of the plasmidial genes *caf1,* encoding the structural sub-unit of the F1 antigen (10), *pla* encoding coagulase and the plasminogen activator (19) *lcrV* encoding the V antigen (16), and the chromosomal *irp2* gene (11)**.** The primer sequences used are described in Table 2.

The reaction mixture, in a total volume of 25 µl, contained: 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.001 % gelatin (wt/vol), 200 mM of each dATP, dCTP, dGTP, and

Table 2: Description of the primers used.

Primer sequences	Target gene	Localization of the gene	Size of the PCR product
5'- CGG GAA TTC GAG GTA ATA TAT GAA AAA AAT CA-3' 5'-CCG CTG CAG ATT ATT GGT TAG ATA CGG-3'	*caf*1	Plasmid pFra	506 bp
5'- AGA GCC TAC GAA CAA AAC CCA C - 3' 5'- GCA GGT GGT GGC AAA GTG AGA T- 3',	*lcr*V	Plasmid pYV	800 bp
5'- AAG TTC TAT TGT GGC AAC C -3' 5'- GAA GCG ATA TTG CAG ACC -3',	*pla*	Plasmid pPst	920 bp
5'- AAG GAT TCG CTG TTA CCG GAC –3' 5'- TCG TCG GGC AGC GTT TCT TCT -3'	*irp*2	Chromosome	300 bp

dTTP (Pharmacia), 20 pmol of each primer, 20 ng of DNA, and 1 U of Taq DNA polymerase (CENBIOT, RS, BR).

Amplifications were performed in a DNA thermal cycler (Perkin-Elmer) programmed for 25 cycles composed of 1 minute at 94ºC, 2 minutes at 50ºC, 3 minutes at 72ºC and a final step of 7 minutes at 72ºC. After amplification, 3 µl of each product was submitted to electrophoresis in 1% agarose gels as described, stained with ethidium bromide and photographed under UV. Then, DNA was precipitated by ethanol (-70ºC) and 500 ng of each probe was labeled using Dig-DNA Labeling and Detection Kit, as specified by the manufacturer (Boehringer Mannheim).

Prehybridization, hybridization, washes and detection were performed in a hybridization oven following the protocol provided with the kit.

RESULTS AND DISCUSSION

With the intent of disclosing any relationship among extra-cryptic DNA bands and any of the classical *Y. pestis* plasmids, 12 cultures with different plasmid content were analyzed by Southern blotting (20) with probes directed at the pFra, pYV and pPst plasmids. Figs. 1 and 2 show the plasmid content of the *Y. pestis* cultures as analyzed in agarose gels, and Figs. 3 and 4 show the DNA bands recognized by the probes.

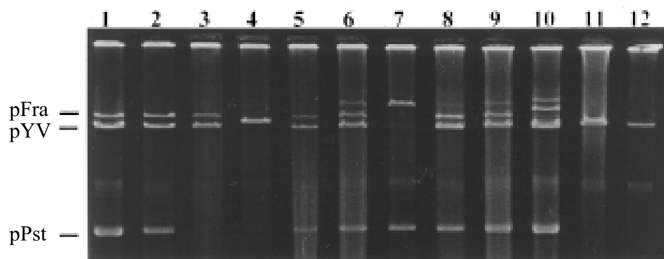

Figure 1. Plasmid content of representative Brazilian strains of *Y. pestis*, in 0,6% agarose gel. Lane 2: P.EXU 208, lane 3: P.EXU 248, lane 4: P.EXU 324, lane 5: P.EXU 281, lane 6: P.EXU 554, lane 7: P.EXU 674, lane 8: P.EXU 767, lane 9: P.EXU 778, lane 10: P.EXU 807, and controls, lane 1: *Y. pestis* EV76, lane 11: YP2952, lane 12: Ye37.

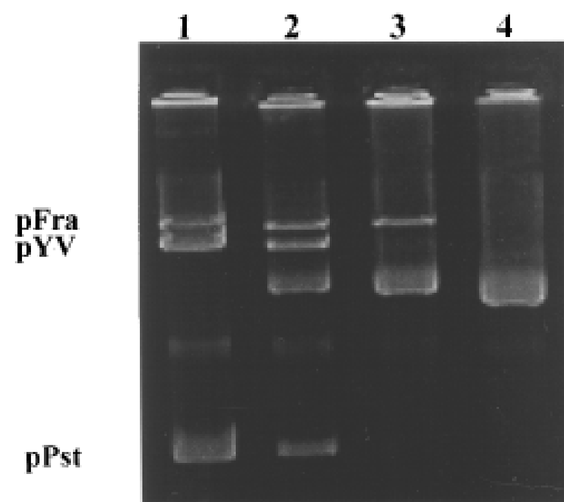

Figure 2. Plasmid content of variants derived from *Y. pestis* isolates, in 0,6% agarose gel. Lane 2: Yp 216/80, lane 3: Yp 216/89, lane 4: Yp 228/89, and control, lane 1: *Y. pestis* EV76.

