MICROBIAL PATHOGENICITY

New genes involved in *Yersinia pestis* fraction I biosynthesis

V. A. FEODOROVA and Z. L. DEVDARIANI

Laboratory of Hybridomas, Russia State Antiplague Research Institute 'Microbe', Saratov, Russia

Antigenic and immunochemical properties of *Yersinia pestis* fraction I (FI) preparations extracted by different methods were studied with polyclonal and monoclonal antibodies. The existence of mature FI in a form of a complex antigen whose subunits have different genetic control was demonstrated. Galactolipid was shown, with *cafI* product, to be the second species-specific component of the FI complex molecule and is probably encoded by chromosomal genes. It, like *cafI* product, was expressed in higher quantities at 37°C than at 28°C. Among FI subunits there were at least two proteins of 28 ± 2 kDa and 43 ± 2 kDa which were not specific for *Y. pestis* but were found also in all *Yersinia* spp. and some other bacteria. These proteins were synthesised independently of the incubation temperature (4°–40°C) and are possibly encoded chromosomally but outside the *caf* operon and galactolipid-encoding genes. Both proteins together with galactolipid comprise an envelope antigen found in pFra[−] or plasmidless *Y. pestis* strains. Organisation of *Y. pestis* FI (mature capsular antigen) in the form of a complex of the envelope antigen and the *cafI* product is discussed.

Introduction

Fraction I (FI antigen, FI) is encoded by a unique *Yersinia pestis* 110-kb plasmid – pFra [1]. The structural gene for FI (*cafI*) and the associated genes (*cafIM*, *cafIA* and *cafIR*) have been cloned and sequenced and shown to be responsible for synthesis of a monomeric form of FI protein of 15–16 kDa [1, 2]. FI is considered to be one of the species-specific antigens [1–12] and many serological diagnostic kits are based on detection of either FI antigen or antibodies to FI in serum [1, 3].

Mature FI can form polymers of *c.* 2 MDa that can dissociate into subunits or oligomers [1, 4, 6, 8, 11]. At present, a non-specific character of some FI components has been reported [7, 13–16] that may be connected with lipopolysaccharide (LPS) contamination of FI preparations [3, 5, 17]. On the other hand, FI is considered to be a glycoprotein [1, 7, 17] whose synthesis is determined not only by *caf* plasmid genes, but also chromosomal genes. Furthermore, it is still unclear whether *Y. pestis* cells produce a morphologically distinct capsule (capsular antigen) or undifferentiated

envelope (envelope antigen) [3]. The aim of this study was the immunochemical characterisation of subunits composing the FI antigen and their probable genetic control.

Materials and methods

Bacterial strains

The *Y. pestis* strains used in this study are listed in Table 1. In addition, 32 strains of other bacteria (*Escherichia coli*, 10; *Shigella*, 7; *Salmonella*, 6; *Proteus*, 2; *Staphylococcus*, 4; *Y. pseudotuberculosis*, 1; *Y. enterocolitica* 1; *Vibrio cholerae* strain 569B,1) were used.

Culture of strains for preparation of FI antigen

Y. pestis EV-76 was grown in an apparatus for submerged cultivation, HEMAP (Switzerland), in 150 L of Hottinger broth (pH 7.2) supplemented with glucose for 40–46 h at 37°C with aeration. The broth culture was then centrifuged at 70 000 *g* for 30 min, the supernate and cells were disinfected separately by the addition of sodium azide to 0.02% and FI was extracted according to Veinblat *et al.* [18] for FI_V and according to Serdobintsev *et al.* [4, 11] for FI_S. *Y. pestis* EV-76 was also grown in an apparatus constructed previously (ACM-Sh) [19] on 60 L of Hottinger agar (pH 7.2) for

Received 9 Oct. 2000; revised version accepted 11 April 2001.

Corresponding author: Dr V. A. Feodorova (e-mail: microbe@san.ru)

Table 1. *Y. pestis* strains studied

Strains	Plasmid profile	Reference or source
79035	pFra ⁺ ;pCad ⁻ ;pPst ⁻	Institute 'Microbe' collection
U-2377	pFra ⁺ ;pCad ⁻ ;pPst ⁺	Institute 'Microbe' collection
A-1108	pFra ⁺ ;pCad ⁻ ;pPst ⁻	Institute 'Microbe' collection
Otten	pFra ⁻ ;pCad ⁻ ;pPst ⁺	2, 13
EV-76	pFra ⁺ ;pCad ⁺ ;pPst ⁺	7, 25
A-1450	pFra ⁻ ;pCad ⁺ ;pPst ⁻	Institute 'Microbe' collection
A-819	pFra ⁺ ;pCad ⁻ ;pPst ⁻	Institute 'Microbe' collection
A-1122	pFra ⁺ ;pCad ⁻ ;pPst ⁻	6
U-2422	pFra ⁻ ;pCad ⁺ ;pPst ⁺	Institute 'Microbe' collection
C-534	pFra ⁺ ;pCad ⁺ ;pPst ⁻	Institute 'Microbe' collection
A-100	pFra ⁺ ;pCad ⁺ ;pPst ⁺	Institute 'Microbe' collection
A-1824	pFra ⁻ ;pCad ⁺ ;pPst ⁺	Institute 'Microbe' collection
EV-2	pFra ⁻ ;pCad ⁻ ;pPst ⁺	Institute 'Microbe' collection
C-533	pFra ⁺ ;pCad ⁺ ;pPst ⁻	Institute 'Microbe' collection
PKR-133	pFra ⁻ ;pCad ⁻ ;pPst ⁻	2, 9
Yava	pFra ⁻ ;pCad ⁻ ;pPst ⁻	2, 9
KM-215	pFra ⁻ ;pCad ⁺ ;pPst ⁺	Institute 'Microbe' collection
KM-216	pFra ⁻ ;pCad ⁻ ;pPst ⁺	25
KM-217	pFra ⁻ ;pCad ⁺ ;pPst ⁻	Institute 'Microbe' collection
KM-218	pFra ⁻ ;pCad ⁻ ;pPst ⁻	9, 25
KM-225	pFra ⁻ ;pCad ⁻ ;pPst ⁻	9
KM-226	pFra ⁺ ;pCad ⁺ ;pPst ⁻	Institute 'Microbe' collection
KM-227	pFra ⁻ ;pCad ⁻ ;pPst ⁺	Institute 'Microbe' collection
M-957	pFra ⁻ ;pCad ⁺ ;pPst ⁺	Institute 'Microbe' collection
M-493	pFra ⁻ ;pCad ⁻ ;pPst ⁺	9

