An *aroA* mutant of *Yersinia pestis* is attenuated in guinea-pigs, but virulent in mice

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This study describes a PCR-based approach for the production of a rationally attenuated mutant of *Yersinia pestis*. Degenerate primers were used to amplify a fragment encoding 91·45% of the *aroA* gene of *Y. pestis* MP6 which was cloned into pUC18. The remainder of the gene was isolated by inverse PCR. The gene was sequenced and a restriction map was generated. The *Y. pestis* *aroA* gene had 75·9% identity with the *aroA* gene of *Yersinia enterocolitica*. The cloned gene was inactivated *in vitro* and reintroduced into *Y. pestis* strain GB using the suicide vector pGP704. A stable *aro*-defective mutant, *Y. pestis* GB Δ *aroA*, was isolated and its virulence was examined *in vivo*. The mutant was attenuated in guinea-pigs and capable of inducing a protective immune response against challenge with the virulent *Y. pestis* strain GB. Unusually for an *aro*-defective mutant, the *Y. pestis* *aroA* mutant was virulent in mice, with a median dose which induced morbidity or death similar to that of the wild-type, although time to death was significantly prolonged.

Keywords: *Yersinia pestis*, *aroA*

INTRODUCTION

Yersinia pestis is the causative organism of bubonic and pneumonic plague and results in a high rate of mortality. It is capable of infecting man and a wide range of animals, with transmission occurring primarily by flea bites. Virulence of the bacterium is due to the expression *in vivo* of a complex array of factors (Straley & Perry, 1995). The prevention of plague is based upon vaccination, but existing whole-cell plague vaccines produce a range of side-effects, making them unsuitable for widespread use (Marshall *et al.*, 1974; Meyer *et al.*, 1974a; Reisman, 1907). Candidate subunit vaccines based on the *Y. pestis* F1 and V antigens have been shown to induce protection against plague (Oyston *et al.*, 1995; Leary *et al.*, 1995). However, F1-defective mutants of *Y. pestis* have recently been identified which are still virulent (Davis *et al.*, 1996; Drozdov *et al.*, 1995). A rationally attenuated *Y. pestis* strain would enable the presentation of a wider variety of antigens, including F1 and V, to the immune system, thus enhancing protective efficacy. The existing whole-cell vaccines currently licensed for human use may leave vulnerable mucosal surfaces unprotected as they are

administered parenterally and may not effectively induce a mucosal immune response (McGhee *et al.*, 1992). The pneumonic form of plague is highly infectious, due to the large numbers of plague bacilli released in aerosols (Cowling & Moss, 1994). A live attenuated mutant strain would be amenable to oral dosing and thus should effectively induce mucosal immunity to protect against pneumonic plague.

Bacteria possess a linear biochemical pathway for the synthesis of aromatic amino acids. The *aroA* gene encodes 5-enolpyruvylshikimate-3-phosphate synthase, which is involved in the conversion of shikimic acid into chorismic acid, a common intermediate in the synthesis of several compounds, including aromatic amino acids, *para*-aminobenzoic acid (PABA), 2,3-dihydroxybenzoic acid (DHB) and *para*-hydroxybenzoic acid. Interruption of this pathway produces a requirement for PABA and DHB, which are not available in mammalian tissues. Therefore the bacteria are only capable of limited replication before being cleared from the host.

Attenuated strains of a range of bacterial species have been produced by inactivation of genes in the aromatic acid biosynthetic pathway. Aromatic-dependent (Δ *aro*) mutants of *Salmonella*, *Pasteurella multocida*, *Aeromonas salmonicida*, *Yersinia enterocolitica*, *Bordetella pertussis*, *Bacillus anthracis* and *Listeria monocytogenes* (Hoiseth & Stocker, 1981; Levine *et al.*, 1987; Homchampa *et al.*, 1992;

Abbreviations: DHB, dihydroxybenzoic acid; PABA, *para*-aminobenzoic acid.

The EMBL/GenBank/DBJ accession number for the sequence reported in this paper is L46372.

