Adhesive properties conferred by the plasminogen activator of *Yersinia pestis*

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A genomic library of *Yersinia pestis* EV76c created in a cosmid vector was screened for clones capable of binding type IV collagen. An unexpectedly high number of such clones was observed. One recombinant plasmid was selected for further study, and the locus controlling collagen binding was mapped by subcloning, transposon mutagenesis and exonuclease digestion. The outer-membrane protein profiles of transposon insertion mutants were correlated with phenotype to implicate a 36 kDa polypeptide in type IV collagen binding. Fine substructure restriction mapping and limited DNA sequence analysis showed the cloned locus to be identical to the locus (*pla*) for the plasminogen activator, previously characterized genetically and biochemically. The *pla* locus is resident on a 9.5 kb plasmid in wild-type *Y. pestis* strains. Curing of this plasmid resulted in negligible reduction in collagen-binding capacity, implying the existence of a chromosomally located determinant for collagen binding. The affinity of the plasminogen activator for collagen was relatively weak. When the cloned *pla* locus was introduced into *E. coli*, it conferred upon the cell the ability to bind to cells from a number of cell lines. Binding to glycolipids separated by thin-layer chromatography demonstrated that the receptor was a member of the globo-series of glycolipids. Since it has been reported that mutation of *pla* dramatically reduces virulence, we propose that this hitherto undescribed function of the gene product could contribute to the biological activities necessary for full virulence.

Introduction

The genus *Yersinia* contains three species pathogenic for man, *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* (Brubaker, 1991). All three species are capable of adhesion to and invasion of eukaryotic cells *in vitro* and *in vivo* (Portnoy *et al.*, 1981; Vesikari *et al.*, 1981). A common prerequisite for full pathogenesis is the virulence plasmid (Heesemann *et al.*, 1984; Portnoy *et al.*, 1983; Wolf-Watz *et al.*, 1985). This plasmid encodes a number of proteins, many of which have been directly implicated in virulence (Bölin *et al.*, 1982; Portnoy *et al.*, 1981, 1984). We have previously demonstrated that the major outer-membrane protein YADA (formerly YOP 1) is responsible for specific binding to several types of collagen (Emödy *et al.*, 1989). We also reported that *Y. pseudotuberculosis* has a second mechanism for collagen binding, presumably chromosomally encoded, since curing of the virulence plasmid was not sufficient to abolish collagen binding.

DNA hybridization studies have shown that *Y. pestis* and *Y. pseudotuberculosis* are extremely closely related, with an homology of 90% (Bercovier *et al.*, 1980). One of the incentives for the present study was therefore to determine if *Y. pestis* also has a chromosomal determinant for collagen binding. In contrast to other members of the genus, *Y. pestis* normally harbours two plasmids other than the virulence plasmid. The larger of these is 110 kb, and has been associated with the production of the murine toxin and Fraction 1 antigen (Protsenko *et al.*, 1983; Brubaker, 1984). The small 9.5 kb plasmid is known to encode at least three polypeptides, responsible for pesticin, pesticin immunity, plasminogen activation and weak coagulase activity (Beesley *et al.*, 1967; Brubaker & Surgalla, 1962; Brubaker *et al.*, 1965; Ferber & Brubaker, 1981; Sodeinde *et al.*, 1988). Plasmid-free strains lack all of these activities, and the ability to cause systemic infection following subcutaneous inoculation...
into guinea pigs or intraperitoneal injection into mice (Brubaker et al., 1965). The 9.5 kb plasmid of \textit{Y. pestis} carrying the locus (pla) encoding the plasminogen activator has recently been characterized at the molecular level (Sodeinde & Goguen, 1988, 1989; Sodeinde et al., 1988; McDonough & Falkow, 1989). The loci encoding the pesticin and pesticin immunity were carrying the locus (Brubaker tide with both plasminogen-activating and coagulase mapped, and a closely linked gene encoding a polypeptide with both plasminogen-activating and coagulase activity was also defined. It was proposed that proteolysis by the plasminogen activator (Pla) was responsible for the degradation of outer-membrane proteins encoded by the virulence plasmid when the bacterium is grown in vitro. Most recently, it has been reported that specific mutations inactivating the pla gene result in a 100000-fold increase in LD₅₀ values for \textit{Y. pestis} after subcutaneous inoculation in mice (Goguen et al., 1990).