24 h at 28°C. Then 60 L of Hottinger broth (pH 7.2) were added and cultivation was continued for 24 h at 37°C. The bacterial cells were used as a source of FI_B [5]. *Y. pestis* EV-76 and other *Y. pestis* strains were grown on Hottinger agar (pH 7.2–7.4) at 28°C for 24 h and then incubated at 37°C for 24 h. *Y. pestis* strain 231 grown on Hottinger agar (pH 7.2–7.4) at 28°C for 24 h was used for acute challenge of mice. Both *Y. pseudotuberculosis* serovar I and *Y. enterocolitica* serovar O3 strains were grown on Hottinger agar (pH 7.2–7.4) at 37°C for 48 h. *V. cholerae* strain 569B was grown in Luria-Bertani broth (LB) or on LB agar at 37°C for 18 h. The other bacteria were grown on Hottinger agar (pH 7.2–7.4) at 37°C for 48 h.

Purification of FI preparations

FI_S was purified in Ultragel AcA-22 (LKB) from the supernate of the *Y. pestis* EV-76 broth culture grown in HEMAP. For FI_V extraction, a cell-free culture supernate was treated successively with ammonium sulphate 40% saturated solution, then 25% and finally 40% saturated solution, dialysed, purified by gel filtration through Ultragel Aca-22 and lyophilised [4]. FI_B was extracted from acetone-dried cells according to Baker *et al.* [5]. FI_S and FI_B were treated with proteinase K (PK) according to Hitchcock and Brown [20] to give FI_{SP} or FI_{BP} and these were used for immunisation of animals and in ELISA.

Antigens

Lipopolysaccharides (LPS) from *Y. pestis* EV-76, *Y. pseudotuberculosis* serovar I and *Y. enterocolitica* serovar O3 strains were purified by the procedures of

Westphal and Jann [21] and Galanos *et al.* [22]. Pure lipid A or core polysaccharide from *Y. pestis* EV-76 was prepared according to Galanos *et al.* [23]. O antigen was isolated from *Y. pestis* EV-76 by the method of Boivin *et al.* [24].

Specific polyclonal and monoclonal antibodies

Different groups of 10, 6–8-week-old female BALB/c mice, treated intraperitoneally (i.p.) with Pristane (Sigma) 0.5 ml were given five doses i.p. at 2-week intervals of 25–50 µg of FI_S or FI_V or FI_B or FI_{SP} and FI_{BP} or (2–5) × 10⁷ cells of *Y. pestis* PKR-133, Yava or KM-218 strains killed with formaldehyde 2%. For some experiments, the mice were re-immunised twice more according to the same schedule with 2-month intervals between re-immunisations. A group of three Wistar rats was immunised intramuscularly with 10 µl of FI_{SP} at 5 mg/ml in distilled water. Monoclonal antibodies (MAbs) against FI_B produced by hybridoma Yp.FI.B₂.D₃.Sp. were obtained from the ascitic fluids of BALB/c mice inoculated i.p. with hybridoma cells. MAb was purified by affinity chromatography as described previously [25].

SDS-PAGE and immunoblotting

PK-treated whole-cell lysates of *Y. pestis* strains (5 × 10⁸ cells/lane) [20], *V. cholerae* 569B and *Y. pestis* EV-76 LPS (20 µg/lane) were analysed by SDS-PAGE in acrylamide 12.5% gels [26] with protein mol. wt markers. The gels were either silver stained [27] or electrotransferred to nitrocellulose membranes (Schleier-Schuell) for Western blot analysis [25]. The membranes were incubated with murine antibodies to

FI_S, FI_{SP}, *Y. pestis* PKR-133, Yava or MAb to FI_B. Specific bands were visualised with peroxidase-labelled rabbit anti-mouse IgG (Gamaleya Institute, Russia).

ELISA

Several modifications of ELISA were used.

Indirect ELISA was used for measurement of serum antibody titre to FI_S, FI_V, FI_B, FI_{SP}, FI_{BP} and formaldehyde-treated cells of *Y. pestis* strains PKR-133, Yava and KM-218. Briefly, ELISA plates (96-well) were sensitised with 100 µl/well of either each FI preparation at a concentration of 10 µg/ml in PBS or a suspension of 1×10^9 cells/ml in PBS of the different *Y. pestis* strains, and incubated overnight at 4°C. The wells were washed three times with a washing buffer (PBS containing Tween-20 0.05%). The wells were then saturated with bovine serum albumin (BSA) 1% in PBS for 1 h at 37°C and washed as before. Then, two-fold serial dilutions of mouse sera from 1 in 40 to 1 in 10^6 were applied in triplicate and the plates were re-incubated for 1 h at 37°C. After washing, peroxidase-labelled rabbit anti-mouse IgG (Gamaleya Institute, Russia), diluted in diluting buffer (BSA 1% in PBS) was added and the plates were incubated for 1 h at 37°C. The wells were washed six times with a washing buffer and then, 100 µl of 2,2'-azinobis (3-ethyl benzthiazoline sulphonic acid) (Sigma) 22.3 mg/ml in 0.05 mM citrate buffer (pH 4.0) containing H₂O₂ 0.0003% were added. The OD₄₀₅ was measured in a Titertec Multiscan Spectrophotometer (Flow Laboratories). An OD of 0.2 above background was considered a positive result.