Vaughan *et al.*, 1993; Bowe *et al.*, 1989; Roberts *et al.*, 1990; Ivins *et al.*, 1990; Alexander *et al.*, 1993) have all been shown to be avirulent and to stimulate protective immunity. *Salmonella typhimurium* Δ aroA administered orally to mice was capable of invasion from the gut, followed by colonization of the Peyer's patches, liver and spleen (Maskell *et al.*, 1987). Multiplication was self-limiting and the salmonellae were cleared after a period of several weeks. However, *Y. enterocolitica* Δ aroA could establish only a short-lived colonization of the reticulo-endothelial system and required repeated inoculations to induce immunity (Bowe *et al.*, 1989).

Y. pestis auxotrophic mutants with a requirement for exogenous supplies of amino acids, including phenylalanine, have been isolated previously and observed to be virulent in mice (Burrows, 1960, 1963). However, the exact mutation to produce phenylalanine dependence was not characterized and it is not possible to know whether genes other than those involved in the production of this aromatic amino acid were expressed normally. In this study, we have constructed a defined isogenic Δ aro mutant and tested its stability, immunogenicity and protective efficacy *in vivo*.

METHODS

Bacteria, plasmids, media and growth conditions. *Y. pestis* laboratory strain MP6 and the human isolate strain GB were routinely cultured aerobically at 28 °C in Blood Agar Base (BAB) broth or on BAB agar. *Yersinia* selective agar base (YSA) was obtained from Oxoid.

S. typhimurium SL3261 (Hoiseth & Stocker, 1981) was provided by B. A. D. Stocker, Stanford University, CA, USA. *Escherichia coli* S17- λ pir was provided by A. Forsberg, Umea, Sweden. *S. typhimurium* and *E. coli* strains were cultured and stored as described by Sambrook *et al.* (1989).

Defined media were prepared as described by Straley & Bowmer (1986), omitting, when necessary, the aromatic amino acids tyrosine, tryptophan and phenylalanine, which were normally present at a final concentration of 1 mM, 0.1 mM and 1 mM, respectively. For all media, ampicillin was added as required at a final concentration of 25 μ g ml⁻¹.

Plasmid pUC18 was obtained pre-digested with *Sma*I and pre-treated with calf intestinal alkaline phosphorylase (Boehringer Mannheim). The vectors pGP704 (Miller & Mekalanos, 1988) and pNJ5000 (Grinter, 1983) were kindly provided by M. Tully, CAMR, Salisbury, UK, and B. Wren, St Bartholomew's Hospital, London, UK, respectively.

Preparation and manipulation of DNA. DNA was isolated from *Y. pestis* by the method of Marmur (1961). Large- and small-scale plasmid isolations, restriction enzyme digests, blunt-ending of DNA fragments and ligations were performed as described by Sambrook *et al.* (1989). Restriction endonucleases, T4 DNA ligase, DNA polymerase I, large fragment (Klenow enzyme), sequencing-grade *Taq* polymerase and polynucleotide kinase were obtained from Boehringer Mannheim.

Electroporation. *E. coli* strains were transformed following electroporation as described by Dower *et al.* (1988), using a Gene Pulser apparatus (Bio-Rad) set at 2.5 kV, 25 μ F and 800 Ω . Following electroporation, bacteria were incubated at 37 °C for 1 h without selection and then plated onto the appropriate selective media.

Bacterial conjugation. Plasmids were introduced into *Y. pestis* by conjugation in a three-way mating (Grinter, 1983), using a *Y. pestis* recipient, a donor strain comprising *E. coli* S17- λ pir containing the plasmid of interest and *E. coli* S17- λ pir.pNJ5000. *E. coli* strains were grown overnight at 37 °C, with shaking, in LB containing the appropriate antibiotic. *Y. pestis* strain GB was grown at 28 °C overnight with shaking in BAB. A 1 ml aliquot of each strain was washed in LB and resuspended to 100 μ l in LB. Equal volumes (50 μ l) of the three strains were mixed and 100 μ l was spotted onto an L-agar plate. After incubation at 28 °C for 4 h, the bacteria were removed from the plate using a sterile swab and resuspended in 1 ml LB. The cells were washed twice in LB, diluted and plated onto YSA containing 25 μ g ampicillin ml⁻¹ and incubated at 28 °C. *E. coli* was unable to grow on the YSA plates as quickly as *Y. pestis*, and grew very pale pink, while the *Y. pestis* colonies were a very dark pink.