In this paper, we present data showing that the pla locus, when cloned in \textit{Escherichia coli}, conferred the ability to bind to type IV collagen. Additionally, it allowed laboratory strains of \textit{E. coli} to adhere to eukaryotic cells grown in tissue culture. In an in vitro binding assay, it was shown that the Pla protein mediated binding to a range of glycolipids in which the globo-side series are dominant components. Binding to immobilized collagen IV and cultured cells could be substantially reduced by treatment with galactosidase, showing that these properties may be mediated by a lectin-like interaction with carbohydrate molecules covalently linked to the collagen molecule.

**Methods**

**Bacterial strains.** The \textit{Yersinia pestis} strain EV76c (Rosqvist et al., 1988) is a former vaccine strain lacking the large virulence plasmid. A derivative of this strain lacking the 9.5 kb plasmid encoding Pla was provided by H. Wolf-Watz, Department of Cell and Molecular Biology, University of Umeå, Sweden. \textit{Yersinia pseudotuberculosis} strain YPIII was described by Bölín et al. (1982). \textit{E. coli} strains TBl and HB101 were employed as hosts for recombinant plasmids (Yanisch-Perron et al., 1985; Bachmann, 1972). Culture conditions for \textit{Yersinia} were as previously described (Emödy et al., 1989).

**Recombinant DNA methodology.** Standard molecular cloning techniques were employed (Sambrook et al., 1989). Total genomic DNA of \textit{Y. pestis} was prepared according to the method of Hull et al. (1981). The cosmid vector pJB8 was described by Ish-Horowicz & Burke (1981). The plasmid vectors pUC18 and pUC19 (Yanisch-Perron et al., 1985) and pK18 and pK19 (Pridmore, 1987) were routinely employed. Sequence analysis was performed on denatured plasmid templates using T7 polymerase (US Biochemicals) in the Sanger chain termination technique. Primers consisted of the forward and reverse sequencing primers on each side of the multiple cloning site in the plasmid vector. Transposon mutagenesis by Tn5, using E467 as donor, was performed as described by Coleman & Foster (1981).

**Collagen binding assays.** Bovine type I collagen was from Collagen Corporation, Palo Alto, Calif., USA, and murine type IV collagen was from Collaborative Research Inc., Bedford, Mass, USA. A tube assay for collagen binding by bacterial cells was employed, according to standard protocols (Emödy et al., 1989). Specificity of type IV collagen binding in this assay was determined using a panel of serum proteins as previously described (Emödy et al., 1989). As an alternative, a quantitative solid phase assay was developed. Wells of standard ELISA plates (Nunc, Denmark) were coated by overnight incubation at 4°C with collagen at a concentration of 20 µg ml⁻¹. The plates were then washed three times with phosphate-buffered saline (PBS; 0.1 M-sodium phosphate, pH 7.2) and blocked with bovine serum albumin (BSA; 2% w/v, in PBS). A standardized bacterial suspension (10⁹ bacteria ml⁻¹) was prepared from cells grown overnight at 37°C on colonization factor antigen agar (CFA agar; Nowicki et al., 1986) and 0.1 ml of this suspension was added to the wells. After 90 min at 37°C the wells were washed repeatedly with PBS, then fixed by adding 2% (v/v) formalin in PBS for 1 min. Attached bacteria were stained for 20 min with crystal violet (0.13% crystal violet, 5% v/v, methanol in PBS). The plate was then washed three times with PBS, allowed to dry in air, and bound dye released by addition of 200 µl SDS (1%, w/v, SDS, 5% v/v, ethanol in PBS) per well, with 20 min incubation for full lysis. Absorbance at 595 nm was read by automated ELISA plate reader (LKB), and relative values calculated with reference to a positive parental strain. Alternatively, tritium-labelled bacterial cells could be used as described below for the cell adhesion assay.

**Solid phase assay for collagen binding.** Wells of microtitre plates (8-well strip plates, Costar) were coated with 100 µl of a solution of collagen IV (20 µg ml⁻¹ in PBS). The wells were then washed with PBS containing 0.2% (w/v) BSA, and blocked by incubation for 1 h with PBS containing 2% (w/v) BSA. A standard curve for coating efficiency was constructed, based upon quantification of radiolabelled collagen bound to the wells. Following blocking, wells were washed three times with PBS containing 0.2% BSA. Increasing amounts of Pla, radio-iodinated according to published procedures (Emödy et al., 1989), were added to a series of coated wells in a constant volume of 100 µl, and incubated for 1 h at ambient temperature. Wells were then washed five times with PBS containing 0.2% BSA. The amount of Pla protein bound was extrapolated from counts of retained radioactivity with reference to the specific activity of the labelled protein added. The background value was subtracted, based upon non-specific retention of Pla by wells coated with BSA.

