

Macromolecular organisation of recombinant *Yersinia pestis* F1 antigen and the effect of structure on immunogenicity

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Abstract

Yersinia pestis, the causative organism of plague, produces a capsular protein (fraction 1 or F1 antigen) that is one of the major virulence factors of the bacterium. We report here the production, structural and immunological characterisation of a recombinant F1 antigen (rF1). The rF1 was purified by ammonium sulfate fractionation followed by FPLC Superose gel filtration chromatography. Using FPLC gel filtration chromatography and capillary electrophoresis, we have demonstrated that rF1 antigen exists as a multimer of high molecular mass. This multimer dissociates after heating in the presence of SDS and reassociation occurs upon the removal of SDS. Using circular dichroism, we have monitored the reassociation of monomeric rF1 into a multimeric form. Mice immunised with monomeric or multimeric rF1 develop similar immune responses, but mice immunised with monomeric rF1 were significantly less well protected against a challenge of 1×10^8 cfu of *Y. pestis* than mice immunised with multimeric rF1 (1/7 compared with 5/7). The significance of this result in terms of the structure and the function of rF1 is discussed. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Yersinia pestis*; F1 antigen; Polypeptide capsule

1. Introduction

Yersinia pestis is the causative agent of plague, a disease which is associated with a high level of mortality in man [1]. Although the bacterium no longer causes pandemics of disease, the WHO estimates that there are approximately 3000 cases of plague annually world-wide, mainly in N. America, S.E. Asia and Africa [2]. The fraction 1 (F1) antigen is a major

component of the surface of *Y. pestis* [1] and forms a capsule-like structure which is visible after negative staining of bacteria [3]. Production of the 15.5-kDa F1 subunit is encoded by the *cafI* gene [4], and the *cafIM* [5] and *cafIA* [6] genes that encode, respectively, a chaperone which allows export of the F1 antigen subunit to the surface and a protein which anchors the F1 antigen into the outer membrane. The F1 subunit is thought to be assembled on the surface of the bacterium to form the capsule. The production of F1 antigen is temperature regulated by the product of the *cafIR* gene [7], and the induc-

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tion of expression at 37°C is thought to be related to its role in protecting the bacterium from killing by host phagocytic cells [8], possibly by preventing complement-mediated opsonisation or by disrupting phagocytic cell membranes [9]. The *cafI*, *cafIR*, *cafIM* and *cafIA* genes are organised into the *caf* operon which has previously been cloned and expressed in *Escherichia coli* resulting in encapsulated bacteria [3].

Intramuscular immunisation with rF1, purified from *E. coli* has previously been shown to induce a protective immune response against *Y. pestis* [10,11] and a subunit vaccine composed of *Y. pestis* F1 antigen and recombinant V antigen (a secreted *Y. pestis* protein) shows potential as a replacement for the current killed whole cell vaccine [12,13]. Although the killed whole cell vaccine contains significant levels of F1 antigen, it is less effective as a vaccine than purified F1 antigen, leading to the suggestion that the partial denaturation of the F1 antigen results in changes to the antigenic structure [11].

A hypothetical model for the macromolecular organisation of F1 antigen from *Y. pestis* has been reported which suggested that the multimeric F1 antigen is composed from dimers of F1 antigen joined in a single plane [14]. The purpose of this study was: to evaluate the physical state of rF1; to investigate the *in vitro* assembly of rF1 monomers; and to examine whether assembly of the rF1 affected the degree of protection afforded after immunisation with rF1.

2. Materials and methods

2.1. Bacterial strains and fermentation

E. coli JM101 containing plasmid pAH34L, encoding the *caf* operon from *Y. pestis* strain GB [3], was cultured in L-broth containing 50 µg ml⁻¹ kanamycin [15]. Cultures were incubated with shaking (170 rpm) at 37°C for 19 h.

2.2. Protein assay

Protein concentration was determined using the BCA protein assay method [16]. The protein levels in column eluates were monitored at 280 nm.