Cloning and sequencing of the *aroA* gene of *Y. pestis*. Degenerate oligonucleotide primers were designed by comparison of the nucleotide sequences of *E. coli*, *S. typhimurium*, *B. pertussis* and *Y. enterocolitica*. These primers corresponded to nucleotides 4–20 (GAATCCCTGACKTTACA, where K = G or T) of the *E. coli* *aroA* gene, with the complementary primer (CACATCGCCATVCGGTG, where V = G, C or A) corresponding to nucleotides 1105–1121 (Fig. 1). Using these oligonucleotide primers in PCR (Dybvig *et al.*, 1992), a DNA fragment was obtained after 35 cycles of amplification (95 °C, 15 s; 50 °C, 15 s; 72 °C, 30 s; Perkin Elmer 9600 GeneAmp PCR System). The fragment was purified, blunt-ended and ligated into pUC18 to produce pYPA7. Synthetic oligonucleotide primers were used to sequence the fragment by the dideoxy chain-termination method, using a PRISM Ready Reaction Dye Terminator Cycle Sequencing kit (Applied Biosystems). Samples were loaded on an ABI 373A automated DNA sequencer and electrophoresed through a 6% (w/v) gel, and chromatograms were analysed using SeqEd (ABI). The remaining bases of the *aroA* gene sequence were obtained by inverse PCR (Havard *et al.*, 1992) following digestion of whole-cell *Y. pestis* DNA with *Sac*II or *Cfo*I.

Determination of virulence for mice and guinea-pigs. BALB/c female mice (6 week old) and female (young, Duncan Hartley) guinea-pigs were used throughout. The median doses of the wild-type and mutant strains which induced morbidity or death in mice were assessed by subcutaneous injection of groups of five animals with serial dilutions of exponential-phase broth cultures grown at 28 °C. Humane end-points were strictly observed, and animals deemed incapable of survival were humanely sacrificed by cervical dislocation. The median lethal dose which induced morbidity or death was determined by the method of Reed & Muench (1938).

The virulence of the strains in guinea-pigs was assessed with cultures grown at 37 °C. Guinea-pigs challenged with 19.1 c.f.u. *Y. pestis* strain GB surviving to 21 d were killed by cervical dislocation and autopsied. One animal which had been challenged with 26.2 c.f.u. GB Δ aroA was also sacrificed, bled by cardiac puncture, post-mortemed and organ smears were made. Sera were titrated by ELISA (Oyston *et al.*, 1995) for the presence of antibodies to F1 and V antigens. Guinea-pigs inoculated with the *aro*-defective mutant were challenged with *Y. pestis* GB on day 24. Fourteen days after this second inoculation, the guinea-pigs were sacrificed and examined for the presence of internal abscesses.

Survival of *Y. pestis* GB Δ aroA *in vivo*. Two groups of 35 mice were inoculated subcutaneously with either 7.28 $\times 10^3$ c.f.u. *Y. pestis* GB or 7.77 $\times 10^3$ c.f.u. GB Δ aroA. At 6, 12, 24, 48, 72 and 96 h, five mice from each group were sacrificed by cervical dislocation and spleens were removed and homogenized in

10 ml PBS (Dulbecco's A recipe; Oxoid) using a stomacher (Seward Medical) on maximum setting for 2 min and diluted in PBS. Spleen homogenates were plated in duplicate on Congo red and YSA plates to determine the numbers of plague bacilli present.

Growth of *Y. pestis* GB Δ aroA in defined media. *S. typhimurium* SL3261 and *Y. pestis* GB Δ aroA were grown overnight in defined medium. Aliquots (1 ml) of each were used to inoculate 20 ml defined medium. The cultures were grown at 28 °C (*Y. pestis*) or 37 °C (*S. typhimurium*) with shaking and the OD₆₀₀ was monitored at hourly intervals using a Philips PU8800 spectrophotometer. At stationary phase, a 1 ml aliquot was used to inoculate a fresh 20 ml culture, and the process was repeated. When SL3261 was not growing in fresh defined media, 100 μ l 1 mg folate ml⁻¹ was added to the broth. After a further 3 h, 100 μ l 1 mg PABA ml⁻¹ and 1 mg DHB ml⁻¹ were added and the OD₆₀₀ was monitored.