**Cloning a \textit{Y. pestis} collagen receptor.** A gene library was constructed in the cosmid vector pJB8 by ligating EV76c DNA which had been partially digested with \textit{SacI} to vector DNA linearized with \textit{BamHI}. The genomic DNA digest was adjusted to yield products predominantly in the 20–40 kb range. The ligated DNA was packaged in vitro (Packagene vials, Promega), and transfected into strain HB101. The yield was 1 × 10⁵ recombinants per µg ligated DNA. Plates containing approximately 300 colonies were replicated onto nitrocellulose filters which were then treated as for normal colony blotting (Helfman et al., 1983). The filters were incubated in a solution of radiolabelled collagen IV, radio-iodinated as described previously (Emödy et al., 1989). After 2 h incubation, the filters were washed for three 10 min periods in blotting buffer, dried, and subjected to autoradiography. Colonies appearing positive in this assay were picked, and their phenotype was verified by both tube and solid-phase assays described above. One such recombinant was chosen for further study, and the cosmid it contained designated pC4001.

**Subcloning and localization of the collagen receptor.** From the original recombinant insert in pJB8 of approximately 23 kb, a variety of progressively smaller fragments carrying the putative receptor gene were subcloned, using collagen binding to monitor each new
An alternative method employed radiolabelled bacterial cells, grown overnight in LB broth incorporating appropriate antibiotic and [methyl-\(^3\)H]thymidine [20 μCi ml\(^{-1}\) (740 kBq ml\(^{-1}\))]. The cells were washed twice in PBS, and attachment performed as above with the exception that the eukaryotic cells were cultured in EIA/RIA strips (8-well strip plates; Costar), facilitating cutting out for counting. Confluent cell cultures were selected to minimize background adsorption. Counting of bound radioactivity was performed with the Beckman LS1800 system.

Isolation of the putative collagen-binding protein. An outer-membrane fraction of \(E. coli\) strain TBl harboured pC4003 was prepared, and extracted with Triton-X100 following standard procedures (Owen, 1985). The Triton-insoluble fraction was electrophoresed on a preparative SDS-polyacrylamide gel (12.5%, w/v, acrylamide). The 36 kDa species of the Pla protein was localized by staining a strip of the gel, and the corresponding area was excised from the main gel. The gel matrix was macerated by repeated passage through a syringe, and protein eluted by overnight gentle shaking in PBS at 4°C. The suspension was clarified by centrifugation at 45000 \(g\) for 30 min and the protein content established (Bio-Rad Protein assay).

Immunization. Antiserum to the \(Y. pestis\) collagen-binding protein was generated by immunization of rabbits (New Zealand White) with the 36 kDa polypeptide isolated by preparative gel electrophoresis. The excised band was macerated in PBS to form a homogenous suspension, then mixed with Freund’s complete adjuvant, and administered subcutaneously. After 3 weeks, a booster injection of the original suspension, without addition of adjuvant, was injected. Serum was collected after a further three weeks, and had an ELISA titre of 4000 against the immunogen, using an absorbance value equal to twice that of a negative control as cut-off.

Glycolipid extractions. A small scale procedure which yielded material suitable for thin-layer chromatography (TLC) from low amounts of starting material was employed as described by Linstedt et al. (1991).

\textit{In vitro adhesion assay}. A standard assay was employed (Linstedt et al., 1991). Briefly, glycolipids were first separated on Kieselgel 60, alumina-backed HPTLC plates (Merck) using chloroform/methanol/water (60:35:8, by vol.). Bacteria were labelled by overnight growth in Luria broth containing 1 mM-CaCl\(_2\) and 50 μCi (1.85 MBq) \(^{[35]S}\)methionine, washed repeatedly, and then incubated with the thin layer chromatogram which had been blocked by incubation with 2% BSA in PBS. After 2 h, the plate was washed repeatedly in PBS, dried and autoradiographed.