2.3. Polyacrylamide gel electrophoresis

The rF1 preparations were analysed by SDS-PAGE or native PAGE (20% or 8–25% gradient gels; PhastSystem, Pharmacia, St. Albans, UK) followed by Coomassie blue staining. Gels were scanned using an LKB Bromma ultrascan XI laser densitometer. For Western blotting, the proteins were transferred onto polyvinylidenedifluoride (PVDF) membrane and detected using anti-F1 monoclonal antibody (F138G-1; American Type Culture Collection, USA) followed by detection of bound antibody using goat anti-mouse IgA-horse-radish peroxidase conjugate (Sigma, Poole UK). Bound antibody was detected using 3,3'-diaminobenzidine tetrahydrochloride substrate.

2.4. Purification of rF1

All buffers contained 0.5 mM phenylmethanesulfonyl fluoride (PMSF; Sigma, Poole, UK) added from a 0.1 M stock solution in propan-2-ol and were pre-filtered (0.2 µm filter) and degassed before use. Water used in chromatographic procedures was of Milli-Q standard.

E. coli JM101/pAH 34L was cultured in 3 × 1 l media, as described above. The cultures were centrifuged at 14 000 × g for 45 min at 4°C and the cell pellet and flocculant layer resuspended in 200 ml phosphate-buffered saline (PBS), pH 7.2 and incubated with gentle rolling at room temperature for 30 min. The resuspension was centrifuged at 14 000 × g for 30 min and the supernatant was adjusted to 50% ammonium sulfate saturation. After stirring for 1 h, the fractionate was centrifuged at 14 000 × g for 30 min at 4°C. The ammonium sulfate pellet was resuspended in PBS and dialysed against 4 × 2 l of the same buffer, overnight. The dialysed crude extract was centrifuged at 27 000 × g to remove insoluble material, followed by filter sterilisation using a 0.22-µm disposable filter (Amicon, Stonehouse, UK).

Aliquots of 200 µl of the dialysed extract were applied to an FPLC Superose 12 HR 10/30 column that had been previously equilibrated with PBS. The rF1 was eluted with the same buffer at a flow rate of 0.5 ml min⁻¹. Peak fractions were collected and analysed for rF1 by SDS-PAGE, Native PAGE and

Western blotting (using a monoclonal antibody raised to F1). An enzyme-linked immunosorbent assay was performed to determine the concentration of rF1 antigen produced, using purified F1 antigen from *Y. pestis* as a standard [12].

2.5. Structural characterisation of rF1

2.5.1. FPLC analysis

Purified rF1 was heated for 10 min at 100°C. At 0, 2 and 24 h post-cooling on ice, 200- μ l aliquots were loaded onto an FPLC Superose HR 10/30 gel filtration column in PBS at a flow rate of 0.5 ml min⁻¹ and the elution profile at 280 nm was recorded. The procedure was repeated in the presence of 0.1% (w/v) SDS. Molecular weight markers were loaded to determine the approximate molecular weight of the treated rF1 samples. These included ribonuclease (13 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa) and BSA (67 kDa) (Pharmacia, St. Albans, UK). The void volume of the column was determined using Blue dextran.

2.5.2. Capillary electrophoresis

SDS dynamic sieving capillary electrophoresis was employed to determine the molecular weight of SDS-treated rF1. Separations were performed using a Bio-Focus 3000 capillary electrophoresis system (Bio-Rad Laboratories). Purified rF1 and standard proteins (lysozyme, 14.4 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 45 kDa; serum albumin, 66.2 kDa; phosphorylase B, 97 kDa; β -galactosidase, 116 kDa; and myosin, 200 kDa), were loaded at 100 psi onto a 24 cm \times 50 μ m uncoated fused capillary containing SDS dynamic sieving separation medium, separations were performed at 15 kV with detection by UV absorbance at 220 nm.

2.5.3. Circular dichroism

CD spectra were recorded from 20 accumulative scans at 20°C using a Jobin-Yvon CD6 spectrometer (Longjumeau, France). Mean residue delta epsilon values ($\Delta\epsilon$) were calculated using concentrations derived from absorption measurements at 280 nm on a Cary 4E absorption spectrometer. Samples of rF1 in 25 mM phosphate buffer, pH 7.0, were used at a concentration of 1.2 mg ml⁻¹. Boiled samples were heated in 1.5 ml plastic reaction tubes in a heated

block at 95–100°C for 10 min, followed by cooling. CD spectra was recorded immediately and at 20 h post-boiling. The procedure was repeated in the presence of 2% (w/v) SDS.

