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A *Yersinia pestis* *guaBA* mutant is attenuated in virulence and provides protection against plague in a mouse model of infection

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Abstract

There is a need to develop effective countermeasures for *Yersinia pestis*, the etiologic agent of plague and a potential bioterrorism agent. *Salmonella* and *Shigella* spp. deleted in the *guaBA* genes involved in guanine biosynthesis have been shown to be attenuated in vivo. In this study, we sought to determine whether deletion of the *guaBA* operon would render *Y. pestis* auxotrophic for guanine and avirulent; such a strain could serve as a live attenuated plague vaccine candidate. A *Y. pestis* *guaBA* mutant was generated by specific deletion of a segment of the *guaBA* operon, producing a guanine auxotroph that was highly attenuated in a mouse model of *Y. pestis* infection. Furthermore, mice vaccinated with a single dose of 7×10^4 CFU via the intravenous route were fully protected against subsequent lethal challenge with the *Y. pestis* parental strain. These findings identify *guaBA* as a target for deletion to generate a live attenuated plague vaccine.

Keywords

Yersinia pestis; plague; guanine; mutant; live attenuated vaccines

1 Introduction

Yersinia pestis is the etiologic agent of bubonic plague, a zoonotic infection transmitted to humans from natural animal reservoirs usually via the bite of an infected flea. Bubonic plague can develop into the pneumonic form of the disease, which is highly infectious by airborne transmission of the pathogen and has a high mortality rate [1]. *Y. pestis* is credited for millions of deaths worldwide and considered a potential bioterrorism agent [2]. A number of antibiotics are active against *Y. pestis*, but their successful use is dependant upon

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early intervention, particularly for the treatment of pneumonic plague [1], the likely consequence of a deliberate release. Plague vaccines with varying efficacies have been developed over the years, including killed and live attenuated organisms [3,4]. One of the best known, the live attenuated *Y. pestis* vaccine strain EV76, is not fully attenuated and poses major safety risks for use in humans [4], whilst the plague vaccine USP, consisting of formaldehyde-killed whole cell organisms, is relatively ineffective in providing protection against pneumonic plague [5]. Improved vaccines based upon the F1 and V protective antigens of *Y. pestis* have been described [6,7]. However, these vaccines may not be effective against respiratory-acquired disease and F1 antigen-negative strains of *Y. pestis* have been reported [8,9]. Overall, there is an urgent need for alternative and flexible medical countermeasures for plague, and a greater understanding of the virulence of *Y. pestis*.

We have previously constructed an *aroA* mutant of *Y. pestis* that was attenuated in the guinea-pig model of infection [10]. This work demonstrated that, like *Salmonella* [11] and *Shigella* [12], the aromatic metabolic pathway is required for full virulence of the pathogen. Other studies have shown that *Salmonella* spp. interrupted in the *guaA* and *guaB* genes encoding enzymes involved in guanine nucleotide biosynthesis were reduced in virulence [13]. A *Shigella flexneri* *guaBA* mutant is also attenuated *in vitro* and *in vivo* [14] and is safe and immunogenic in human volunteers [15,16]. More recently, *guaA* and *guaB* mutants of *Francisella tularensis* were reported to be attenuated *in vivo* [17]. In this study, we sought to determine whether deletion of the *guaBA* operon would render *Y. pestis* auxotrophic for guanine and attenuated for virulence. In the *Y. pestis* genome *guaB* and *guaA* are adjacent genes that form an operon governed by a single promoter upstream of *guaB* [18]. Herein we describe the construction and initial characterisation of a *Y. pestis* *guaBA* mutant generated by specific deletion of a segment of the *guaBA* operon.

2 Results and discussion

It has long been known that *Y. pestis* exhibits multiple auxotrophies (Reviewed in [19]), including strains with requirements for methionine, phenylalanine, proline, arginine and uracil. However, these strains retained full virulence, indicating that these compounds are freely available to the pathogen *in vivo*. Other auxotrophies were attenuating *in vivo*, such as the requirement for purines [20]. The effect of inactivation of guanine biosynthesis has not previously been determined for *Y. pestis*, although it is an attenuating mutation in other members of the Enterobacteriaceae [13,14,15,16]. Allelic replacement was used to generate a *Y. pestis* *guaBA* mutant, which contained a deletion spanning from the 3' end of the *guaB* gene to the 5' end of the *guaA* gene and including the intergenic space. The *guaBA* genes were replaced by a kanamycin-resistance cassette. Unlike wild-type *Y. pestis*, the *Y. pestis* *guaBA* mutant was unable to grow without supplementation with guanine, but was able to grow in the presence of guanine, confirming the phenotype of the mutant.