Glycosidase treatment. Glycosidases were from Sigma. \(\beta\)-Galactosidase, endoglycosidase H, endoglycosidase F and sialidase treatment of immobilized type IV collagen and cultured eukaryotic cells was performed as follows. The coated and blocked wells were washed twice with PBS for the \(\beta\)-galactosidase, or acetate buffer (0.1 M, pH 5.5) for the endoglycosidases and sialidase. Enzyme was added to a final concentration of 10 U ml\(^{-1}\) in PBS for \(\beta\)-galactosidase, or 10 U ml\(^{-1}\) in acetate buffer for endoglycosidase H, endoglycosidase F and sialidase. All reactions were performed at 37°C for 2 h, and were terminated by repeated washing in PBS. A normal binding assay was then performed.

\section*{Results}

\textbf{Cloning and localization of a determinant encoding a collagen binding protein}

A gene library constructed in the cosmids vector pJBS was screened for collagen binding clones by colony blotting.
with radiolabelled type IV collagen. The Sau3A-digested genomic DNA to be cloned was not size fractionated, but digestion conditions had been optimized for production of fragments in the 20–40 kb range. Collagen-binding clones were detected at a rate of about 1 per 100 clones, i.e. about three times the expected frequency for a single copy locus in such a cloning system. One primary clone, designated pC4001, had an insert of around 23 kb and was chosen for further study. This recombinant cosmid directed the expression of collagen binding as measured in both tube and solid-phase assays. By a series of successive subcloning experiments, the relevant sequence was reduced to 3·6 kb of Y. pestis DNA cloned in pK18, called pC4003 (Fig. 1). The insert size was further reduced by progressive deletion with exonuclease BAL31 to generate pC4004, which harboured a 2·4 kb fragment. This plasmid was subjected to transposon mutagenesis by Tn5, using a suicide phage delivery system. Phenotypes arising from transposon mutagenesis of the cloned DNA were checked by collagen-binding assays. Physical mapping of the site of Tn5 insertion in mutants failing to bind collagen implicated a region of about 1500 bp centred around a unique BamHI site in the cloned fragment. This area could be conveniently subcloned into pUC19 on a 1200 bp fragment generated by digestion with the enzymes PstI and HindIII, creating the recombinant plasmid pC4006 (Fig. 1).

Identification of the cloned collagen binding protein

The outer-membrane protein profiles of parental collagen-binding clones, transposon insertion mutants and BAL31 deletion mutants were compared (Fig. 2). From consideration of the phenotypes of these derivatives, it was readily apparent that a prominent polypeptide migrating with an apparent molecular mass of 36 kDa was present both in Y. pestis and collagen-binding E. coli clones, but was absent in Y. pestis lacking the 9·5 kb plasmid and E. coli transposon-insertion mutants which did not bind type IV collagen. Since the outer-membrane proteins of Y. pestis are relatively well characterized, and the polypeptide we were focusing on was a conspicuous component, we investigated the possibility that the collagen-binding protein had been previously identified or analysed. Fine structure restriction mapping of the cloned fragment in pC4006, followed by comparison with the literature for Y. pestis outer-membrane proteins, indicated similarity to the locus encoding the plasmino-
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Gen-binding assay and the ability to attach to McCoy cells. Adhesion could also be seen with other cell lines including Hep2. The binding activity mediated by Pla was specific for type IV collagen, since tube assay with type I collagen and a panel of serum proteins was consistently negative (data not shown).

Carbohydrate residues are established target structures for a variety of prokaryotic adhesion mechanisms. To test their possible involvement in this system, immobilized collagen and McCoy cell monolayers were treated with a small selection of specific endoglycosidases prior to performance of the adhesion assay. Significant inhibition was achieved only with β-galactosidase in either assay (Table 1). Endoglycosidases F and H were equally poor inhibitors of adhesion to McCoy cells, while the latter had almost no effect on binding to immobilized collagen. Sialidase had no inhibitory affect in either assay.

Table 1. Effect of glycosidase treatment on Pla-mediated binding to McCoy cells and immobilized type IV collagen

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Percentage inhibition of binding by Pla to:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>McCoy cells</td>
</tr>
<tr>
<td>Endoglycosidase F</td>
<td>15</td>
</tr>
<tr>
<td>Endoglycosidase H</td>
<td>17</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>46</td>
</tr>
<tr>
<td>Sialidase</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 2. Binding of E. coli cells with or without the cloned pla gene to mammalian glycolipids