2.6. Immunisation with SDS-treated and untreated rF1 and challenge of immunised mice

Purified rF1 (1 mg ml⁻¹) was diluted in PBS and emulsified with an equal volume of incomplete Freund's adjuvant (IFA) to a final concentration of 100 μ g ml⁻¹. Six- to eight-week-old female BALB/c mice, raised under specific-pathogen-free conditions (Charles River Laboratories, UK) were used in this study. Mice in group 1 ($n=22$) each received intramuscularly (i.m.) 100 μ l of SDS treated rF1 (10 μ g). A second group of 22 mice, each received i.m. 100 μ l of untreated rF1 (10 μ g). Mice were boosted on day 21. On day 64, 13 mice were challenged subcutaneously with 100 μ l aliquots containing 10⁵ or 10⁶ colony-forming units (cfu) of *Y. pestis* strain GB (the LD₅₀ dose of strain GB is approximately 1 cfu per mouse [17]). The remaining mice in each immunisation group were anaesthetised intraperitoneally with a 100- μ l cocktail of Domitor (Norden Laboratories, SmithKline Beecham, Surrey) (6 mg dose⁻¹) and Ketalar (Parke-Davis) (27 μ g dose⁻¹); blood was then removed by cardiac puncture. A control group of age-matched mice were not immunised with rF1.

2.7. Measurement of serum antibody titres

Serum antibody titre was measured by a modified ELISA [12]. Microtitre plates were coated with either SDS-treated rF1 (5 μ g/ml in PBS containing 0.1% (w/v) SDS) or untreated rF1 (5 μ g ml⁻¹ in PBS). Test sera were serially diluted, in duplicate, across the plate. Bound antibody was detected with peroxidase labelled conjugates of anti-mouse polyvalent immunoglobulin. Pooled sera from each group were tested for the titre of total IgG and isotypes IgG₁, IgG_{2a}, IgG_{2b} and IgG₃. The titre of specific antibody was estimated as the maximum dilution of serum giving an absorbance reading at 414 nm of greater than 0.1, after subtraction of the absorbance due to non-specific binding detected in the control sera.

2.8. Statistical analysis

A Student's *t*-test was applied to determine the significance of the difference between treatment group means.

3. Results

3.1. Purification of rF1

The centrifugation of *E. coli* JM101/pAH34L cultures resulted in the sedimentation of bacterial cells above which a layer of flocculated material was visible. In preliminary experiments, this flocculated material was shown to be mainly rF1. Additional rF1 could be removed from the surface of the bacterial cells by gentle washing in PBS. The rF1 was purified by ammonium sulfate fractionation followed by FPLC Superose gel filtration chromatography, yielding approximately 50 mg of rF1 per litre of culture. Based on scanning densitometry of SDS-PAGE gels, the purified rF1 antigen was judged to be at least 95% pure and the purified protein reacted with a monoclonal antibody against the *Y. pestis* F1 antigen (Fig. 1). Native PAGE showed that rF1 existed as a

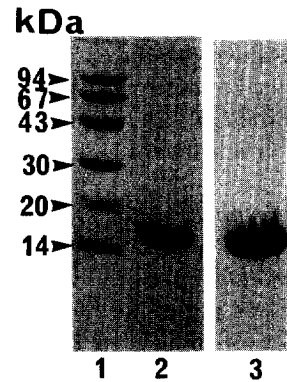


Fig. 1. Analysis of purified rF1 antigen by SDS-PAGE. Molecular mass marker proteins (track 1, molecular mass $\times 10^3$ kDa arrowed) and purified rF1 antigen (track 2) were separated through 20% homogenous gels and stained with Coomassie blue R250. In some experiments, the gels were Western blotted using anti-F1 monoclonal antibody (track 3).

high molecular weight form, too large to enter the gel (data not shown). During FPLC Superose gel filtration chromatography, rF1 eluted in the void volume (6 ml) of the gel filtration column, indicating a molecular weight which was higher than the molecular weight predicted from the deduced amino acid sequence of the *caf1* gene (15.5 kDa). The

