BACTERIAL VIRULENCE

Virulent non-capsulate *Yersinia pestis* variants constructed by insertion mutagenesis

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Summary. Insertion mutagenesis with the help of the plasmid pFS23 was used to generate *Yersinia pestis* *fra* mutants. The results of pFra[−] strain production under non-selective conditions suggested that such *Y. pestis* variants may be generated in natural plague foci at high frequency and may participate in supporting the epizootic process. Present data suggest that the reduction of virulence in Fra[−] strains reported by the majority of investigators was connected with the use of *Y. pestis* variants carrying additional unidentified mutations. It was shown that the loss of the ability to produce capsular antigen (FI) alone or in combination with absence of murine toxin production did not lead to an increase in LD50 absolute values. Simultaneous loss of these two virulence determinants did not influence the duration of survival of the infected animals. However, absence of only FI antigen production in the infecting strain resulted in prolonged survival of the infected animals. Conversion of plague infection from acute to chronic form is probably dependent on the animal host species and the *Y. pestis* parent strain subjected to mutagenesis.

Introduction

It has been suggested that the ability of non-capsulate *Yersinia pestis* variants to cause chronic infection¹ can play an important role in supporting the epizootic process in natural plague foci.² The laboratory-constructed non-capsulate strains usually showed reduced virulence.³ The known methods of construction of non-capsulate *Y. pestis* strains were based on spontaneous or induced mutant selections that were of low productivity⁴ or led to many unforeseen mutations.⁵ Thus, the incubation of *Y. pestis* cultures on magnesium-oxalate agar at 37°C led not only to *fra* locus deletions of the plasmid pFra⁵ but also to insertions or deletions of the plasmid pCad and to *Y. pestis* chromosomal mutations.⁶ However, the treatment of *Y. pestis* cells by ethidium bromide failed to achieve directed mutagenic action.⁷ These mutations resulted in uncontrolled changes of the biological properties of the *fra* mutants.

Recently the plasmid-located *fra* operon encoding capsular antigen (fraction I; FI) was cloned and sequenced.^{8–12} It was shown that the *fra* operon

consists of four¹¹ or five⁸ open reading frames able to encode proteins. The FI structural gene *ycaF* (*cafI*) was localised near the *ClaI* site.^{8,10}

The objective of the present study was to construct *ycaF* (*cafI*) mutants and pFra[−] variants of virulent *Y. pestis* strains with the help of insertion mutagenesis and to study their biological properties.

Materials and methods

Bacterial strains

Y. pestis strains used in the study are listed in the table.¹³ *Escherichia coli* strain HB101¹⁴ was used for genetic engineering manipulations.

Media and culture conditions

The cultures were grown on Luria Broth medium (LB; tryptone 10 g, yeast extract 5 g, NaCl 10 g) supplemented with agar 2%, pH 7.2, at 37°C (*E. coli*; *Y. pestis* for FI immunochemical testing) and at 28°C (*Y. pestis* in all the other cases). Antibiotics were used in the following concentrations: kanamycin 50 mg/L and ampicillin 100 mg/L.