Concur ELISA was performed for comparison of serum antibody titres to FI_S and FI_{SP}. The wells coated with 100 µl of FI_S at 10 µg/ml in PBS and blocked as above were incubated with two-fold serial dilutions of the mouse serum to FI_S or with two-fold serial dilutions of the same serum previously incubated with FI_{SP} for 30 min at room temperature with continuous shaking. Anti-mouse conjugate and substrate were employed as described above.

Indirect dot-ELISA was used for locating FI_{SP} and FI_{BP} epitopes and studying their biochemical nature. Several drops of *Y. pestis* LPS, O antigen, lipid A, core polysaccharide, FI_S, FI_B, FI_{SP}, FI_{BP}, *Y. pseudotuberculosis* LPS and FI_{SP} treated with 25 mM sodium periodate in water for 30 min at room temperature, and FI_{SP} treated with 1 M NaOH in water for 30 min at 37°C, were placed on to nitrocellulose membranes and fixed at 110°C for 10 min. After washing three times in PBS and blocking with skimmed milk 3% for 15 min, the nitrocellulose membranes were incubated for 15 min with polyclonal murine antibodies to FI_S or FI_B or FI_{SP} or FI_{BP} or MAb to FI_B. Peroxidase-labelled anti-mouse IgG was employed as a conjugate. O-Dianisidine (Sigma) 0.01% was used as a substrate.

Finally, the membranes were dried and observed for colour differentiation.

Concur dot-ELISA. Live suspensions of the *Y. pestis* strains as listed in Table 1 (each at a concentration of 1×10^9 cells/ml of PBS) were spotted on to a nitrocellulose membrane and fixed with ethanol 96% for 20 min. After washing and blocking steps, the membrane was incubated with rat antibodies to FI_{SP} for 15 min at 37°C. After washing, the membrane was incubated with MAb; this was followed by a conjugate antibody and substrate as described above.

Indirect dot-ELISA was used for the establishment of genetic control of FI components synthesis. The same bacterial suspensions were placed on to nitrocellulose membranes and fixed, washed and blocked as described for concur dot-ELISA. Then, the membranes were incubated with murine antibodies to FI_{SP}, *Y. pestis* PKR-133, Yava and KM-218 strains for 15 min at 37°C. Subsequent procedures were performed as above.

Normal mouse serum was used as a negative control for all ELISA tests.

Phage inhibition test

Indicator cells of *Y. pestis* EV-76 ($c. 7 \times 10^8$ cells) were plated on to Hottinger agar. Then 10 µl volumes of 10-fold serial dilutions from 1×10^{-1} to 1×10^{-7} of phage L-413 [28] were spotted on to the plates. The same phage dilutions in combination with an equal volume of (i) *Y. pestis* LPS, 2.5 mg/ml in distilled water or (ii) FI_S, 5 mg/ml in distilled water or (iii) MAb, 5 mg/ml in distilled water were also used; 10–15 times higher concentrations of FI_S and MAb were employed in some experiments. The plates were left for 10 min at room temperature and then incubated for 18–20 h at 28°C. The inhibition of phage activity was shown by the absence of lysis of *Y. pestis* indicator cells. Phage with distilled water instead of antigens was the negative control, which showed as a clear spot of lysed *Y. pestis* cells.

Results

Antigenic activity of FI preparations

All FI preparations induced an antibody response in BALB/c mice (Table 2). The antibody titre to FI_S was 1000 times higher than to FI_B. Antibodies to all FI preparations reacted not only with homologous antigens but also with each of the FI preparations independently of their chemical composition and, in particular, their carbohydrate content (Table 3). Antibodies to FI_S, FI_B and FI_V as well as MAb also reacted with FI_{SP} and FI_{BP} (Table 4).

FI_{SP} and FI_{BP} also induced antibodies in BALB/c mice (Table 4). These antibodies reacted with FI_S, FI_B and

Table 2. Comparison by indirect ELISA of specific antibody titres to the FI preparations extracted by different methods

Antigen used for coating wells	Mean (SD) titre with antibodies to		
	FI _B	FI _V	FI _S
<i>Y. pestis</i> EV-76	167 272 (6912.4)	608 000 (32 000)	687 000 (0)
FI _B	1344 (149.3)	22 222 (2228.8)	11 000 (10 000)
FI _V	20 000 (1490.7)	888 000 (7950.9)	349 090 (20 000)
FI _S	40 000 (2981.4)	5500 (500.0)	1 408 000 (128 000)

Table 3. Chemical analysis of FI preparations and their characterisation in immunodiffusion tests

FI preparations	Mean (SD) protein content (% dry weight)	Mean (SD) carbohydrate content (% dry weight)	Number of precipitation lines*
FI _B	90.0 (0.1)	5.0 (0)	1
FI _V	97.6 (0.2)	< 0.1	1
FI _S	99.1 (0.2)	0.01	1

*In immunodiffusion test with commercial polyclonal antiserum.

FI_V to the same titre of 10 000 in ELISA. In concur ELISA, the specific titres of antibodies to FI_S were 256. This was significantly less than that in indirect ELISA.

Specificity of antibodies to FI_{SP} and localisation and biochemical nature of FI_{SP} and FI_{BP} epitopes

In indirect dot-ELISA, antibodies to FI_{SP} reacted with all the *Y. pestis* strains listed in Table 1 independently of their plasmid profile. All the antibodies and the MAb reacted with FI_{SP} in indirect dot-ELISA. Antibodies to FI_B and FI_{BP} also reacted with other main carbohydrate *Y. pestis* antigens, whereas antibodies to FI_V, FI_S, FI_{SP} and MAb did not (Table 4).

