Expression of heterologous O-antigen in *Yersinia pestis* KIM does not affect virulence by the intravenous route

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All strains of *Yersinia pestis* examined have been found to lack an O-antigen. In other members of the *Enterobacteriaceae*, the rough phenotype often results in attenuation. However, *Y. pestis* is the aetiological agent of bubonic plague. In evolving from the ancestral enteropathogenic *Yersinia pseudotuberculosis*, and with the development of an arthropod-vector system, smooth LPS production is not necessary for *Y. pestis* virulence and the metabolic burden has been alleviated by inactivation of the O-antigen biosynthetic operon. To investigate this, *Y. pestis* strain KIM D27 was transformed with a plasmid carrying the operon encoding the O-antigen of *Yersinia enterocolitica* O:3. Expression of the O-antigen could be detected in silver-stained gels. The receptor for bacteriophage ϕ YeO3-12 has been shown to be O-antigen, and infection by this bacteriophage results in lysis of *Y. enterocolitica* O:3. Expression of the O-antigen in *Y. pestis* conferred sensitivity to lysis by ϕ YeO3-12. The O-antigen-expressing clone was shown to be as virulent in mice by the intravenous route of challenge as the rough wild-type. Assays showed no alteration in the ability of *Y. pestis* to resist lysis by cationic antimicrobial peptides, serum or polymyxin.

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INTRODUCTION

LPS is the major component of the Gram-negative outer membrane and the important role it plays in bacterial survival has been well documented (Reeves, 1994; Bayston & Cohen, 1990). *Yersinia pestis*, the causative agent of bubonic and pneumonic plague (Perry & Fetherston, 1997), is unusual when compared with other pathogenic members of the *Enterobacteriaceae* in that it does not possess smooth LPS; instead, its outer membrane is composed mainly of lipo-oligosaccharide (LOS). The LPS of the other pathogenic *Yersinia* species, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, have been shown to contain an O-antigen essential for virulence, the expression of which is regulated by temperature (al-Hendy *et al.*, 1992; Zhang *et al.*, 1997; Karlyshev *et al.*, 2001; Mecsas *et al.*, 2001). The genetic basis for the lack of O-antigen in *Y. pestis* has been shown to be the presence of five pseudogenes in the gene cluster directing the biosynthesis of O-antigen (Skurnik *et al.*, 2000; Prior *et al.*, 2001). In agreement with the genetic data, LOS isolated from *Y. pestis* has been shown to lack O-antigen (Prior *et al.*, 2001; Hitchen *et al.*, 2002). Characterization of the *Y. pestis* LOS showed the core to possess two distinct molecular species of LOS, differing in terminal galactose or

heptose (Hitchen *et al.*, 2002; Vinogradov *et al.*, 2002), and to contain Kdo, like other LPS molecules that have been studied (Ellwood, 1960; Hartley *et al.*, 1974). The ability of Gram-negative bacteria to avoid serum-mediated killing would normally be facilitated by complement binding to the O-antigen of LPS. In other pathogenic bacteria that possess LOS, such as *Neisseria* and *Haemophilus* species, the LOS is sialylated, which confers serum resistance on the bacteria (Mandrell *et al.*, 1992; Vogel *et al.*, 1999), possibly by masking sugar residues that are reactive with the C3 component of complement (Estabrook *et al.*, 1997). The role of LOS in the ability of *Y. pestis* to avoid serum-mediated killing is not immediately apparent from the structure reported (Hitchen *et al.*, 2002), since it lacks both an O-antigen and a sialylated core.

Y. pestis is considered to have evolved from *Y. pseudotuberculosis* serotype O:1b 1500–20 000 years ago (Achtman *et al.*, 1999; Skurnik *et al.*, 2000). *Y. pseudotuberculosis* is a gastrointestinal pathogen and possesses smooth LPS with an O-antigen, which is an essential virulence determinant (Karlyshev *et al.*, 2001). All strains of *Y. pestis* examined to date are rough. This implies that possession of an O-antigen is somehow detrimental to the lifestyle of the flea-transmitted systemic pathogen. However, *Y. pestis* is known to infect by the oral route, indicating that the loss of O-antigen

Abbreviations: LOS, lipo-oligosaccharide; MLD, median lethal dose.

is not linked directly to the change in lifestyle of the pathogen (al-Hendy *et al.*, 1991). It is possible that the biochemical burden placed on the organism as a result of O-antigen expression would be disadvantageous. Alternatively, expression of an O-antigen may interfere with the action of surface-exposed proteins required for virulence and, thus, O-antigen expression would be attenuating *in vivo*.