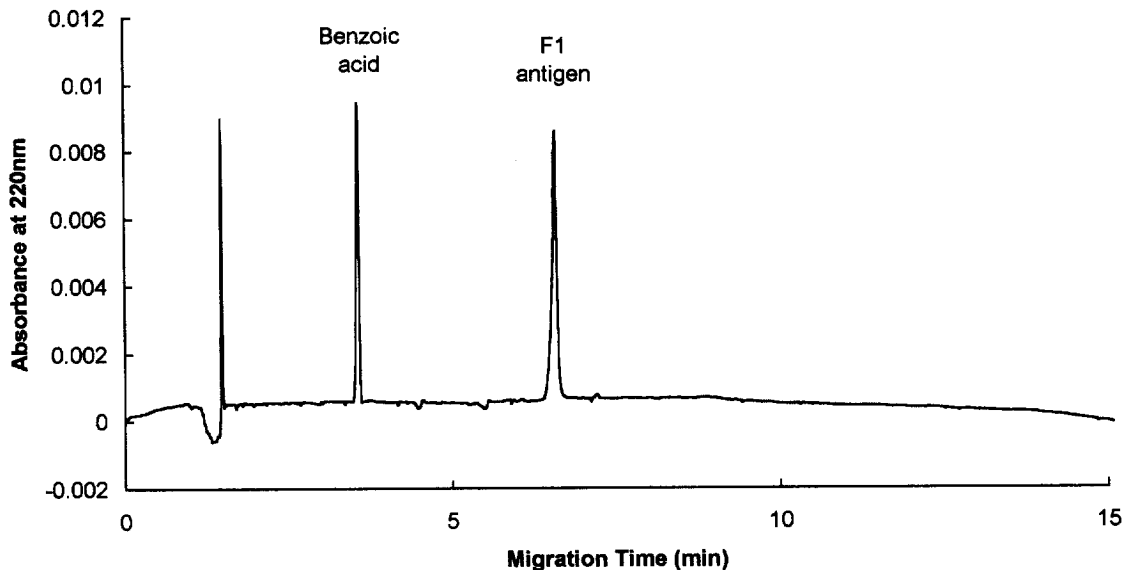


Fig. 2. Molecular weight determination of rF1 by SDS dynamic sieving capillary electrophoresis. Purified rF1, with benzoic acid as an internal reference was heated for 10 min at 100°C in the presence of 0.1% (w/v) SDS and then loaded onto a 24 cm \times 50 μ m fused silica capillary and separated at 15 kV.

nature of the high molecular weight form of rF1 was investigated using a variety of techniques.

3.2. Structural characterisation

3.2.1. Analysis of multimeric and monomeric forms of rF1

When rF1 was heated to 100°C in the presence of 0.1% w/v SDS and then analysed using Superose gel filtration chromatography, the rF1 eluted at a volume of 12 ml indicating a lower molecular weight than the untreated rF1. The low molecular weight form of rF1 antigen was maintained for at least 24 h after cooling to 0°C. When the SDS-treated rF1 antigen was dialysed against PBS, a return to the high molecular weight form of rF1 antigen was observed. SDS dynamic sieving capillary electrophoresis, showed that the molecular weight of the SDS-treated rF1 was 20 kDa (Fig. 2).

3.2.2. Reassociation of rF1

Interconversion of the low and high molecular weight forms of rF1 could also be achieved by heating rF1 in the absence of SDS. When a solution of the purified rF1 antigen, which had been heated to 100°C and then cooled, was applied to the FPLC Superose column it eluted at a position which, on comparison with standard proteins, indicated a molecular weight of 20 kDa (Fig. 3A). After further incubation at 0°C, the rF1 antigen eluted from the FPLC Superose column at positions which indicated multimerisation; after 2 h the rF1 antigen eluted as a peak corresponding to the monomeric form of F1 antigen and also a peak with an apparent molecular size of 40 kDa (Fig. 3B). After 24 h, a broad peak was observed which would encompass multimers in the size range 80–200 000 kDa (Fig. 3C).