Table. *Y. pestis* strains: relevant attributes

Strain no.	Virulence determinants (Phenotype)	Source or reference
231 (708)	Fra ⁺ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁺ Fb ⁺ Cg ⁺ Pgm ⁺ *	CCIM†
231pFra/pFS23	Fra ⁺ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁺ Fb ⁺ Cg ⁺ Pgm ⁺	This study
231pFra ⁻	Fra ⁻ Tox ⁻ Lcr ⁺ V ⁺ Pst ⁺ Fb ⁺ Cg ⁺ Pgm ⁺	This study
231pPst ⁻	Fra ⁺ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁻ Fb ⁻ Cg ⁻ Pgm ⁺	The author's collection
231pPst ⁻ pFra/pFS23	Fra ⁻ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁻ Fb ⁻ Cg ⁻ Pgm ⁺	This study
231Psb ⁻	Fra ⁺ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁺ Fb ⁺ Cg ⁺ Pgm ⁻	13
231Psb ⁻ pFra/pFS23	Fra ⁻ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁺ Fb ⁺ Cg ⁺ Pgm ⁻	This study
358	Fra ⁺ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁺ Fb ⁺ Cg ⁺ Pgm ⁺	CCIM
358pFra/pFS23	Fra ⁺ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁺ Fb ⁺ Cg ⁺ Pgm ⁺	This study
358pFra ⁻	Fra ⁻ Tox ⁻ Lcr ⁺ V ⁺ Pst ⁺ Fb ⁺ Cg ⁺ Pgm ⁺	This study
358pPst ⁻	Fra ⁺ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁻ Fb ⁻ Cg ⁻ Pgm ⁺	The author's collection
358pPst ⁻ pFra/pFS23	Fra ⁻ Tox ⁺ Lcr ⁺ V ⁺ Pst ⁻ Fb ⁻ Cg ⁻ Pgm ⁺	This study

*Fra, synthesis of capsular antigen; Tox, synthesis of mouse exotoxin; Lcr, low calcium response; V, synthesis of V antigen; Pst, synthesis of pesticin; Fb, fibrinolysin activity; Cg, coagulase activity; Pgm, storage of exogenous haemin.

†CCIM, The Culture Collection of Institute "Microbe".

Fig. 1. Construction of pFS23. Isolation of plasmid DNA by alkali-lysis method and genetic engineering methods was performed essentially as described by Maniatis *et al.*¹⁴ Plasmid pFS21 was constructed by deletion of the *ClaI*-*ClaI* fragment from previously constructed plasmid pFS2.¹⁰ The *Bam*HI-*Bam*HI fragment with *kan* (*Km*^R) locus from pUC4K¹⁶ was isolated and cloned into *Bam*HI site of pFS21. The plasmid generated was named pFS23. The vector fragment of the plasmid pFS23 carried the *bla* (*Ap*^R) gene and the replication region of the plasmid ColE1. This ColE1 *ori* could not provide stable preservation of recombinant plasmids constructed on its basis in *Y. pestis* recipient cells under the non-selective conditions.¹⁷

Main phenotypic determinants

The main phenotypic determinants encoded by *Y. pestis* plasmids and chromosome were analysed as described previously¹⁵ (FI antigen production was tested by haemagglutination tests for FI antigen detection).

Construction of the pFS23 plasmid

The scheme of the pFS23 plasmid construction is shown in fig. 1. Plasmids were isolated from *E. coli* and *Y. pestis* by the alkali-lysis method of Birnboim and Doly.¹⁴ Protocols for restriction enzyme digestions, and the use of T4 DNA ligase, were essentially as

described by Maniatis *et al.*¹⁴ All enzymes were used as recommended by the manufacturers.

Plasmid pFS21 was constructed from previously constructed plasmid pFS2¹⁰ by deleting the 1986-bp *ClaI*-*ClaI* fragment and ligating the two ends. This deletion includes the 173-bp region from the beginning of the FI structural gene *ycaF*. The *Bam*HI-*Bam*HI fragment with *kan* (Km^R) locus from pUC4K¹⁶ was isolated and cloned into the *Bam*HI site of pFS21. The plasmid generated was named pFS23. The vector fragment of the plasmid pFS23 carried the *bla* (Ap^R) gene and the replication region of the plasmid ColE1. This ColE1 *ori* could not provide stable preservation of recombinant plasmids constructed on its basis in *Y. pestis* recipient cells in unselective conditions.¹⁷