In this study, we have expressed an O-antigen in *Y. pestis* in order to determine whether the recombinant organism is attenuated for systemic virulence.

METHODS

Bacterial strains, plasmids, chemicals and growth conditions.

The strains used in this study are listed in Table 1. *Y. pestis* was routinely cultured on Blood base II (BAB) agar (Oxoid; 40 g l⁻¹) containing 0.25% (w/v) haemin or in BAB broth. *Y. enterocolitica* O:3 and *Escherichia coli* were grown on Luria-Bertani (LB) medium. Ampicillin and tetracycline were used at respective final concentrations of 55 and 20 µg ml⁻¹ as required. All chemicals were purchased from Sigma-Aldrich. Unless otherwise stated, plasmid extractions, restriction enzyme digestions, DNA ligations and transformations were performed by standard procedures (Sambrook *et al.*, 1989) using enzymes provided by Roche.

Plasmid pAY100 (Table 1) carried ampicillin- and tetracycline-resistance markers and the O-antigen operon of *Y. enterocolitica* 6471/76-c O:3 (al-Hendy *et al.*, 1991). As tetracycline is an antibiotic used in therapy of plague, the tetracycline-resistance marker was inactivated by digestion with *Sall* and *Bam*HI and filling of the generated ends with the Klenow fragment of DNA polymerase I (Sambrook *et al.*, 1989). The blunt ends were then religated and the plasmid transformed in *E. coli* JM109. Ampicillin-resistant colonies were screened for tetracycline sensitivity. The isolated plasmid was designated pAY100.1.

PCR for confirmation of the presence of pAY100.1. Oligonucleotide primers were designed from the sequence of pBR322 (JP3, 5'-AAATTGCTAACGCAGTCAGG; JP4, 5'-TGGAGTGGTGAATCCGT TAG) and the *wbbX* gene (JP1, 5'-ATGTTGGCAGCAATAATG; JP2, 5'-TAGCGCTTCCATTACAGC) (GenBank accession no. Z18920) and synthesized by Cruachem Ltd. The PCR was performed for 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min.

Gel electrophoresis, silver staining and proteinase K minipreparations. Glycine gel electrophoresis was performed with

the buffers of Laemmli (1970) using a 12.5% separating gel with a 4.5% stacking gel. Aliquots (10 µl) of each sample were electrophoresed for approx. 2 h at 100 mV in the Mini-protein II slab system (Bio-Rad). Proteinase K minipreparations of LPS were produced and gels were silver-stained according to the method of Chart (1994).

Bacteriophage lysis assays. Bacteriophage ϕ YeO3-12 (Pajunen *et al.*, 2000, 2001) was propagated by adding a small volume of bacteriophage to 100 µl of an overnight culture of *Y. enterocolitica* O:3 diluted in 5 ml fresh broth for 3 h at room temperature and filtered with a 0.22 µm filter. The filtrate contained the bacteriophages, which were stable for several months when stored at 4 °C.

E. coli and *Y. enterocolitica* strains were cultured overnight and serially diluted in sterile PBS (pH 7.4). To 100 µl aliquots of each diluted culture was added 10 µl of a suspension of 1×10^6 p.f.u. bacteriophage ml⁻¹ and 4 ml molten top agar (tryptone soy agar solidified with 0.7% agarose; Oxoid) supplemented with antibiotic as required. This was poured onto BAB plates and incubated at ambient temperature overnight. The plates were examined for the development of plaques. Alternatively, the bacteria were mixed with top agar, poured onto the plate and 10 µl undiluted bacteriophage was then dropped onto the centre of the plate.

For lysis experiments using *Y. pestis*, bacteria were grown on fresh LB agar containing 100 µg ampicillin ml⁻¹. A single colony was used to inoculate 5 ml tryptone soy broth (TSB) supplemented with 100 µg ampicillin ml⁻¹ in a 15 ml Falcon tube, which was incubated at 26 °C for 30 h with gentle rocking to obtain a culture in middle to late exponential phase. A 150 µl aliquot of the culture was mixed with 3 ml molten soft agar (TSB supplemented with 133 µg ampicillin ml⁻¹, solidified with 0.4% Bacto agar, cooled to 50 °C) and poured onto lambda agar plates (10 g Bacto tryptone l⁻¹, 2.5 g NaCl l⁻¹, 15 g Bacto agar l⁻¹ and 150 µg ampicillin ml⁻¹). The plates were incubated at 26 °C for 90 min and 20 µl aliquots of dilutions of bacteriophage ϕ YeO3-12 stock were then pipetted onto the surface. The plates were incubated overnight at 26 °C and examined for plaque formation.