3.2.3. Circular dichroism

Secondary structure determination of rF1, using Far-UV-CD, gave a signal that is not characteristic of any of the three common forms of secondary structure, and, in fact, was almost a mirror image of that expected for random coil. Often, unusual CD spectra are due to a strong aromatic residue contribution [18]. However, rF1 has a low aromatic residue content (containing four tyrosine and no

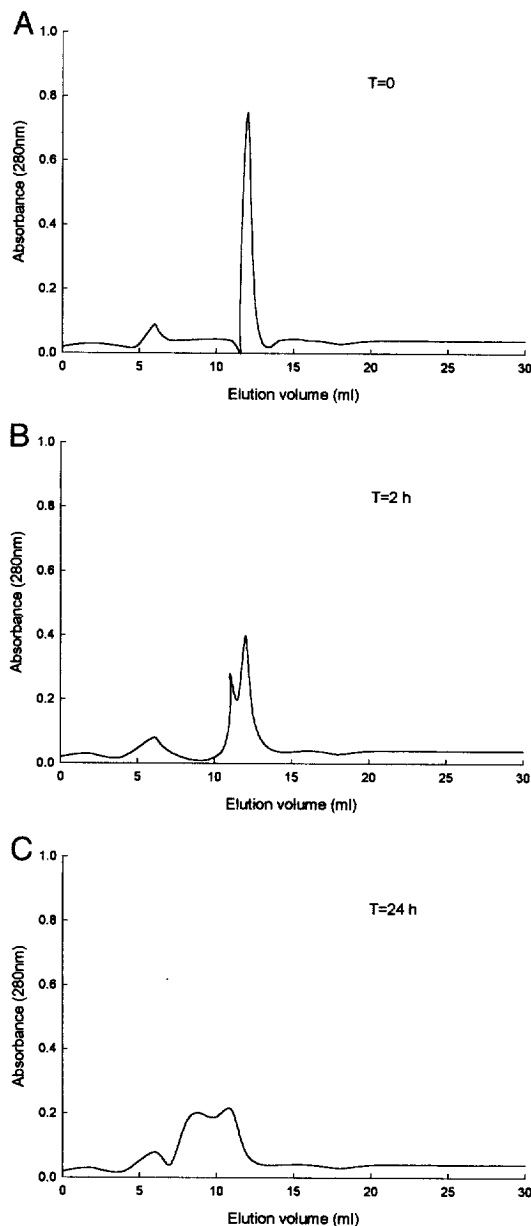


Fig. 3. Conversion of rF1 from low to high molecular weight forms. Purified rF1 (0.5 mg ml^{-1}) was heated to 100°C for 10 min, cooled on ice and samples applied to a Superose 12 column at 0 h (A), 2 h (B) or 24 h (C) post-cooling.

tryptophan residues), which gives an estimated molar extinction coefficient of $5120 \text{ M}^{-1} \text{ cm}^{-1}$. The spectral maximum at 200 nm, normally exists in type II β -turns, anti-parallel β -sheet and even short helices

Table 1
IgG titres in mice immunised with SDS treated or untreated rF1

| Immunisation group | IgG titres (\log_{10}) when tested using plates coated with | |
|--------------------|---|-----------------|
| | rF1 | SDS-rF1 |
| rF1 | 5.43 ± 0.15 | 5.44 ± 0.28 |
| SDS-rF1 | 5.09 ± 0.32 | 5.23 ± 0.44 |

Figures shown are mean values from groups of 6 mice \pm S.E.M.

[18,19]; however, the corresponding characteristic minima do not appear in rF1. The spectrum observed for rF1 could result from β -structure, superimposed on a random coil signal.

After heating in the presence of 25 mM sodium phosphate buffer, pH 7.0, the protein exhibited a pure random coil spectrum which returned partially to the original signal after 20 h (Fig. 4). When heated in SDS the protein adopted a pure α -helical conformation, which was stable.

3.3. Measurement of serum antibody titres

Preliminary studies were undertaken with rF1 (1 mg ml^{-1}), which had been heated for 10 min at 100°C in the presence of 10% (w/v) SDS, cooled and then diluted with PBS and emulsified with IFA, to a final concentration of $100 \mu\text{g ml}^{-1}$ rF1, 0.2% (w/v) SDS. After incubation at 22°C , samples removed and analysed by SDS-PAGE showed that the rF1 mixed with adjuvant remained in the monomeric form for at least 24 h. For immunisation studies, either rF1 (untreated rF1) or rF1 which had been boiled in 10% SDS and then mixed with adjuvant as described above (SDS-treated rF1) were used.