Transformation of *Y. pestis*

Y. pestis was transformed with plasmid DNA by electroporation as described by Yezhov *et al.*¹⁸

Construction of the *ycaF* *Y. pestis* mutants

Recipient *Y. pestis* cells were transformed by the specially constructed target plasmid pFS23. The plasmid pFra *Sal*GI-*Eco*RI fragment cloned in pFS23 had a deletion of the *ClaI*-*ClaI* region consisting of a part of *ycaF* gene and insertion of *kan* gene into the *Bam*HI site of *fra* operon. The Km^R marker flanked by the regions homologous to the pFra *fra* operon was able to enter into homologous recombination with the latter. This allowed generation of homologous recombinants with the intact DNA sequence substituted by a *ycaF*-defective DNA fragment of the plasmid pFS23 harbouring the Km^R marker. Cultivation of recombinant cells on ampicillin-free medium did not prevent elimination of the pFS23 vector region and allowed selection of *Y. pestis* $Km^R Ap^S Fra^- Tox^+$ variants. The advantages of this method were the absence of translocations and the stability of the recombinants.

Selection was performed in the following way. Km^R clones of the *Y. pestis* transformants were examined for expression of the Ap^R phenotype. $Km^R Ap^S$ variants were found at a frequency of 0.1–15% of the whole recombinant cell population.

Preliminary passage of the Km^R transformant mixture in previously immunised mice (10^8 cfu/animal) increased the proportion of the tested $Km^R Ap^S$ clones in the *Y. pestis* cultures from dead animals up to 50–100%. These mice were immunised by FI antigen (20 µg/animal) 21 days before injection. The $Km^R Ap^S$ clones were tested for FI antigen production by haemagglutination. $Km^R Ap^S Y. pestis$ cultures were shown to be Fra^- in all clones analysed.

Selection of pFra⁻ (pFra/pFS23⁻) clones

The main difficulty in the construction of *Y. pestis* pFra⁻ variant is the absence of a convenient marker for negative selection. In the selective method de-

scribed below, the use of *Y. pestis* strains carrying pFra/pFS23 plasmids with the Km^R marker provided a solution to this problem.

The *Y. pestis* 231pFra/pFS23 and 358pFra/pFS23 cultures were grown on LB agar plates containing kanamycin at subinhibitory concentrations. These concentrations were defined experimentally for every strain. About 300 cells were sown on agar plates containing kanamycin (1–10 µg/ml) and grown at 28°C. The loss of Km^R was manifest by inhibition of cell growth. Two variants of bacterial colonies were seen on the plates containing kanamycin (1–2 µg/ml) after incubation for 3–5 days; these were 0.5–1.5 mm and 2.0–3.5 mm in diameter respectively. The clones from the first group were analysed on agar plates containing a completely inhibitory concentration of kanamycin (50 µg/ml). Km^S mutations of these strains arose at the frequency of $(2.5 \times 10^{-5} - 5.0 \times 10^{-5})$. Km^S clones examined by plasmid screening¹⁴ were shown to be pFra⁻ in all clones analysed.

Definition of bacterial virulence for mice and guinea-pigs

Outbred mice weighing *c.* 20 g or outbred guinea-pigs weighing *c.* 250 g were given suspensions of 48-h agar cultures in NaCl 0.89% solution in a total volume of 0.2 ml (with 10-fold dilutions) by subcutaneous injection. The animals were observed for 21 days. LD50 values and confidence intervals were measured according to the method of Kärber modified by Ashmarin and Vorob'ov.¹⁹ The confidence interval was determined for a probability of 95%.

Results

Comparative analyses of *Y. pestis* Fra⁺ and Fra⁻ strains

The main biological properties of the strains were compared. There were no differences between wild strains and their Fra⁻ variants in: (i) cultural properties; (ii) nutritional requirements; (iii) fermentation of glycerol, rhamnose and urea; (iv) phage sensitivity. The parent strains and their Fra⁻ derivatives did not differ in other virulence-related properties such as low calcium response, synthesis of V-antigen, synthesis of pesticin, fibrinolysin activity, coagulase activity or storage of exogenous haemin. Synthesis of murine exotoxin was retained by the Fra⁻ strains generated with the help of the insertion mutagenesis but not in the pFra⁻ variants.