Determination of virulence in mice. The median lethal doses (MLD) of *Y. pestis* carrying either pBR322 or pAY100.1 were assessed by intravenous challenge of groups of six female 6-week-old BALB/c mice (Charles River Laboratories) with serial dilutions of exponential phase broth cultures grown at 28 °C. The MLD was determined by the method of Reed & Muench (1938). Prior to challenge and daily after that, the mice were dosed subcutaneously with 50 µl 100 mg ampicillin ml⁻¹ (Intervet) to stabilize the plasmids. Humane end-points were strictly observed and animals deemed incapable of survival were killed humanely by cervical dislocation. Spleens were removed from all culled

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Comments	Reference
Strain		
<i>Y. pestis</i> KIM D27	Lcr ⁺ , pgm ⁻ , pst ⁺	Garcia <i>et al.</i> (1999)
<i>E. coli</i> JM109	Cloning strain	
<i>Y. enterocolitica</i> 6471/76-c	Serotype O:3	Skurnik <i>et al.</i> (1984)
Bacteriophage ϕ YeO3-12		al-Hendy <i>et al.</i> (1991)
Plasmid		
pAY100	pBR322 carrying the O-antigen operon of <i>Y. enterocolitica</i> 6471/76-c O:3; amp ^r , tet ^r	al-Hendy <i>et al.</i> (1991)
pAY100.1	Derivative of pAY100 with the tetracycline-resistance marker inactivated; amp ^r	This study
pBR322	Cloning vector; amp ^r , tet ^r	Bolivar <i>et al.</i> (1977)

and some dead mice, homogenized in BAB broth and plated on BAB with and without supplementation with ampicillin to confirm maintenance of plasmids.

Determination of MICs. *Y. pestis* KIM carrying either pBR322 or pAY100.1 was grown in BAB broth statically at 28 °C for 18 h followed by 4 h at 37 °C. Cultures were diluted 1 : 100 in fresh BAB broth for MIC assays using polymyxin B, cecropin P1 and mastoparan at final concentrations of 100–0.78 µg ml⁻¹ and guinea pig serum at 10–0.078 % (v/v) (Sigma) in 96-well polypropylene microtitre plates. The MIC was determined as the lowest concentration of the compound that did not allow visible growth after 24 h at 28 °C. These assays were performed in triplicate and significance was determined by Student's *t*-test in Microsoft Excel.

RESULTS

Expression of LPS in *Y. pestis*

Y. pestis KIM was transformed with plasmid pAY100.1, expressing the O-antigen from *Y. enterocolitica* O:3. Transformants were selected on BAB/ampicillin and the presence of the plasmid was confirmed by PCR. Expression of the O-antigen in *E. coli* JM109 and *Y. enterocolitica* O:3 allows infection by the bacteriophage ϕ YeO3-12 and subsequent lysis, which can be observed as plaque formation in bacterial lawns. Plaques were also observed when the bacteriophage plaque assay was performed with *Y. pestis* KIM/pAY100.1. Proteinase K minipreparations were prepared from *Y. enterocolitica* O:3 or *Y. pestis* KIM carrying either pBR322 or pAY100.1 and these were separated by SDS-PAGE and visualized by silver staining (Fig. 1). The preparation from *Y. enterocolitica* showed an O-antigen smear rather than a typical LPS ladder, which was also observed for the *Y. pestis* KIM/pAY100.1 sample. This smear is typically produced by this O-antigen, which does not form the ladders seen after electrophoresis of LPS preparations from other bacteria (Skurnik *et al.*, 1995, 1999). The profile of *Y. pestis* KIM/pBR322 did not show the O-antigen smear. In some of the preparations (Fig. 1; lanes 1 and 2), there appeared to be

Fig. 1. SDS-PAGE analysis of LPS isolated from *Y. enterocolitica* O:3 (lane 1), *Y. pestis* KIM/pAY100.1 (2) and *Y. pestis* KIM/pBR322 (3).

some resistant protein bands present, and these were not removed even after prolonged proteinase K digestion. The extract from *Y. pestis* KIM/pBR322 (Fig. 1; lane 3) shows the typical profile of *Y. pestis*, with no O-antigen being expressed while the lipid A and core bands are visible.