RESULTS

Sequence analysis of the *Y. pestis* aroA gene

The sequence (Fig. 1) contained an open reading frame of 1275 nucleotides which encoded a protein of 425 amino acids. The nucleotide sequence exhibited 75.9% identity to the aroA gene of *Y. enterocolitica* (O'Gaora *et al.*, 1989). A high degree of identity was also identified between the predicted amino acid sequence of the aroA gene from *Y. pestis* and the aroA genes from *E. coli*, *S. typhimurium*, *B. pertussis* and *Y. enterocolitica*, which showed 79.5%, 79.1%, 52.2% and 90.1% identity, respectively (Fig. 2). When examining the sequence published here, it should be borne in mind that it was derived from a PCR product rather than a directly cloned gene and may therefore contain PCR-introduced errors. To minimize such errors, the pooled PCR products of the aroA gene amplified from chromosomal DNA preparations were sequenced three times in each direction to confirm the sequence obtained from the cloned PCR-derived gene.

Construction of the *Y. pestis* Δ aroA mutant

A restriction map was generated from the sequence of the *Y. pestis* aroA gene. A unique *Sac*II site and two *Cla*I sites were identified, allowing the deletion of an internal 293 bp fragment. Plasmid pYPA7 was digested with *Cla*I and *Sac*II, blunt-ended and re-ligated. Loss of the 293 bp fragment was confirmed by PCR, restriction digests and sequencing. The deleted aroA gene fragment was excised from pUC18 by digestion with *Sac*I and *Sal*I and cloned into *Sac*I/*Sal*I-cut pGP704 to produce pGPYP Δ A7. The plasmid was electroporated into *E. coli* S17- Δ pir, mobilized into *Y. pestis* GB and transconjugants were selected on YSA plates containing 25 μ g ampicillin ml⁻¹. Colonies which appeared on the selective plates resulted from integration of pGPYP Δ A7 into the chromosome by a single cross-over event. Five colonies were picked and replated twice on nonselective media before ampicillin-sensitive revertants appeared due to a second cross-over event. As either the wild-type copy of the gene originally present on the *Y. pestis* chromosome or the plasmid-borne mutated gene could have been excised, the deletion was confirmed by PCR (Fig. 3). The mutant, strain GB Δ aroA, was unable to grow on defined media without supple-

ments of aromatic amino acids, whereas the wild-type grew on both types of media.

Growth of *Y. pestis* GB Δ aroA in defined media

The growth of *S. typhimurium* SL3261 (*aroA* his) in defined media containing aromatic amino acids and histidine was measured. By the third subculture in defined media, the culture showed no increase in optical density after incubation for up to 24 h at 37 °C. Supplementing the media with PABA and DHB, but not folate alone, resulted in rapid growth of the organism. *Y. pestis* GB Δ aroA was also able to grow in the same batch of media; however, repeated subculture did not result in limitation of growth, even by the seventh subculture into fresh media. Therefore, unlike *S. typhimurium* SL3261, the aroA-defective *Y. pestis* mutant was not starved of PABA and DHB in the defined media.

Virulence of *Y. pestis* GB Δ aroA in vivo

Y. pestis GB was virulent in BALB/c mice, with a median dose which induced morbidity or death of approximately 1 c.f.u., confirming the virulence of this strain observed previously (Russell *et al.*, 1995). The median dose of GB Δ aroA required to produce morbidity or death was 1.66 c.f.u., but the mean time to death of 158.9 ± 8.6 h was significantly longer than with strain GB (108.3 ± 7.75 h; $P < 0.001$). *Y. pestis* was isolated from organ smears from the mice challenged with GB Δ aroA. PCR amplification of the aroA gene of the recovered organisms showed that the mutant had not reverted to the wild-type.