No consistent differences were found in the LD50 values for mice and guinea-pigs of parent Fra⁺ strains and their Fra⁻ derivatives. The only exception was a pair of strains, 231Psb⁻ and 231Psb⁻pFra/pFS23, whose LD50 values for guinea-pigs were 10 (2–24) cfu and 267 (67–966) cfu, respectively (fig. 2). Mice infected with strain 231 or its derivatives died in approximately the same periods of time (fig. 3).

Fig. 2. LD50 of *Fra*⁻ strains and their parent variants for mice (A, B) and guinea-pigs (C, D). 1, strain no. 231; 2, 231pFra/pFS23; 3, 231pFra⁻; 4, 231pPst⁻; 5, 231pPst⁻pFra/pFS23; 6, 231Psb⁻; 7, 231Psb⁻pFra/pFS23; 8, 358; 9, 358pFra/pFS23; 10, 358pFra⁻; 11, 358pPst⁻; 12, 358pPst⁻pFra/pFS23. Confidence interval was measured for probability of 95%.

Fig. 3. Average life duration in mice (A, B) and guinea-pigs (C, D) infected with *Fra*⁻ strains and their parent variants. 1, strain no. 231; 2, 231pFra/pFS23; 3, 231pFra⁻; 4, 231pPst⁻; 5, 231pPst⁻pFra/pFS23; 6, 231Psb⁻; 7, 231Psb⁻pFra/pFS23; 8, 358; 9, 358pFra/pFS23; 10, 358pFra⁻; 11, 358pPst⁻; 12, 358pPst⁻pFra/pFS23. Confidence interval was measured for probability of 95%.

However, infection of animals with *Fra*⁻Tox⁺ derivatives of strain 358 caused their death after significantly longer periods. All *Fra*⁻Tox⁺ variants of the strains analysed, except strain 231pFra/pFS23, caused the death of guinea-pigs after substantially longer periods of time than their parent strains.

Discussion

Elucidation of mechanisms that allow pathogenic micro-organisms to resist the immune systems of host species is one of the fundamental medical and biological problems. Resolution of this problem may be

achieved by identification of the genetic determinants encoding immunogenic proteins, clarification of mechanisms of regulation of their expression, and modelling their antigenic and immunogenic activity in experiments. *Y. pestis* Fra⁻ strains, in comparison with the wild strains, had a selective survival advantage in mice immunised with the live plague vaccine.²⁰ Such strains might be produced by insertion of *Y. pestis* insertion elements^{21,6} into the region of the *fra* operon or by spontaneous elimination of the plasmid pFra. The results of creating pFra⁻ strains under non-selective conditions suggested that such *Y. pestis* variants could be generated in natural plague foci at fairly high frequency and may participate in supporting the epizootic process.

It was reported previously that *Y. pestis* mutations to Fra⁻ phenotype caused a significant reduction of virulence.^{3,4} Present data suggest that this may have been due to the fact that the majority of investigators used *Y. pestis* variants carrying additional unidentified mutations. The present study, with isogenic strain sets, showed that the loss of FI production ability alone or in combination with the absence of murine toxin production did not lead to an increase in LD50 absolute values. Simultaneous loss of these two virulence determinants did not influence the duration of

survival of the infected animals. However, in the case of absence of FI antigen production alone in the infecting strain, the duration of survival in the infected animals increased. Conversion of plague infection into its chronic form depended on the animal species and the *Y. pestis* parent strain subjected to mutagenesis.

These data allow us to speculate that loss of the ability to produce antigen FI may lead to a decrease of the propagation rate of *Y. pestis* in the host organism. This would be manifest as an increased duration of survival of the infected animals. The loss of both virulence determinants encoded by the plasmid pFra could lead to a further increase of duration of infection and even to an increase in LD50. However, it is compensated by conversion of energy sources of the bacterial cell from synthesis of the plasmid pFra products to synthesis of other pathogenicity factors and to intensification of cell division. This, respectively, leads to the reduction of duration of survival in the infected animals corresponding to that in animals infected with the wild strains of *Y. pestis*.

These findings are in agreement with the data of Shanina²⁰ who described the highly virulent pFra⁻ strain 358/12.

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