Sodium periodate-treated FI_{SP} reacted with all anti-

bodies and MAb, whereas NaOH-treated FI_{SP} did not (Table 4).

Defining non-specific protein FI components

In concur dot-ELISA with the relevant strains, rat antibodies to FI_{SP} made the specific colour signal less intense but did not completely inhibit specific interaction of MAb with *Y. pestis* strains either bearing or devoid of pFra or any other plasmid, irrespective of the cultivation temperature of the strains (Fig. 1).

Antibodies to *Y. pestis* strains lacking *fra* operon genes (Table 1) [2, 9] specifically reacted to a higher titre with all FI preparations and *Y. pestis* EV-76 from cultures grown at 37°C than those from cultures grown at 28°C. No reaction with LPS from *Y. pestis*, *Y. pseudotuberculosis* or *Y. enterocolitica* was observed.

In immunoblotting, the same antibodies reacted with two proteins of 28 ± 2 and 43 ± 2 kDa in *Y. pestis* EV-76, PKR-133 and Yava. However, in *Y. pestis* EV-76 grown at 28°C, the bigger immunoreactive protein was of 44 ± 2 kDa.

Specificity of antibodies to FI_S and MAb

In immunoblotting of FI_S and MAb, strong reactions with proteins of 15 (*cafI* product), 43, 60 and 70 kDa and two glycoproteins of *c.* 90 kDa were seen, together

Table 4. Specific reaction of antibodies and MAb with different *Y. pestis* carbohydrate antigens in indirect dot-ELISA

<i>Y. pestis</i> antigens	Reaction with antibodies to					
	FI _B	FI _V	FI _S	FI _{SP}	FI _{BP}	MAb
LPS isolated by the method of Westphal and Jann [21]	+	—	—	—	+	—
LPS isolated by the method of Galanos <i>et al.</i> [22]	+	—	—	—	+	—
O-antigen	+	—	—	—	+	—
Lipid A	+	—	—	—	+	—
Core polysaccharide	—	—	—	—	—	—
FI _{SP}	+	+	+	+	+	+
FI _{BP}	+	+	+	+	+	+
Sodium periodate-treated FI _{SP}	+	+	+	+	+	+
NaOH-treated FI _{SP}	—	—	—	—	—	—

+ positive reaction;
— no reaction.

Fig. 1. Indirect dot-ELISA of *Y. pestis* strains with MAb (A) and the concur dot-ELISA of the same strains with antibodies to FI_{SP} (B). The *Y. pestis* strains used in both analyses from the left to the right and top to bottom correspond to those listed in Table 1.

with a less strong reaction with a 28-kDa protein (Fig. 2A). In immunoblotting of FI_{SP} with MAb only the two glycoproteins were seen. Immunoblotting of FI_S and antibodies to FI_S obtained after one immunisation cycle (five doses, see *Materials and methods*) gave the same pattern. Antibodies to FI_S obtained after three immunisation cycles reacted with proteins of 43, 60 and 70 kDa. The 15-kDa protein and the two glycoproteins gave weaker reactions (Fig. 2B).

Fig. 2. Immunoblotting of FI_S and MAb (A) or antibodies to FI_S (B) obtained after three immunisation cycles. Number on the left indicates the position and mass of standard proteins.

Changes of *Y. pestis* carbohydrate components depending on the cultivation temperature

In indirect ELISA, FI_{SP} from the *Y. pestis* strain EV-76 cultured at 37°C reacted with antibody more strongly than that from the strain cultured at 28°C (Table 5).

The carbohydrate profiles of the PK-digested *Y. pestis* EV-76 strain grown at 28°C and 37°C were similar but not identical (Fig. 3, lanes B and C). Both profiles possessed three predominant components – one lower mol. wt band and two bands of intermediate mol. wt together with two minor bands of intermediate mol. wt and a single high mol. wt component. However, two additional bands of intermediate mol. wt and a ladder pattern of higher mol. wt, typical of bacteria with S-type LPS, e.g., *V. cholerae* (Fig. 3, lane D), appeared in the profile of *Y. pestis* EV-76 grown at 37°C (Fig. 3, lane C). This profile differed greatly from that of the R-type LPS preparation isolated from the relevant strain (Fig. 3, lane A), which showed two components of low and high mol. wt.

Carbohydrate profiles of the other *Y. pestis* strains cultivated at 28°C showed two similar high and low mol. wt components and a single component of intermediate mol. wt. (Fig. 4A). The same *Y. pestis* strains cultivated at 37°C showed two additional predominant bands of 13.2–16.5 kDa and 25.1–34.0 kDa, except *Y. pestis* strains M-957, PKR-133 and M-493, which showed a ladder pattern typical of bacteria with S-type LPS (Fig. 4B, lanes G, F, A, respectively) and the 13.2–16.5-kDa component was not seen in *Y. pestis* PKR-133 (Fig. 4B, lane F).

The results of the phage inhibition test are shown in Table 6. Equal amounts of *Y. pestis* LPS did not prevent phage lysis of bacteria grown at 28°C but inhibited lysis of bacteria grown at 37°C. In contrast, FI_S and MAb prevented lysis of bacteria grown at 28°C but did not inhibit lysis of bacteria grown at 37°C. Lysis of bacteria grown at 37°C was seen when higher concentrations of FI_S and MAb were used.