The *Y. pestis* *guaBA* mutant was evaluated for virulence in mice following subcutaneous and intravenous injection with ten-fold serial dilutions of bacterial culture. Even at the highest dose tested, 7×10^4 CFU, the *guaBA* mutant was not lethal by either route of infection, demonstrating a high level of attenuation. In similar conditions, the wild-type *Y. pestis* strain GB is known to have an MLD of 1 CFU by the subcutaneous route [4]. Thus, the *guaBA* deletion reduced the virulence of the parent *Y. pestis* strain by at least 10 000-fold. This compares favourably with the performance of an *aroA* mutant of the same strain, which retained virulence in mice [10]. Interestingly, this attenuating target was not identified when a signature-tagged mutant library was screened in mice by the subcutaneous route of infection [21], although other purine biosynthesis mutants were identified. An empirically derived purine auxotroph was unable to grow in macrophages [22], confirming that insufficient purines are available intracellularly to facilitate growth. Transcriptional profiling

revealed *guaBA* expression to be repressed when shifted from 26°C to 37°C *in vitro* [23]. Although purine biosynthesis was repressed, expression of the pyrimidine biosynthetic pathway was unaffected. F1 and V antigen-specific serum IgG antibodies were measured in mice that received the undiluted culture, the 10⁻² and 10⁻⁶ dilutions (Figure 1). The highest responses were seen in mice inoculated intravenously with the undiluted culture. The F1 mean titers increased with the dose, although the differences among group did not reach statistical significance. No trends were seen in mice immunized subcutaneously. F and V-specific antibodies were detected in mice that received the 10⁻⁶ dilution, likely induced by antigenic material in the culture media since no organisms were contained in this dilution. Antibodies to F1 and V antigen have been associated with protection and can serve as a measure of vaccine immunogenicity [24]

Historically, various live attenuated strains of *Y. pestis* have been used in humans in an attempt to prevent plague. It was known from early studies that an effective vaccine strain had to express the F1 and V antigen in order to induce a protective immune response. For example, immunisation with strain Tjiwidej, which does not produce V antigen, did not reduce the incidence of pneumonic plague, whereas strain EV, which does produce V antigen, was able to induce a protective immune response [19]. Sera from vaccinated individuals were able to protect passively immunised mice against plague. The EV series of strains has been most widely used for human vaccination, primarily in the former Soviet Union. These strains are descended from an attenuated strain isolated early in the 20th century. Although laboratory passage led to heterogeneity in the strains [25], the primary basis for attenuation was loss of the pigmentation locus, and the associated loss of an adjacent pathogenicity island. There were several problems associated with using such live vaccines. The immune response varied between individuals, and some vaccinees failed to respond, even to multiple inoculations. In addition the side-effects associated with the use of such strains could be severe, even to the point of a significant proportion of recipients requiring hospitalisation [25]. For these reasons, use of live attenuated strains of *Y. pestis* declined in the 1960s. However, with the advent of modern molecular techniques, the production of defined, well-characterised live attenuated mutants may be able to produce attenuated strains with appropriate safety and immunogenicity profiles, offering an alternative approach to the use of defined sub-units as vaccines [26,27]. Such an approach may offer increased flexibility in protecting against genetic variants of *Y. pestis*. To determine the ability of the *Y. pestis guaBA* mutant to provide protection against subsequent infection with virulent *Y. pestis*, the mice inoculated intravenously or subcutaneously with the mutant were subjected to challenge with approximately 66 MLD of the wild-type *Y. pestis* strain GB (Figure 2). The *Y. pestis guaBA* mutant afforded high levels of protection following administration by the intravenous route (Figure 2A). Particularly, inoculation with 7 × 10⁴ CFU provided 100% protection against *Y. pestis* infection. Some of these protected mice did, however, develop small abscesses visible on their spleens following their removal post-mortem (Table). In comparison, subcutaneous administration of *Y. pestis guaBA* afforded lower levels of protection against subsequent infection with virulent *Y. pestis*. This could be due to further attenuation of the mutant resulting from subcutaneous administration or higher efficiency of the intravenous immunization. A live attenuated vaccine for plague would likely require multiple deletions and would need to be administered via a route other than intravenous for practical delivery. With regards to the abscesses observed in some of surviving mice after challenge, while we cannot be certain that administration of the *guaBA* mutant did not contribute to the formation of these abscesses, this is considered unlikely given the length of time that passed between administration of the attenuated mutant and the removal of spleens. Against this background, our findings are still highly relevant as they identify *guaBA* as a candidate target for deletion in *Y. pestis* to generate a live attenuated plague vaccine.