Effect of O-antigen expression on virulence in the mouse model of infection

The effect of expressing the O-antigen in *Y. pestis* on the virulence of the organism was evaluated. *Y. pestis* KIM is capable of killing mice when administered by the intravenous route. The MLDs of the recombinant *Y. pestis* KIM/pAY100.1 and the control strain of *Y. pestis* KIM/pBR322 were compared. To stabilize the plasmid *in vivo*, mice were dosed subcutaneously with veterinary-grade ampicillin for the course of the experiment. The MLD for *Y. pestis* KIM/pBR322 was calculated to be 199.78 c.f.u., while that for *Y. pestis* KIM/pAY100.1 was calculated as 221 c.f.u. Plating of spleen homogenates taken from mice that had died showed that the plasmids had been stably maintained in *Y. pestis* KIM.

Effect of O-antigen expression on resistance of *Y. pestis* to lysis

The effect of a range of surface-active compounds was evaluated against *Y. pestis* expressing the O-antigen versus the wild-type control. The MIC of polymyxin against both strains was 250 µg ml⁻¹ (Fig. 2a). Absolute inhibition of growth was not achieved with either of the cationic antimicrobial peptides, cecropin and mastoparan, although, at higher concentrations, there was some inhibition of growth (Fig. 2b). There was no difference in the susceptibility of *Y. pestis* KIM carrying plasmids pAY100.1 or pBR322 to these peptides. *Y. pestis* KIM/pBR322 was unable to grow in the presence of 0.3125 % guinea pig serum, was partially inhibited at one dilution step lower and was resistant to 0.078 % serum (Fig. 2c). The expression of the O-antigen by *Y. pestis* did not result in any difference in sensitivity to serum killing.

DISCUSSION

We have expressed the O-antigen of *Y. enterocolitica* in *Y. pestis* strain KIM. Expression of the O-antigen was proven by observation of the O-antigen smear observed after SDS-PAGE analysis, which is the expected profile of this O-antigen (Ogasawara *et al.*, 1985). The undigested protein bands observed in some of the samples have been reported previously for *Y. enterocolitica* O:3 (al-Hendy *et al.*, 1992). Bacteriophage ϕ YeO3-12 was able to lyse *Y. pestis* expressing the O-antigen, showing that the O-antigen was expressed by *Y. pestis* and was exposed correctly to the phage, allowing initiation of phage infection.

Many virulence factors have been described for *Y. pestis* (Perry & Fetherston, 1997), but one of the surprising aspects of this pathogen is that it does not possess an O-antigen, its

Fig. 2. Effects of polymyxin (a), cationic antimicrobial peptides (b) and serum (c) on growth of *Y. pestis* KIM with and without O-antigen expression. A BAB broth control (◆) was included in each experiment. (a) Growth of *Y. pestis* KIM/pBR322 in the absence (□) and presence (■) of polymyxin and *Y. pestis* KIM/pAY100.1 in the absence (△) and presence (▲) of polymyxin. (b) Growth of *Y. pestis* KIM/pBR322 alone (□) and in the presence of mastoparan (○) or cecropin (■) and *Y. pestis* KIM/pAY100.1 alone (△) and in the presence of mastoparan (●) or cecropin (▲). (c) Growth of *Y. pestis* KIM/pBR322 in the absence (□) and presence (■) of guinea pig serum and *Y. pestis* KIM/pAY100.1 in the absence (△) and presence (▲) of guinea pig serum.

outer membrane instead containing LOS (Hitchen *et al.*, 2002). *Y. pestis* is the only species of the pathogenic *Yersinia* to lack an O-antigen (Brubaker, 1991). The LPS O-antigen has been shown to be essential for virulence of other pathogenic *Yersinia* species (al-Hendy *et al.*, 1992; Zhang *et al.*, 1997; Karlyshev *et al.*, 2001; Mecsas *et al.*, 2001) as well as for other members of the *Enterobacteriaceae*, as it is required for resistance to complement lysis, cationic antibacterial

peptides and phagocytosis (Porat *et al.*, 1987; Makela *et al.*, 1988). In *Y. enterocolitica* O:3, the O-antigen is required for resistance to killing by the alternative complement pathway (Wachter & Brade, 1989). A rough mutant of *Y. enterocolitica* O:3 was resistant to bacteriophage ϕ YeO3-12 lysis and showed reduced virulence in mice (al-Hendy *et al.*, 1992). The expression of an O-antigen in *Y. pestis* KIM had no significant effect on virulence following intravenous challenge of mice. Virulence was neither enhanced, which would have indicated a role *in vivo*, nor reduced, which would have been an indication of metabolic burden.