Y. pestis infection in guinea-pigs caused a protracted disease compared to that seen in mice. Typically the animals appeared ruffled, lost weight and became less active. In some cases, there was asymmetrical paralysis of the rear limbs, followed by an apparent depression of the respiratory rate. The animals were humanely killed at this time. Autopsy revealed abscesses in the liver and spleen, and the organs were enlarged. *Y. pestis* GB produced disease in guinea-pigs. One animal died within 21 d and the livers and spleens of all but one of the remaining animals were shown to be covered by abscesses upon post-mortem on day 21. The guinea-pigs challenged with GB Δ aroA all survived to day 21, and autopsy of one animal at this time revealed no apparent internal abscesses. Serum from the autopsied animal had a specific anti-F1 antigen titre of 1:1600 and an anti-V antigen titre of 1:800. Guinea-pigs from both challenge groups had a subcutaneous lesion at the site of injection.

The remaining guinea-pigs which had originally received GB Δ aroA were challenged with *Y. pestis* GB (9.26×10^3 – 9.26×10^6 c.f.u.). All the animals survived the challenge with no apparent ill-effects over 14 d. Post-mortem after this interval revealed all the surviving animals to be healthy, with no internal abscesses. No *Y. pestis* was isolated from smears of livers and spleens.

Survival of the aro-defective mutant in vivo

Following subcutaneous inoculation of *Y. pestis* GB or GB Δ aroA in mice, no bacteria were isolated from the

Fig. 1. Nucleotide and deduced amino acid sequences of the *aroA* gene of *Y. pestis*. The internal region of the gene deleted to generate the inactivated gene in pGPY Δ A7 is underlined by a solid bar from base 312 to base 605. Arrows under the sequence indicate the bases corresponding to the oligonucleotide primers used to amplify the fragment cloned into pUC18 to produce pYPA7.

blood during the first 24 h. Bacteraemia then increased over several days. The spleens of both groups of mice were colonized by 24 h, with numbers of bacteria increasing to over 10^8 c.f.u. g^{-1} at the termination of the experiment at 96 h. The mean times to death for two groups of five mice challenged with a high dose of approximately 7×10^7 c.f.u. of either strain GB or GB Δ *aroA* were 82.9 ± 6.3 h and 117.1 ± 9.6 h, respectively.

DISCUSSION

One of the earliest descriptions of an attenuating mutation in a bacterium concerned purine dependence of *Y. pestis* (Burrows, 1963). This mutant was generated by classical clonal selection techniques. Other spontaneous mutants of *Y. pestis* are currently available as vaccines for the prevention of plague, e.g. strains EV76 and Haffkine. However, the exact nature of these attenuations has not

been characterized and the side-effects of vaccinating with these strains were sufficiently severe to hospitalize 2 out of 12 volunteers in one study (Meyer, 1970). Formalin-killed whole-cell vaccines are also available, but these are highly heterogeneous with variable endotoxin content and their side-effects make them unsuitable for general use (Marshall *et al.*, 1974; Meyer *et al.*, 1974a; Reisman, 1907). The advantages of rationally attenuated auxotrophic strains of other bacterial species have been described previously by Clements *et al.* (1992) and a rationally attenuated strain of *Y. pestis* may overcome some of the problems encountered with existing vaccines, since the exact nature of the mutation is known and the degree of attenuation can thus be controlled (Sigwart *et al.*, 1989; MacFarland & Stocker, 1987).

The *aroA* gene has been sequenced from a range of bacterial species. This enabled degenerate PCR primers to be designed from regions of identity. This study found a high level of identity between the deduced amino acid