Discussion

In this study, the antigenic and immunochemical properties of *Y. pestis* FI antigen preparations extracted by the classical method of Baker *et al.* [5] and those of Veinblat *et al.* [18] and Serdobintsev *et al.* [4, 11] were compared. In contrast to FI_B, the other two preparations contained significantly less carbohydrate – 50 times less in FI_V and 500 times less in FI_S (Table 3). Although all the antigens induced an antibody response in BALB/c mice, FI_S raised the highest antibody titres detected by ELISA, especially with the homologous antigen. This could lead to a conclusion that the FI protein provided the immune response. However, antibodies to all FI preparations as well as MAb reacted not only with native (protein-containing) anti-

Table 5. Titres of antibodies to FI_{SP} and *Y. pestis* strains lacking *caf* operon genes with different *Yersinia* antigens in indirect ELISA

Antigen	Mean (SD) titre with antibodies to		
	FI _{SP}	<i>Y. pestis</i> Yava	<i>Y. pestis</i> PKR-133
<i>Y. pestis</i> EV-76 (28°C)*	672 (74.6)	120 (13.3)	152.7 (7.3)
<i>Y. pestis</i> EV-76 (37°C)*	10 500 (1166.6)	1111.1 (1047.5)	9000 (666.6)
FI _B	3000 (333.3)	608 (32)	11 000 (905.2)
FI _V	10 769.2 (770.2)	704 (64)	168 (18.6)
FI _S	10 076.9 (984.8)	746.6 (72.1)	173.3 (13.4)
<i>Y. pestis</i> LPS	—	—	—
O-antigen	—	—	—
Lipid A	—	—	—
<i>Y. pseudotuberculosis</i> LPS	—	—	—
<i>Y. enterocolitica</i> LPS	—	—	—

*Temperature of cultivation.

—, no reaction.

Fig. 3. SDS-PAGE of *Y. pestis* LPS (A), PK-digested whole-cell lysates of *Y. pestis* EV-76 grown at 28°C (B) or 37°C (C) and *V. cholerae* 569B (D).

gens but also with PK-digested (protein-free) antigens (Table 4). Moreover, as shown by concur ELISA based on the use of an FI preparation with minimal carbohydrate content (FI_S), antibodies to FI carbohydrate predominated over those to FI protein and gave 39–3900 times higher titres.

The carbohydrate components of FI_B, FI_V and FI_S appeared to be different and all contained phospholipid or galactolipid but not glycolipid (Table 4). Antibodies to FI_B and FI_{BP} reacted with cell wall components and protein-free FI (FI_{SP}), whereas antibodies to FI_S, FI_{SP} and FI_V and MAb gave a positive reaction with only protein-free FI in dot-ELISA (Table 4). Thus, FI

isolated according to the procedure of Baker *et al.* [5] was contaminated by polysaccharide components of the *Y. pestis* cell wall, especially LPS, that possibly provided the cross-reactions of the 'envelope' antigen with antisera to *Y. pseudotuberculosis*, *Y. enterocolitica* and *Salmonella* [7]. In contrast to FI_B, FI_V and FI_S contained only specific capsular polysaccharide. *Y. pestis* FI antigen is a glycoprotein [1, 4–8, 17, 18] and *Y. pestis* probably synthesised capsular polysaccharide in addition to LPS and O antigen [3, 7, 17]. These data confirmed the hypothesis of Chen and Meyer that *Y. pestis* has a carbohydrate microcapsule located just under a protein envelope [10]. The presence of specific carbohydrate in the FI antigen possibly explains positive haemagglutination tests of antibodies to FI with specimens from the remains of animals which died of plague 30–40 years ago [29]. In contrast to FI specific protein which can be destroyed easily by decay in natural conditions, the carbohydrate part is more resistant. This indicates the significant role of the carbohydrate component for serological activity of FI antigen and retrospective diagnosis of plague. Thus, the phospholipid is an essential subunit of the FI antigen and not a contaminant.

Study of the specificity of antibodies to FI_{SP} in dot-ELISA with *Y. pestis* strains with different plasmid profiles demonstrated their ability to interact with both pFra-containing and plasmidless strains. Although the structural gene for FI capsule (*cafI*) and associated genes *cafIM*, *cafIA* and *cafIR* were shown to be encoded by pFra, the results of the present study strongly indicated that the essential carbohydrate component of FI antigen was encoded by chromosomal genes. This finding is supported by a report demonstrating the possibility of extraction of a substance reacting with MAbs to FI from plasmidless *Y. pestis* strains [13]. In this case the integration of pFra into the *Y. pestis* chromosome could be supposed [9], but in the current study the plasmidless *Y. pestis* strains used were shown by DNA–DNA filter hybridisation [2], to be without the *caf* operon genes including *cafI*, yet the specific reaction of antibodies to FI_{SP} with them was

Fig. 4. SDS-PAGE of PK-digested whole-cell lysates of *Y. pestis* strains grown at 28°C (**A**) or 37°C (**B**). Lane **A**; M-493; **B**, A-1450; **C**, A-1108; **D**, A-100; **E**, U-2377; **F**, PKR-133; **G**, M-957; **H**, 79035.

Table 6. The inhibition of phage activity with different substances

Phage L-413 and antigen/MAb combination	Zone of lysis of bacteria cultivated at	
	28°C	37°C
Phage	+	+
Phage + LPS	+	—
Phage + FI _S	—	+
Phage + MAb	—	+

+ positive reaction;
— no reaction.

readily observed. This confirms the existence of chromosomal genes responsible for FI complex antigen biosynthesis [2, 9, 13, 14, 30].