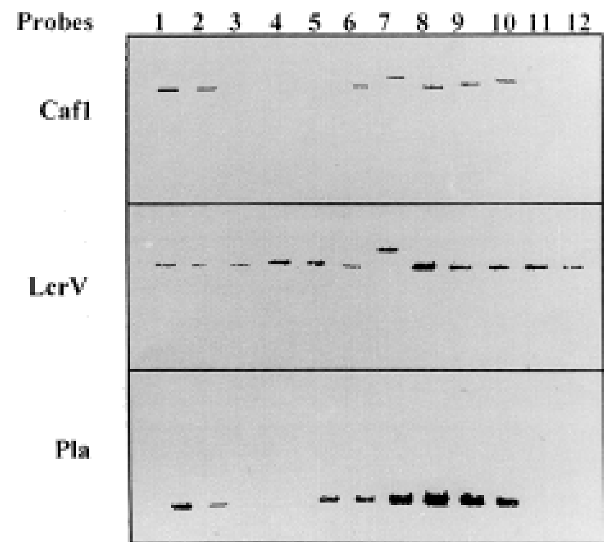

Figure 3. Southern blot hybridization of the probes Caf1, LcrV and Pla with DNA from *Y. pestis* cultures. Lane 1: *Y. pestis* EV76, lane 2: P.EXU 208, lane 3: P.EXU 248, lane 4: P.EXU 324, lane 5: P.EXU 281, lane 6: P.EXU 554, lane 7: P.EXU 674, lane 8: P.EXU 767, lane 9: P.EXU 778, lane 10: P.EXU 807, and controls, lane 11: YP2952, lane 12: Ye37. For record, the membrane was photographed immediately after the development and later digitized.

The Pla probe recognized its target (pPst) in all the strains harboring pPst analyzed (Figs. 3, 4) and the bands sized between pPst and pYV plasmids, present on the variants (Fig. 4) suggesting some homology among these bands and pPst. Furthermore, all the strains carrying these bands expressed the pPst encoded properties coagulase and pesticin. Accordingly with these results, Chu *et al*. (7) showed that an additional-small plasmid of about 19 kb is likely a dimer of pPst.

The Caf1 probe hybridized only with its target (pFra) or with larger bands, suggesting that they are related. Surprisingly the LcrV probe recognized its target (pYV) or bands larger than pFra or sized between pFra and pYV. These results could suggest that these large bands can represent some rearrangement, open circular or linearized forms of the pFra and pYV plasmids.

Moreover, the strains had also been probed with a probe directed at the chromosomal *irp*2 gene. This gene is located on

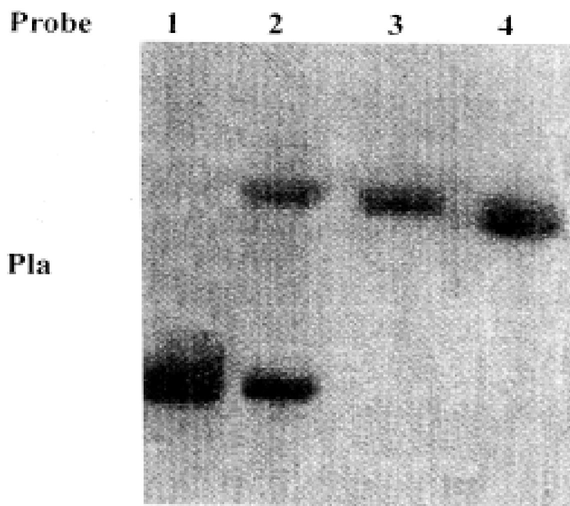

Figure 4. Southern blot hybridization of the probe Pla with DNA from *Y. pestis* cultures. Lane 1: *Y. pestis* EV76, lane 2: YP 216/80, lane 3: YP 216/89, lane 4: YP 228/89. For record, the membrane was digitized immediately after the development.