M - - L E S L T L O P I A L V N G T V N L P G S K S V S N R A L L A A L A E G T T Q L N N V L D S I	Y. pestis
M - - L E S L T L H P I A L I N G T V N L P G S K S V S N R A L L A A L A E G T T Q L N N L D S I	Y. enterocolitica
M - - E S L T L Q P I A R V D G T I N L P G S K S V S N R A L L A A L A H G K T V L T N L L D S I	E. coli
M - - E S L T L O P I A R V D G T I N L P G S K S V S N R A L L A A L A P C G K T A L T N L L D S I	S. typhimurium
M S G L A Y L D L P A A R L A R G E V A L P G S K S I S N R V L L A A L A E G S T E I T G L L D S I	B. pertussis
DD I R H M L N A L Q A L G V D F R L S A D R T C C E V D G L G G K L V A E Q P L S L F L G N A G T	Y. pestis
DD I R H M L N A L Q A L G V K Y R L S A D R T R C E V D G L G G K L V A E Q P L E L F L G N A G T	Y. enterocolitica
DD V R H M L N A L T A L G V S Y T L S A D R T R C E I I G N G G P L H A E G A L E L F L G N A G T	E. coli
DD V R H M L N A L S A L G I N Y T L S A D R T R C D I T G N G G A L R A P G A L E L F L G N A G T	S. typhimurium
DD T R V M L A A L R Q L G V S V G E V A D G - - C V T I E G V A R F P T E Q A - E L F L G N A G T	B. pertussis
A M R P L A A V L C L G N S D I V L T G E P R M K E R P I G H L V D A L R Q G G A Q I D Y L E Q E N	Y. pestis
A M R P L A A A L C L G K N D I V L T G E P R M K E R P I G H L V D A L R Q G G A Q I D Y L E Q E N	Y. enterocolitica
A M R P L A A A L C L G S N D I V L T G E P R M K E R P I G H L V D A L R Q G G A K I T Y L E Q E N	E. coli
A M R P L A A A L C L G Q N E I V L T G E P R M K E R P I G H L V D S L R Q G G A N I D Y L E Q E N	S. typhimurium
A F R P L I T A A L A L M G G D Y R L S G V P R M H E R P T G D L V D A L R Q F G A G T E I Y L G Q A G	B. pertussis
Y P P L R L R G G F R - - G G E L T V D G R V S S Q F L T A L L M T A P L - - - A E Q D T Y I R I	Y. pestis
Y R R - C T A G G F R - - G G K L T V D G S V S S Q F L T A L L M T A P L - - - A E Q D T E I Q I	Y. enterocolitica
Y P P L R L Q G G F T - - G G N V D V D G S V S S Q F L T A L L M T A P L - - - A P E D T V I R I	E. coli
Y P P L R L R G G F T - - G G D I E V D G S V S S Q F L T A L L M T A P L - - - A P K D T I I R V	S. typhimurium
Y P P L R I G G G S I R V D G P V R V E G S V S S Q F L T A L L M A I A P V L A R R S G O D I T I E V	B. pertussis
M G D L V S K P Y I D I T L H L M K A F G I D V G H E N Y Q I F H I K G G Q T Y R S P G T Y L V E G	Y. pestis
Q G E L V S K P Y I D I T L H L M K A F G I D V V H E N Y Q I F H I K G G Q T Y R S P G T Y L V E G	Y. enterocolitica
K G D L V S K P Y I D I T L N L M K T F G V E I E N Q H Y Q Q F V V K G G Q S Y Q S P G T Y L V E G	E. coli
V G E L V S K P Y I D I T L N L M K T F G V E I A N H H Y Q Q F V V K G G Q Q Y H S P G R Y L V E G	S. typhimurium
V G E L T S K P Y I E T I T L N L M A R F G V S V I R R D G W R A F T T A R D A V Y R G P R M A I E G	B. pertussis
D A S S A S Y F L A A A A I K G G T V R V T G I G K K S V Q G D T K F A D V L E R M G A R V T W G D	Y. pestis
D A S S A S Y F L A A A A I K G G T V R V T G I G K Q S V Q G D T K F A D V L E K M G A K I S W G D	Y. enterocolitica
D A S S A S Y F L A A A A I K G G T V K V T G I G R N S M Q G D I R F A D V L E K M G A T I C W G D	E. coli
D A S S A S Y F L A A A I K G G T V K V T G I G R K S M Q G D I R F A D V L E K M G A T I T W G D	S. typhimurium
D A S T A S Y F L A L G A I G G G P V R V T G V G E D S I Q G D V A F A A T L A A M G A D V R Y G P	B. pertussis
D Y I E C S R G - - - - - E L Q G I D M D M N H I P D A A M T I A T T A L F A T G P T T Y R N I	Y. pestis
D Y I E C S R G - - - - - E L Q G I D M D M N H I P D A A M T I A T T A L F A D G P T V I R N I	Y. enterocolitica
D Y I S C T R G - - - - - E L N A I D M D M N H I P D A A M T I A T A L F A K G T T R L R N I	E. coli
D F I A C T R G - - - - - E L H A I D M D M N H I P D A A M T I A T A L F A K G T T T L R N I	S. typhimurium
G W I E - T R G V R V A E G G R L K A F D A D F N I L T P D A A M T A A T L A L Y A D G P C R L R N I	B. pertussis
Y N W R V K E T D R L T A M A T E L R K V G A E V E E G E D Y I R V V P P L Q - - L T A A D I G T Y	Y. pestis
Y N W R V K E T D R L S A M A T E L R K V G A E V E E G Q D Y I R V V P P A Q - - L T A A E I G T Y	Y. enterocolitica
Y N W R V K E T D R L F A M A T E L R K V G A E V E E G H D Y I R I T P P E K - - L N F A E I A T Y	E. coli
Y N W R V K E T D R L F A M A T E L R K V G A E V E E G H D Y I R I T P P A K - - L Q H A D I G T Y	S. typhimurium
G S W R V K E T D R I H A M H T E L E K L G A G V Q S G A D W L E V A P P E P G G W R D A H I G T W	B. pertussis
D D H R M A M C F S L V A L S D T P V T I L D P K C T A K T F P D Y F E Q F A R - - - - K	Y. pestis
N D H R M A M C F S L V A L S D T P V T I L D P K C T A K T F P D Y F E Q L A R L - S Q I A	Y. enterocolitica
N D H R M A M C F S L V A L S D T P V T I L D P K C T A K T F P D Y F E Q L A R I - S Q A A	E. coli
N D H R M A M C F S L V A L S D T P V T I L D P K C T A K T F P D Y F E Q L A R M - S - T P A	S. typhimurium
D D H R M A M C F L L A A F G P A A V R I L D P G C V S K T F P D Y F D V Y A G L L A A R D	B. pertussis