Interestingly, the synthesis of the carbohydrate component of FI appeared to be temperature regulated. This substance, like the *cafI* product, was expressed by the plague bacillus in higher quantities at 37°C than at 28°C. This was shown first by the results of ELISA in which antibodies to FI_{SP} reacted with *Y. pestis* strains cultivated at 37°C at significantly higher ($\times 15.6$) titre than with the same strains cultivated at 28°C; the reaction with *Y. pestis* lacking *caf* operon genes had the same tendency (Table 5). Secondly, in phage inhibition tests, larger quantities of FI or MAb inhibited specific phage lysis of *Y. pestis* bacteria grown at 37°C than of those grown at 28°C (Table 6). It is known that phage

receptors on bacteria have a polysaccharide nature [31] and the phage receptor on *Y. pestis* is a galactolipid [7]. Furthermore, SDS-PAGE and immunoblotting showed that lengthening of carbohydrate components in bacteria grown at 37°C (optimal for FI synthesis) but not at 28°C occurs regularly in *Y. pestis* EV-76 (Fig. 3, lanes B and C), *Y. pestis* strains with different plasmid profiles and plasmidless strains (Fig. 4A and B). These components were not detectable in a *Y. pestis* LPS preparation profile in an SDS-PAGE gel loaded with three times the amount of extract. Some of these components reacted with both antibodies and MAb to FI_S in immunoblotting and were probably species-specific (Fig. 2). These data provide strong evidence that the components that appeared were FI phospholipid which, together with the *cafI* product, was probably the second specific component of the FI complex antigen. This seems quite possible as higher specificity for galactolipid in comparison with native FI antigen was shown previously in a haemagglutination test [32]. However, it is unclear whether all the remaining carbohydrate components are related to the short or shortened O-side chains of *Y. pestis* LPS or to the envelope antigen [7]. The carbohydrate profiles of all the *Y. pestis* strains used in this study, irrespective of the cultivation temperature, were not typical of bacteria with an R-type LPS, as high and intermediate mol. wt carbohydrate components (usually corresponding to O-chains of S-LPS) were seen (Fig. 4A and B). Bacteria cultivated at 37°C synthesised these components in

larger quantities, as the relevant bands were significantly more intense and some of the strains produced a variable number of repeating saccharide units typical of S-LPS (Fig. 4B, lanes A, F, G). If these components are related to O-chains of *Y. pestis* LPS, these data suggest that *Y. pestis* does not produce a typical R-LPS, as was postulated previously [1, 3], but an SR-LPS with short O-chains that show some degree of variability in different strains. Furthermore, both phospholipid and LPS synthesis is temperature regulated and strongly modified at 37°C – the *in-vivo* temperature – as demonstrated by the results of the phage inhibition test where phage L-413 in combination with *Y. pestis* LPS was used (Table 6). Therefore, LPS appears not to be a conserved antigen as was postulated earlier [3]. On the other hand, these components may be parts of other carbohydrate-containing *Y. pestis* antigens.

If FI is a glycoprotein comprising only the protein *cafI* product and galactolipid, antibodies to one of the components should prevent interaction of MAb with *Y. pestis* strains lacking *caf* operon genes. However, in concur dot-ELISA, xenogenic antibodies to FI_{SP} only partly blocked FI antigenic epitopes located on the bacterial cell surface (Fig. 1). Moreover, in addition to the 15-kDa protein, *cafI* product and the 91–93-kDa carbohydrate component, other proteins were visualised in immunoblotting with both MAb and antibodies to FI_S (Fig. 2A). These data lead us to the conclusion that the FI complex antigen (mature FI molecule) contains additional protein subunits, probably encoded chromosomally but outside *caf* operon or galactolipid-encoding gene regions. This was confirmed by the ability of antibodies to *Y. pestis* strains lacking *caf* operon genes to react with FI antigen in indirect ELISA (Table 5) and proteins of 28 and 43 kDa in immunoblotting; these were the proteins visualised in immunoblotting with both MAb and antibodies to FI_S (Fig. 2A). Both proteins appeared to be non-specific for *Y. pestis*. They were also found in outer membranes of all yersiniae – *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. kristensenii*, *Y. frederiksenii* and *Y. intermedia* [33] – and many other representatives of the Brucellaceae and the Enterobacteriaceae [13]. The adhesive properties of these proteins probably explain the adhesive and lectin properties of *Y. pestis* FI [34] and FI haemagglutination activity [7]. Furthermore, synthesis of these proteins is independent of cultivation temperature (4–40°C) [16, 33] and probably provides psychrophility to *Y. pseudotuberculosis* [35] and *Y. pestis* [16]. However, they are synthesised in smaller quantities than *cafI* product because *Y. pestis* pFra⁺ strains grown at 37°C give a significantly stronger positive reaction in immunofluorescence tests [13, 14, 25] or ELISA with MAb (data not shown) than pFra[–] strains grown at ≤28°C which demonstrate only colour background [13, 14, 25]. Because of the higher sensitivity of dot-ELISA than immunofluorescence and haemagglutination tests, a positive reaction of MAb with plasmidless *Y. pestis* strains was registered (Fig. 1). It should be

emphasised that high specificity of the MAb is well proven [1, 9, 12–14, 16, 25]. At the same time, antibodies to these proteins revealed only homologous antigens in immunoblotting of *Yersinia* whole-cell lysates and reacted in ELISA to a high titre [33] with a wide spectrum of gram-negative bacteria probably, because both proteins with mol. wts of 28 and 43 kDa together with the 18-kDa protein (identified as *cafI* product) were isolated by the procedure of Baker *et al.* [5] from capsulate (*Klebsiella pneumoniae* and *Citrobacter freundii*) and non-capsulate (*Shigella dysenteriae*, *P. mirabilis*, *E. coli*, *Y. pseudotuberculosis*, *Y. enterocolitica*) bacteria including plasmidless *Y. pestis* strains [13]. Thus, they seem to be chromosomally encoded components of an envelope but not a capsular antigen. Furthermore, these two proteins probably provided the positive reaction of *Y. pestis* strains, which lack or lost pFra, with MAb to FI in haemagglutination tests [9, 30]. Previously this phenomenon was explained as the integration of pFra into the host chromosome and the ability of such strains to produce minute amounts of FI antigen [9]. However, in the current study, antibodies to plasmidless *Y. pestis* PKR-133, Yava and KM-218 lacking *cafI* gene [2] gave strongly positive reactions with FI preparations in ELISA (Table 5) and immunoblotting with these two proteins. The 28-kDa (envelope) protein could not be a 28.7-kDa *cafIM* product [1] because DNA–DNA hybridisation showed the absence of the relevant sequence in the DNA of all the strains [12]. At the same time this protein was seen in the profiles of whole-cell lysates of all *Y. pestis* strains by SDS-PAGE (data not shown). This indicates that neither *cafI* nor *cafIM* products but rather the envelope proteins (at least two of them) provide specific interaction of MAb with pFra[–] strains. This envelope antigen is isolated from pFra⁺ *Y. pestis* strains within FI complex antigen and is its obligatory part. The presence of the above-described non-specific protein components in FI can possibly explain positive reactions of FI with intact animal sera [15]. This seems very likely as the 43-kDa protein was reported to mimic human tissue antigens [36].