3 Conclusions

This study has constructed and evaluated a *Y. pestis guaBA* that is highly attenuated *in vivo* and affords high levels of protection against subsequent infection with virulent *Y. pestis*. This data supports *guaBA* as a target for live attenuated vaccines for bacterial infections including plague.

4 Materials and Methods

4.1 Bacterial strains, plasmids, growth conditions and chemicals

Unless otherwise stated the chemicals were purchased from Sigma-Aldrich (Poole, United Kingdom) and the isolation of DNA, DNA digestions, ligations and transformations into *E. coli* were using standard procedures [28] using enzymes provided by Promega Ltd (Southampton, United Kingdom), Roche (United Kingdom) or Amersham Pharmacia (United Kingdom). *E. coli* was cultured in LB broth or on LB agar at 37°C. *Y. pestis* was routinely cultured at 28°C in Blood Agar Base (BAB) broth, on BAB agar [10], or on *Yersinia* selective agar (YSA; Oxoid, Basingstoke, United Kingdom). Media was supplemented with antibiotics kanamycin or ampicillin, where required, to final concentrations of 50 µg/ml or 55 µg/ml and 2-aminopurine was added to growth media to final concentrations of 200 µg/ml to 400 µg/ml. Media was supplemented, as needed, with bile salts and sodium deoxycholate to a final concentration of 1 % w/v. TMH medium was used for evaluation of the guanine-requirement of *Y. pestis*. To prepare 500 ml TMH medium, 250 ml 2X stable amino acids (5 mM DL-alanine, 2 mM L-isoleucine, 2 mM L-valine, 2 mM phenylalanine, 2 mM tyrosine, 10 mM DL-threonine, 2 mM L-arginine, 10 mM L-proline, 10 mM L-glutamate, 2 mM L-lysine, 10 mM glycine, 2 mM L-histidine, 4 mM DL-aspartate, 10 mM L-serine) was mixed with 50 ml 10X salt solution (50 mM K₂HPO₄, 100 mM citric acid, 100 mM NH₄Cl, 1 mM FeCl₂, 0.1 mM MnCl₂), 10 ml 1M MgCl₂, and 100 ml dH₂O, then sterilised by autoclaving before addition of 12.5 ml 1M HEPES and 5M NaOH to pH7.4. Finally, 50 ml 10X vitamin solution (5ml 1M D-gluconate, 5 ml 10 mM tryptophan, 10 ml 125 mM L-asparagine, 2.5 ml 200 mM L-glutamine, 5 ml 250 mM Na₂S₂O₃, 5 ml 100 mM L-methionine, 17.5 ml dH₂O). Plasmids were introduced into *E. coli* by electroporation [29] and into *Y. pestis* by conjugation [10] with *E. coli* 19851 as the donor strain.

4.2 Construction of a *guaBA* suicide plasmid

Two DNA segments of the *Y. pestis guaBA* operon, identified in the *Y. pestis* genome [18], were amplified using the PCR. The PCR primers (restriction sites underlined) yps36 (GCCTCTAGATCATTCCCA CTCAATCGTTGC) and yps37k (GATGTTTCGCACTAGTCCATACGACATGCTGTACCGCC) were used to amplify the 5' terminus of the *guaBA* allele and primers yps38k (GTCGTATGGACTAGTGCGAACATCACGGCCAGTGATG) and yps39 (GGCCTCGAGATGCTACGTATCGCGAAAGAAGC) were used to amplify the 3' terminus of the *guaBA* allele. An overlapping PCR was subsequently performed using primers yps36 and yps39, resulting in a fragment complementary to the 5' end (codons 1-140) of the *guaB* gene and the 3' end (codons 394-525) of the *guaA* gene, but lacking codons 141-347 of *guaB*, the intergenic region between *guaB* and *guaA*, and codons 1-393 of *guaA*. In addition, a *SpeI* site was introduced at the junction between the two fused PCR products. The amplified DNA fragment, Δ *guaBA*, was digested using the unique restriction sites that were included in the external primers, *XbaI* and *XhoI*, and subsequently ligated into suicide vector pDM4, which confers sucrose sensitivity [30], that had been digested with *XbaI* and *XhoI*, yielding pSL144. Next, a kanamycin-resistance cassette was introduced into the *SpeI* site of pSL144. To accomplish this, pACYC177 (New England Biolabs) was

PCR amplified using the kana 3 (GCCACTAGTAGCGCTGAGGTCTGCCTCGTG) and kana 4 (GCCACTAGTTTAGAAAACTCATCGAGCATC) primers. The PCR product was digested with *Spe*I and cloned into pSL144, thereby yielding pSL148.