The induction of antibodies to rF1 antigen in mice inoculated i.m. with either SDS-treated rF1 or untreated rF1 in IFA was examined on day 64 post-immunisation. The antibody titres in sera from mice immunised with rF1 or SDS-treated rF1 were similar when tested using ELISA plates coated with rF1 or SDS-treated rF1 (Table 1). The distribution of antibody across the IgG subclasses was investigated. The profile from mice immunised with rF1 or SDS-treated rF1 was similar to that previously reported after immunisation with F1 purified from *Y. pestis* [13], with an IgG₁ response predominating and lower levels of IgG_{2a}, IgG_{2b} and IgG₃.

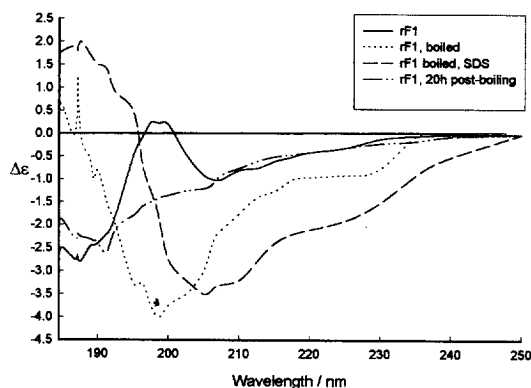


Fig. 4. CD analysis of treated and untreated rF1. Each curve represents the average of 20 accumulated spectra measured at a concentration of 1.2 mg ml^{-1} rF1 (0.2 mm pathlength cell). The sample contained 25 mM sodium phosphate, pH 7.0 with and without 2% w/v SDS. —, rF1 untreated; ···, rF1, 100°C for 5 min; - - -, rF1 100°C for 5 min followed by incubation at RT for 20 h; (- - -) rF1, 100°C for 5 min in the presence of 2% w/v SDS. Each spectrum was corrected by subtraction of a comparable blank. The abscissa is in units of $\Delta\epsilon (\text{M}^{-1} \text{ cm}^{-1})$ where M is the molar concentration of amino acid residues.

3.4. Protection of immunised mice against challenge with a virulent strain of plague

To compare the protective efficacy of immunisation with SDS-treated rF1 and untreated rF1, mice were challenged with *Y. pestis* GB strain in the dose range 1×10^5 – 1×10^6 cfu per mouse, by subcutaneous injection. All of the mice in the untreated control group died following injection with 1×10^5 cfu (Table 2). There were no mortalities in either immunised group at this challenge dose. At a challenge dose of 1×10^6 cfu, only 1 of 7 of the mice immunised with SDS-treated rF1 survived, compared with 5 of 7 mice given untreated rF1 (14.29% compared with 71.42%).

Table 2
Protection of mice immunised with rF1 or SDS treated rF1 (SDS-rF1) against challenge with *Y. pestis*

| Group | Challenge dose | Number of survivors |
|---------|----------------|---------------------|
| Control | 10^5 cfu | 0/6 |
| rF1 | 10^5 cfu | 6/6 |
| rF1 | 10^6 cfu | 5/7 |
| SDS-rF1 | 10^5 cfu | 6/6 |
| SDS-rF1 | 10^6 cfu | 1/7 |

4. Discussion

We have previously reported the cloning and expression of the *caf* operon in *E. coli*. The rF1 antigen produced by this bacterium has been used in a number of studies to evaluate this protein as a component of an improved vaccine against plague. The method reported here for the purification of the rF1 antigen yielded a protein which was homogeneous when analysed by SDS-PAGE. The expression of the *caf* operon, rather than only the *cafI* gene, in *E. coli*, resulted in the export of the rF1 antigen onto the surface of the bacterium and avoided the disruption of bacterial cells to isolate the protein. The rF1 antigen appeared to be only loosely bound to the surface of the bacterium and could be removed by gentle washing of the cells. Whereas Andrews et al. [11] reported that purified rF1 was in a low molecular mass form, we have found that rF1, which we isolated from cells, was in a high molecular mass form. It is possible that the more complex purification procedure described by Andrews et al. [11], which included treatment of bacterial cells with acetone and toluene, resulted in the monomerisation of rF1.