The subunits of the depolymerised protein portion of FI antigen were considered to be identical in their properties despite being of different mass [1, 6, 7, 11, 13]. However, they differ in their biological activity and are encoded by different genes. For instance, the 15-kDa protein is a product of *caf* operon genes located mainly on pFra, the 28- and 43-kDa proteins are encoded by chromosomal genes, and galactolipid is also encoded chromosomally, but outside the above-mentioned genes. Genetic control of synthesis of other subunits is now unclear. Thus, mature FI complex antigen is formed from products of several genes or operon genes as a result of post-translational modification of the relevant products. These products – subunits of FI, easily visualised in immunoblotting of electrophoresed (depolymerised) FI antigen with antibodies or MAb – formed a common serologically

active specific epitope as native (polymerised) FI preparations gave a single precipitation line in immunodiffusion tests with commercial polyclonal absorbed antiserum, antibodies or MAb (Table 2) or in immuno-electrophoresis with antibodies to FI [4, 6, 7]. If the *cafI* product is synthesised by *Y. pestis*, the bacterium forms a gel-like capsule which can be defined by staining with India ink [3, 10] and FI capsular antigen can be isolated. If a bacterium lacks the *cafI* product, it does not form a capsule but rather an unstainable envelope, and an envelope antigen probably common to a number of bacteria can be isolated. Thus, only one of the protein subunits, namely the *cafI* product, determines *Y. pestis* FI antigen specificity that is confirmed by the absence of *cafI* genes in the genomes of many other bacteria examined by the relevant probe [2]. Furthermore, FI subunits showed different antigenic activity. Thus, antibodies to the 43-kDa, 60-kDa and 70-kDa proteins and two carbohydrate components of FI could be determined only in hyperimmune murine sera, whereas antibodies to the remaining subunits (proteins of 15 and 28 kDa) were raised in mice after one single immunisation cycle (Fig. 2A and B). The data obtained in this study confirm the previously shown heterogeneity of FI antigen [1, 5–7, 11, 13, 17, 18]. Differences in temperature regulation of synthesis of the FI subunits probably explain the fact that FI antigen is found in larger amounts at 37°C than at lower temperatures [1, 3, 16, 25].

Thus, FI complex antigen is encoded by different genes/operons and is formed of several subunits with various biological activities (resistance to phagocytosis, bacterial adhesion, lectin properties, induction of a protective response against *Y. pestis*). Because of its complicated structure this antigen, being surface located, provides the ability of *Y. pestis* to survive in changing environmental conditions, to defend itself against the mammalian host immune system and to contribute significantly to plague pathogenesis.

We thank Professor T.M. Taranenko for the *Y. pestis* FI_B and FI_S preparations, Dr O.V. Gromova for providing the *Y. pestis* FI_V preparation, Mrs Ye.V. Mitina and M.N. Islyayeva for excellent technical assistance and N.Ye. Teryoshkina for help in preparing the manuscript and for continuous encouragement.

References

1. Perry RD, Fetherston JD. *Yersinia pestis* – etiologic agent of plague. *Clin Microbiol Rev* 1997; **10**: 35–66.
2. Goncharov AY. Structural and functional organisation of pYT plasmid DNA fragment responsible for *Yersinia pestis* Fraction I synthesis. PhD thesis, Russia State Antiplague Research Institute “Microbe”, Russia, 1995.
3. Brubaker RR. The genus *Yersinia*: biochemistry and genetics of virulence. *Curr Top Microbiol Immunol* 1972; **57**: 111–158.
4. Serdobintsev LN, Taranenko TM, Verenkov MS, Naumov AV. Capsular antigen isolation by one-step gel filtration. Problems of natural focal infections. Saratov, Russia. 1983: 37–41.
5. Baker EE, Sommer H, Foster LE, Meyer E, Meyer KF. Antigenic structure of *Pasteurella pestis* and the isolation of a crystalline antigen. *Proc Soc Exp Biol Med* 1947; **64**: 139–141.