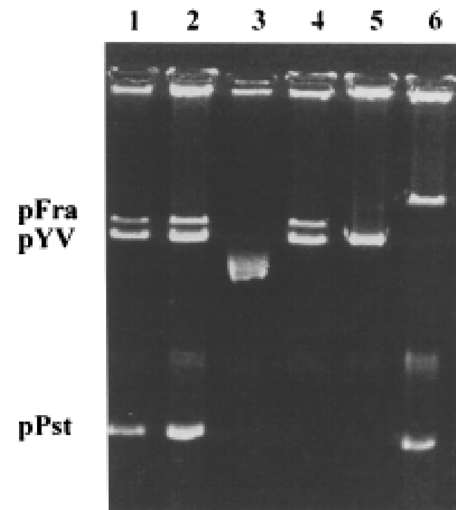

Figure 5. Plasmid content, in 0,6% agarose gel, of the *Y. pestis* cultures passed through the rodent/flea/rodent cycle. Lane 1: Yp EV76 (control), lane 2: P.PB 881, lane 3: YP 228/89, lane 4: YP 228/93, lane 5: P. EXU 547, lane 6: P.EXU 543.

a chromosomal DNA fragment that deletes spontaneously at high frequency in *Y. pestis* (3). Therefore we speculated that the extra-cryptic DNA bands found in some of the Brazilian *Y. pestis* strains could be the *irp*2 fragment. However, the Irp2 probe did not recognize any of the extra bands (data not shown). On one hand this result shows that the extra bands are not chromosomal fragments carrying the *irp*2 gene and on the other hand, it proves that the probes are specific for their targets.

Protsenko *et al.* (18) correlated the appearance of additional plasmids and increase in plasmid molecular mass with handling *Y. pestis* strains in the laboratory. We also observed spontaneous modification of the plasmid profile of some strains, after subculturing. We wondered if the plasmid content of *Y. pestis* strains change through passages "in vivo". Our studies conducted with the five cultures, listed in Table 1, showed no change in their plasmid content after the "in vivo" passages. Fig,. 5 shows the plasmid content of these cultures. All of them displayed a plasmid content identical to those in the cultures inoculated. Contrary to what happens "in vitro", the environment pressure over the bacteria "in vivo" caused no change in their plasmid content, nor selection of typical clones among the atypical cultures inoculated (to be published elsewhere).

The presence of extra-cryptic plasmid bands of varied molecular mass have been reported in wild strains of *Y. pestis* and a correlation with the geographic characteristics of the foci have been put forward (5, 8). Recently, a large extra plasmid band found in strains isolated in Madagascar was associated with antibiotic resistance (9, 12). Extra-cryptic DNA bands have also been found in strains from our collection. However, we could not establish any relevant epidemiological feature associated with geographical or epizootiological characteristics of the strains analyzed neither with antibiotic resistance.

Therefore, we don't believe that the Brazilian *Y. pestis* strains displaying atypical plasmid profiles could represent true wild type spontaneous variants. It is rather probable that these variants displaying extra-cryptic plasmid bands have been selected during prolonged storage (up to 25 years) or handling at the laboratory.

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RESUMO

Homologia entre bandas extras de DNA críptico e os plasmídios típicos em cepas brasileiras de *Yersinia pestis*

Yersinia pestis, o agente causador da peste, possui três plasmídios bem caracterizados: pFra (90-110 kb), pYV (70 kb) e pPst (9.5 kb). Adicionalmente, algumas bandas extras de DNA críptico têm sido observadas em numerosas cepas selvagens em vários focos do mundo. Bandas extras também foram observadas em cepas brasileiras. Para verificar se existe alguma homologia entre as bandas extras de DNA críptico e os três plasmídios típicos, foram analisadas 12 culturas de *Y. pestis* através de hibridização com sondas dirigidas aos genes *caf1*, *lcrV* e *pla*

localizados respectivamente nos plasmídios pFra, pYV e pPst. As sondas foram construídas através de amplificação por PCR e marcadas com digoxigenina. A sonda Pla reconheceu seu alvo (pPst) e bandas de cerca de 35 kb sugerindo que estas últimas podem se tratar de um multímero do pPst. A sonda Caf1 reconheceu seu alvo (pFra) assim como bandas mais altas. A sonda LcrV, além de reconhecer seu alvo (pYV), também hibridizou com bandas maiores que pFra e bandas de tamanho entre as de pFra e pYV. Estes resultados sugerem que as bandas grandes poderiam ser resultantes de algum rearranjo, formas abertas circulares ou linearizadas dos plasmídios pFra e pYV.

Palavras-chave: *Y. pestis*, plasmídios, sondas, genes de virulência

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