The F1 antigen is known to form a capsule-like structure on the surface of *Y. pestis* and the autoassembly of the F1 antigen has been suggested, but this is the first detailed study of this process. The gene products of the *caf* operon include the F1 antigen; the CafIM protein which is thought to act as a chaperone for the F1 antigen; the CafIA protein which is thought to anchor the F1 antigen into the outer membrane of the bacterium and the CafIR protein which regulates gene expression. Our findings suggest that in vitro, the F1 antigen is able to assemble into a high molecular weight form and this might indicate the mechanism by which the antigen on the surface of the bacterium assembles into high molecular weight fimbrial-like structures. The primary amino acid sequence of the F1 antigen does not reveal the presence of cysteine residues which might form disulfide bridges. Our finding that the oligomeric rF1 could be converted to the monomeric form by heating to 100°C, and that SDS prevented reassociation into the multimeric form suggests that hydrogen bonds and hydrophobic interactions play a key role in the oligomerisation of rF1 antigen.

In addition, analysis by circular dichroism shows that rF1 antigen contains β -structure, supporting the proposal by Galyov et al. [4], that F1 antigen had at least 50% β -sheet structure. More recently molecular modelling of the structure of F1 antigen has suggested that the protein adopts mainly a β -sheet structure [20]. In our studies, heating rF1 for 5 min at 100°C caused loss of all native secondary structure, with a return to a conformation similar to, but not identical to the original, after incubation for 20 h. Heating rF1 in the presence of SDS resulted in the formation of an α -helical rich structure. This is due to the amphiphilic environment of the SDS micelle, causing α -helix formation in sequences that do not adopt this formation normally.

Previous workers have shown that oral immunisation with *Salmonella typhimurium* expressing the *caf* operon induced a higher level of protection against plague than oral immunisation with *S. typhimurium* expressing only the *cafI* gene [3,21]. It was suggested that this might be due to differences in the protective efficacy of monomeric or multimeric forms of the F1 antigen [3]. In this study, the treatment of rF1 with SDS, which was shown to monomerise the rF1, did not affect the immunogenicity of the F1 antigen. In addition, we were unable to detect a significant difference in the level of antibody when rF1 or SDS-treated F1 were used to coat ELISA plates. This finding might indicate that the SDS-treated rF1 antigen re-folded when introduced into animals, and whilst we cannot discount this possibility, it is clear that SDS-treated rF1, although equally immunogenic, was less effective in inducing protection against plague. Previous studies with the killed whole cell vaccine or purified F1 antigen vaccine have shown that antibody titres to F1 antigen were higher in mice immunised with the killed whole cell vaccine yet the mice immunised with F1 antigen were better protected against plague [11]. These findings suggested that the F1 antigen in the killed whole cell vaccine had been denatured [11] and the breakdown of F1-antigen in this vaccine has been reported [22]. These findings are similar to those reported for the Vi capsular antigen of *Salmonella typhi*, where high molecular weight forms are reported to induce better protection than low molecular weight forms of the antigen [23].

The peptide ${}_{75}\text{TSQDGNNHQ}_{83}$ has been identi-

fied as the main B-cell epitope, and on the basis of molecular modelling it has been suggested that this epitope is located on a hydrophilic loop which is accessible in monomeric or multimeric forms of F1 antigen [20]. It is possible that either another region of multimeric rF1 is responsible for a protective immune response or that the affinity of antibodies generated against the SDS-treated F1 antigen was lower than those generated against rF1 antigen.

It has previously been reported that F1 antigen purified from *Y. pestis* will be a component of an improved subunit vaccine against *Y. pestis* and 4/5 mice immunised with *Y. pestis* F1 were protected against 2×10^5 cfu of *Y. pestis* given by the s.c. route [12]. The rF1 we have prepared protected 6/6 mice against challenge with 10^5 cfu of *Y. pestis*. These results also extend the findings of Andrews et al. [11] who showed that immunisation with rF1 antigen provided protection against 100 LD₅₀ doses of *Y. pestis*. The method we have described for purification of a highly protective form of F1 antigen will facilitate the development of an improved subunit vaccine against *Y. pestis*. In addition, the results of this study provide insight into the mechanisms of assembly of bacterial polypeptide capsules. Our findings will now permit further studies to investigate the detailed structure of the *Y. pestis* F1 antigen using recombinant F1 antigen.

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