6. Bennett LG, Tornabene TG. Characterization of the antigenic subunits of the envelope protein of *Yersinia pestis*. *J Bacteriol* 1974; **117**: 48–55.
7. Głońska R, Gruszkiewicz E. Chemical composition and biological activity of the *Yersinia pestis* envelope substance. *Infect Immun* 1980; **30**: 506–512.
8. Habig W, Hudson BW, Marshall JD, Cavanaugh DC, Rust JH. Evidence for molecular heterogeneity of the specific antigen (fraction-1) of *Pasteurella pestis*. *Infect Immun* 1971; **3**: 498–499.
9. Protsenko OA, Filippov AA, Kutyrev VV. Integration of the plasmid encoding the synthesis of capsular antigen and murine toxin into *Yersinia pestis* chromosome. *Microb Pathog* 1991; **11**: 123–128.
10. Chen TH, Meyer KF. Studies on immunization against plague. VII. A hemagglutination test with the protein fraction of *Pasteurella pestis*: a serologic comparison of virulent and avirulent strains with observations on the structure of the bacterial cells and its relationship to infection and immunity. *J Immunol* 1954; **72**: 282–298.
11. Vorontsov ED, Dubichev AG, Serdobintsev LN, Naumov AV. Association–dissociation processes and supermolecular organisation of the capsule antigen (protein FI) of *Yersinia pestis*. *Biomed Sci* 1990; **1**: 391–396.
12. Kirillina OV. Physical mapping of *Yersinia pestis* pFra DNA, cloning of a region responsible for capsular antigen synthesis. PhD thesis, Russia State Antiplague Research Institute “Microbe”, Russia, 1992.
13. Kosse LV. Characterization of *Yersinia pestis* capsular antigen preparations isolated from genetically modified strains. PhD thesis, Russia State Antiplague Research Institute “Microbe”, Russia, 1992.
14. Bichul OK, Lebedeva SA, Alexeyeva LP *et al.* Obtaining of monoclonal antibodies to *Yersinia pestis* FI and their use for studying different *Yersinia* strains. Microbiology, laboratory diagnostics and specific prophylaxis of quarantine infections. Saratov, Russia. 1989: 44–49.
15. Basova NN, Kravtsov FE, Naumovich LS. Antibodies to *Yersinia pestis* FI are found in intact animal sera. Problems of serological studies for epidemiological surveillance over natural plague foci of the USSR. Russia, Saratov. 1982: 58–59.
16. Ikimbayev AM, Temiraliyeva GA, Devdariani ZL. Application of monoclonal antibodies for studying of *Yersinia pestis* psychrophility. Materials of a regional scientific conference on particularly dangerous diseases. Russia, Gooryev. 1989: 22–25.
17. Veinblat VI, Gooseva NP, Anayev VV *et al.* Lipid component of *Yersinia pestis* capsular antigen preparations. Microbiology, laboratory diagnostics and specific prophylaxis of quarantine infections. Saratov, Russia. 1989: 90–92.
18. Veinblat VI, Titenko MM, Verenkov MS, Men'shov PI, Kormilitsyn AV. [Characteristics of capsular antigen preparations isolated from a broth culture of *Yersinia pestis* strain EV-76.] *Zh Mikrobiol Epidemiol Immunobiol* 1985; **(4)**: 19–24.
19. Filippov AF, Nikolaev NI, Sesterenko AF. The cultivation of the plague bacillus and cholera vibrios on agar in the AKM-Sh apparatus. Problems of particularly dangerous infections. Russia, Saratov. 1970: 158–163.
20. Hitchcock PJ, Brown TM. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* 1983; **154**: 269–277.
21. Westphal O, Jann K. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. In: Whistler RL, Be Miller JN, Wolfram ML (eds) *Methods in carbohydrate chemistry*. New York, Academic Press 1965: 83–91.
22. Galanos C, Luderitz O, Rietschel ET, Westphal O. A new method for the extraction of R lipopolysaccharides. *Infect Rev Biochem* 1997; **14**: 239–235.
23. Galanos C, Luderitz O, Westphal O. Preparation and properties of antisera against the lipid-A component of bacterial lipopolysaccharides. *Eur J Biochem* 1971 **24**: 116–122.
24. Boivin A, Mesrobian I, Mesrobian L. Technique pour la préparation des polysaccharides microbiens spécifiques. *Compte rend Soc de Biol* 1933; **113**: 490–492.
25. Feodorova VA, Devdariani ZL. Development, characterisation and diagnostic application of monoclonal antibodies against

- Yersinia pestis* fibrinolysin and coagulase. *J Med Microbiol* 2000; **49**: 261–269.
26. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
  27. Tsai C-M, Frasch CE. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* 1982; **119**: 115–119.
  28. Larina VS. On the nature of species specificity of L-413 and Pokrovskaya's plague diagnostic bacteriophages. Diagnostics and prophylaxis of particularly dangerous infections. Russia, Saratov. 1983: 8–13.
  29. Lobachyev VS, Kanatov YuV, Levy MI *et al.* [Detection of specific *Yersinia pestis* antigen in subphosile mammalian bones.] *Zhurn Microbiol* 1972; **7**: 21–27.
  30. Protsenko OA, Anisimov PI, Mozharov OT, Konnov NP, Popov IuA. [Detection and characterization of the plasmids of the plague microbe which determines the synthesis of pesticin I, fraction I antigen and "mouse" toxin exotoxin.] *Genetica* 1983; **19**: 1081–1090.
  31. Knirel' YuA, Kochetkov NK. [Structure of lipopolysaccharides from gram-negative bacteria. II. Core structure: a review.] *Biokhimiya* 1993; **58**: 182–201.
  32. Glosnicka R, Kunikowska D, Dominowska C, Wegner Z, Preyborowski T, Malottke R. [Description of *Yersinia* reservoirs in synanthropic rodents in ports in the Gdansk coastal area.] *Bull Inst Marit Trop Med (Gdynia)* 1976; **27**: 247–254.
  33. Novikova OD, Vostrikova OP, Portnyagina O, Khomenko VA, Solov'eva TF, Ovodov IuS. [Antigenic properties of *Yersinia* outer membrane porins in *Yersinia* genus.] [*Bcull Eksp Biol Med*] 1996; **121**: 657–660.
  34. Serdobintsev LN, Karpunina LV, Konnov NP *et al.* Studying of *Yersinia pestis* capsular antigen preparations hemagglutination activity. Biotechnology, immunology and biochemistry of particularly dangerous infections. Russia, Saratov. 1989: 25–31.
  35. Somov GP, Pokrovskiy VI, Besednova NN (eds) Pseudotuberculosis. Russia, Moscow. 1990: 20–21.
  36. Vidayeva NA. Studying of expression of Yops encoded by *Yersinia pestis* pCad²⁺. PhD thesis, Russia State Anti plague Research Institute "Microbe", Russia, 1992.