In *Klebsiella pneumoniae*, loss of LPS O-antigen side-chain expression had no effect on the ability of the pathogen to cause pneumonia (Cortes *et al.*, 2002). Rather, the capsule was shown to play a key role in resistance to complement deposition and alveolar macrophage killing. *Y. pestis* possesses a capsule composed of F1-antigen (Baker *et al.*, 1952), which is a 17 kDa protein expressed at 37 °C but not at lower temperatures (Bennett & Tornabene, 1974). However, possession of the F1-antigen capsule does not apparently act to compensate for lack of an O-antigen on LPS, as F1-defective strains are still virulent (Welkos *et al.*, 1995). Alternative mechanisms for the avoidance of complement lysis by *Y. pestis* have been proposed based on degradation of the membrane attack complex. *Y. pestis* expresses Pla, a plasminogen activator at 37 °C with coagulase activity at lower temperatures (McDonough & Falkow, 1989). The Pla protein is capable of degrading proteins other than plasminogen, including C3, but inactivating the *pla* gene has variable effects on virulence and the role of Pla is still undetermined to a large extent (Perry & Fetherston, 1997). LPS isolated from the surface of *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* and incorporated into liposomes rendered the liposomes resistant to complement lysis (Porat *et al.*, 1995). This may mimic the effect seen in *Neisseria gonorrhoeae*, where rough LPS confers resistance to complement lysis by aberrant deposition of the membrane attack complex (Joiner *et al.*, 1983). However, this effect was not confirmed by other studies, which suggested that the O-antigen of *Y. enterocolitica* did indeed play a role in resistance to complement-mediated killing (Wachter & Brade, 1989; al-Hendy *et al.*, 1992; Skurnik & Toivanen, 1993).

Not only does *Y. pestis* cause bubonic plague, resulting from the bite of an infected flea, it also causes pneumonic plague following aerosol transmission, and orally acquired infections have been reported both in humans (Christie *et al.*, 1980) and, experimentally, in mice (Butler *et al.*, 1982; Sebbane *et al.*, 2001). It has been postulated that the O-antigen may be associated with functions related to the oral route of infection that may be irrelevant in the case of vector-mediated transmission (al-Hendy *et al.*, 1992). For example, in *Vibrio cholerae*, LPS is required for colonization of the small intestine (Nesper *et al.*, 2001). However, the KIM strain used in this study is virulent only by the intravenous route and not by other peripheral routes (Une & Brubaker, 1984), so it was not possible to determine directly whether possession of an O-antigen had an effect on oral virulence.

In *Salmonella enterica* var. Typhimurium, loss of O-antigen increased susceptibility to the peptide magainin 2, although not to the defensin NP-1 (Groisman, 1994). Resistance to cationic antimicrobial peptides is complex, and *Y. pestis* is highly resistant to those that have been examined (Bengoechea *et al.*, 1998; Hitchen *et al.*, 2002). Expression of the O-antigen did not appear to affect the charge of the bacterial surface in an adverse way, as it did not render the strain susceptible to lysis by cationic antimicrobial peptides.

In conclusion, *Y. pestis* appears to compensate for loss of the O-antigen so that it is highly resistant to many of the stresses and threats that it may encounter. Expression of the O-antigen did not improve the pathogen's ability to resist lysis by serum and had no impact on virulence by the intravenous route. Non-expression of O-antigen would represent a huge saving in energy, as synthesis of such a major macromolecule would be a significant biochemical burden. In addition, an added benefit would be the loss of a molecule used as a receptor by many bacteriophages. However, it must be remembered when interpreting the results that the O-antigen used in this study is different from that which the progenitor strain of *Y. pestis* would have expressed, and the effect of O-antigen expression may be affected by variations in the O-antigen structure.

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