4.3 Construction of a *Y. pestis* *guaBA* mutant

Plasmid pSL148 was electroporated into *E. coli* 19851 cells and introduced to *Y. pestis* strain GB by conjugation in a three-way mating [31] with *E. coli* λ pir pNJ5000 as the helper strain [32]. Briefly, 50 μ l of each strain was mixed and 100 μ l was spotted onto an LB agar plate and incubated at 28°C for 5 hours. The bacteria were then re-suspended in 1 ml of LB broth and plated onto YSA supplemented with chloramphenicol and kanamycin for 48 h at 28°C. Colonies were picked into BAB broth, grown overnight at 28°C, and plated onto BAB agar supplemented with kanamycin and 10 % (w/v) sucrose [10]. To screen for recombinant *Y. pestis* colonies, bacterial lysates were used as template DNA for PCR using primers to amplify the deleted *guaBA* DNA fragment and the kanamycin resistance cassette. A kanamycin-resistant and chloramphenicol-sensitive *Y. pestis* *guaBA* mutant was identified following plating on selective media and used for further studies.

4.4 Determination of requirement for guanine

To determine the guanine-dependence of the *Y. pestis* *guaBA* mutant, the bacteria were cultured statically at 28°C for 2 days in TMH medium with or without 0.001% guanine added, and growth was measured.

4.5 Determination of virulence in mice

The virulence of the *Y. pestis* *guaBA* mutant was assessed by subcutaneous and intravenous injection of groups of 5 female 6-week-old BALB/c mice (Charles River Laboratories) with ten-fold serial dilutions (6 dilutions starting at approximately 7×10^4 CFU) of exponential-phase broth culture grown at 28°C [4], and comparison to known virulence data for the corresponding wild-type *Y. pestis* strain GB [4].

4.6 F1 and V-specific IgG

Serum IgG against *Y. pestis* antigens were measured by ELISA. Immulon II plates were coated with F1 (0.4 μ g/ml in PBS) or V (1.25 μ g/ml in carbonate buffer) for 3 h at 37°C and blocked overnight with 10% dry milk in PBS. The wells were then washed with PBS containing 0.05% Tween 20 (PBST) and serum samples (diluted 2-fold in PBST-milk) added for 1 h at 37°C. HRP-labelled anti-mouse IgG (KPL) was used as conjugated and TMB Peroxidase (KPL) as substrate. After 15 min of incubation, the reaction was stopped by the addition of 1 M H_3PO_4 and absorbances at 450nm were measured. Sera were run in duplicate along with negative and positive calibrated controls. Titers were calculated from regression curves as the inverse of the dilution that produces an absorbance value of 0.2 above the blanks. Results are reported in ELISA Units/ml.

4.7 Protection against virulent *Y. pestis* challenge

In order to determine the protective efficacy of the *guaBA* mutant against wild-type *Y. pestis* infection, the groups of 5 mice inoculated with the 10-fold dilutions of the mutant were subsequently challenged by subcutaneous injection with approximately 66 CFU (equivalent to 66 MLD [4] of *Y. pestis* GB on day 49. Survival was monitored for 14 days. Humane endpoints were strictly observed and animals deemed incapable of survival were euthanized by cervical dislocation. On day 63, survivors were euthanized and spleens were removed for observation.

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Figure 2. Protection conferred by increasing doses of a *Y. pestis guaBA* mutant against virulent *Y. pestis* challenge

Mice were inoculated intravenously (A) or subcutaneously (B) with with ten-fold serial dilutions of *Y. pestis guaBA* as described in Figure 1. Forty nine days after immunization, all mice were challenged subcutaneously with 66 MLD of the parent virulent strain *Y. pestis* GB. Data represent survival curves during the 14 day monitoring period; curves are shown divided in two panels for better visualization.

Table

Presence of abscesses on spleens taken from surviving mice inoculated intravenously with *Y. pestis guaBA* and challenged with virulent *Y. pestis* GB

<i>Y. pestis guaBA</i> inoculum	Number of challenge survivors/total number	Presence of abscesses
Undiluted original culture (approx. 7×10^4 CFU)	5/5	Some spleens with small abscesses
10^{-1} dilution	3/5	All spleens with abscesses
10^{-2} dilution	2/5	Both spleens with abscesses
10^{-4} dilution	1/5	Spleen with large abscess