Fig. 2. Alignment of the deduced amino acid sequences encoded by *aroA* genes from *Y. pestis*, *Y. enterocolitica*, *E. coli*, *S. typhimurium* and *B. pertussis*. Amino acids identical to those of *Y. pestis* protein are boxed.

Fig. 3. Amplification of the *aroA* gene of *Y. pestis* GB (lane 1), *Y. pestis* GBΔ*aroA* (lane 2) and pGYPΔA7 (lane 3). The lengths of the bands on the gel are indicated in bp.

sequence from the *Y. pestis aroA* gene and those from other species (Fig. 2). The *aroA* gene in other species of the *Enterobacteriaceae* has been shown to form part of an operon with *serC*, which is located upstream of *aroA* (O'Gaora *et al.*, 1989; Duncan & Coggins, 1986). The nucleotide sequence upstream from the *aroA* gene was homologous to *serC* of *Y. enterocolitica* (data not shown). Inspection of the sequence of the intergenic region did not reveal any obvious promoters, suggesting that the *aroA* gene of *Y. pestis* is transcribed as part of an operon. A deletion was produced in the cloned fragment of the *aroA* gene and this was used to generate the *aro*-defective mutant strain of *Y. pestis*, GBΔ*aroA*. A single copy of the *aroA* gene containing the lesion was confirmed by PCR. This avoided the need for Southern blots as the DNA of *Y. pestis* GB is resistant to digestion due to DNA-methylation systems, including the *dam* and *dcm* systems found in other enterobacteria (Demidova *et al.*, 1984).

Previous work has evaluated the relative susceptibility of various animal models to identify a suitable model for the evaluation of candidate plague vaccines (Meyer *et al.*, 1974c). Although various nonhuman primates were considered to reflect most closely the disease in humans, mice and guinea-pigs were also identified as being susceptible to *Y. pestis* infection and able to produce an immune response upon vaccination. GB Δ aroA was attenuated in guinea-pigs *in vivo*. Only 1/15 animals appeared sick, there were no deaths and healthy animals appeared to have cleared the bacteria as there were no abscesses on their major internal organs. The induced immunity after a single low dose of the mutant protected against homologous challenge with a dose of 9.26×10^6 c.f.u. of the virulent wild-type strain and significant titres of specific anti-F1 and anti-V antibodies were detected in sera from immunized animals. High antibody titres to F1 antigen and V antigen have been shown to correlate with protection against plague (Meyer *et al.*, 1974b; Leary *et al.*, 1995; Motin *et al.*, 1994). Surprisingly, the aro-defective mutant was still virulent in mice, although the increased time to death of the mice would suggest that the rate of growth *in vivo* was reduced. The virulence of GB Δ aroA in mice contrasts with the behaviour of a Δ aroA mutant of *Y. enterocolitica*, which was not lethal in the mouse model (Bowe *et al.*, 1989). *Y. pestis* is known to possess additional genetic elements encoding a variety of virulence determinants, e.g. unlike *Y. enterocolitica*, *Y. pestis* produces a toxin which is active in mice. This may go some way towards explaining the observed host-dependent nature of the attenuation. The inability of the *Y. enterocolitica* Δ aroA mutant to cause disease was linked with the rapid clearance of the bacteria from the reticulo-endothelial system. It has been suggested that this may indicate an increased susceptibility of *Yersinia* to bacterial clearance mechanisms and that *Yersinia* may rely on rapid and overwhelming growth to kill mice (Bowe *et al.*, 1989). Our results provide a contrasting result; the LD₅₀ of GB Δ aroA was similar to that of the wild-type strain. The ability of the Δ aroA mutant of *Y. pestis* to grow *in vivo* also contrasts with results obtained with Δ aroA mutants of other bacterial species, but is supported by the observation that phenylalanine-dependent mutants of *Y. pestis* are not attenuated in mice (Burrows, 1960, 1963). A previous examination of host specificity of Brazilian isolates of *Y. pestis* found that low levels of free asparagine were responsible for a mouse-virulent strain being nonvirulent in guinea-pigs (Burrows & Gillet, 1971). A similar difference in aromatic amino acid levels may provide an explanation for the attenuation of the *Y. pestis* Δ aroA mutant in guinea-pigs.

The persistence, but inability to grow, in reticulo-endothelial tissues observed with *S. typhimurium* Δ aroA has been attributed to the inability of the bacteria to synthesize PABA and DHB, which are essential for growth. Our results show that growth of *S. typhimurium* Δ aroA ceased after subculturing through media lacking PABA and DHB, whereas the growth of the *Y. pestis* mutant was not limited in the same media. It is therefore possible that *Y. pestis* possesses an alternative biosynthetic

pathway which bypassed the mutation or alternative scavenging mechanisms for metabolites. In addition to preventing folate production, mutation in the *aroA* gene blocks the synthesis of the iron-chelating siderophore enterochelin in *S. typhimurium*. It is possible that after growth in defined media, starvation of PABA and DHB inhibited enterochelin synthesis in *S. typhimurium* SL3261 and therefore iron limitation impaired bacterial multiplication. *Y. pestis* possesses a complex system for scavenging iron, including iron chelation by the siderophore yersiniabactin and a haemin-uptake pathway (Straley & Perry, 1995). It would be expected that yersiniabactin synthesis is prevented in GB Δ aroA, as has been shown for the Δ aroA strain of *Y. enterocolitica* (Heesemann *et al.*, 1993). Although the mutation in the *aroA* gene may have prevented siderophore production in *Y. pestis*, the availability of alternative iron-uptake mechanisms may have been the factor that permitted growth of GB Δ aroA in the defined media by bypassing the blockage in the siderophore iron-transport system.

It has been known for some time that different virulence factors are important in different species; e.g. a classically attenuated mutant, *Y. pestis* EV76, which was classified as 'harmless' in guinea-pigs was subsequently shown to be virulent in nonhuman primates (Meyer *et al.*, 1974c). Our finding that a Δ aroA mutant is able to cause disease in one host animal species while being attenuated in another raises the possibility that Δ aro mutants of other bacteria may be virulent in alternative animal models.

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