A standard overlay assay of glycolipids separated by thin-layer chromatography, and probed with radiolabelled bacteria, was performed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HB101</th>
<th>HB101/pC4006 (Pla+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total glycolipid extract (A498 cells)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Total glycolipid extract (Caco-2 cells)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Total glycolipid extract, meconium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neutral glycolipid (pig erythrocyte)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acidic glycolipid (pig erythrocyte)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactosylceramide</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Total glycolipid, uroepithelial cells</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Total neutral extract dog mucosum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Buccal extract</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Globoside</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Identification of the receptor for Pla

In a standard *in vitro* assay for glycolipid binding, cells harbouring the *pla* gene bound to the total glycolipid extracts from a number of tissue sources, as well as neutral extracts from cells rich in globotetraosylceramide (globoside). Cells carrying the cloned *pla* locus also bound to purified globoside. These novel bindings were in addition to the limited background of compounds recognized by the host strain. (Table 2).

Discussion

The objective of the present study was to use molecular cloning techniques to identify a chromosomally encoded collagen-binding mechanism in *Y. pestis*. The surprising result of this investigation, however, has been to implicate an outer-membrane protein of established function as a possible adhesin. The process of subcloning, deletion and transposon mutagenesis unequivocally linked the collagen-binding phenotype to a region of
polypeptide and immobilized type IV collagen indicated an apparent dissociation constant of 48 amino acid residues. Our finding that the isolated 36 kDa Pla polypeptide bound to collagen supports the probability that it is this protein which is responsible for the activities associated with the cloned fragment. However, it does not unequivocally rule out the possibility that the gene product of the second smaller open reading frame might be responsible for, or contribute to, the adhesive properties we have observed in this study.

Introduction of the cloned pla locus into E. coli conferred upon the recipient the ability to bind to both type IV collagen and to cultured eukaryotic cells. Evidence provided by glycosidase treatment supports the hypothesis that specific sugar recognition is the common denominator for these two binding properties. A unique feature of type IV collagen is the presence of complex carbohydrate, attached through asparagine and hydroxylysine residues in the helical region (Glanville et al., 1985, 1987; Schwarz et al., 1986). The function of these substitutions is unknown. Their presence could well explain the specificity of Pla recognition of type IV collagen from among other collagen types.

Neither endoglycosidase H nor F dramatically reduced collagen binding or McCoy cell binding. Highest inhibition was achieved with β-galactosidase, implicating galactose somewhere in the structure recognized. Further supportive evidence for the importance of sugar residues was provided by a standard glycolipid attachment assay. Cells expressing Pla bound to total glycolipid from two cell lines (known to be rich in globoside), and from uroepithelial cells. Finally, purified globoside was recognized by the bacterial lectin, possibly indicating that this is the target in eukaryotic cells. As yet, however, there is insufficient data to pinpoint an homologous structure in the carbohydrate content of collagen type IV. The globoside recognized by Pla has the structure GalNAcβ1-3Galβ1-4Galβ1-4Glcβ1-1Ceramide (Leffler & Svanborg-Edén, 1981) while the hydroxylysine-linked carbohydrate of collagen type IV has the structure Glcα1-2Galβ1-5Hydroxylysine (Spiro & Spiro, 1970). Further experiments are underway to elucidate the exact sugar specificity of the lectin-like activity of Pla.

Scatchard analysis of the interaction between the Pla polypeptide and immobilized type IV collagen indicated an apparent dissociation constant of 1 μM. Compared to bacterial binding of serum proteins, exemplified by non-immune binding of streptococci and staphylococci to immunoglobulin (reviewed by Boyle, 1990), this affinity is very low (about three orders of magnitude lower). Rather than the high affinity protein–protein interactions which Fc-binding mechanisms seem to typify, the Pla-mediated binding to type IV collagen seems to be a lectin-like interaction of much lower avidity.

The bindings to type IV collagen, separated glycolipids and cultured eukaryotic cells described in this study are reproducible interactions which can be quantitatively monitored in vitro. Whether they have a role in the process of pathogenesis in vivo remains to be proven. Circumstantial evidence suggesting they might is, however, already available. Goguen et al. (1990) reported that mutation in the pla locus resulted in decreased virulence for Y. pestis when administered subcutaneously in mice, but not intravenously. It may arguably be important for establishing a locus of infection that the bacterium be able to bind to connective tissue components and/or eukaryotic cells. In a number of bacterial–host interaction systems, glycolipids are important recognition targets for establishing infection (Karlsson, 1987; Ofek & Sharon, 1990). Additionally, the ability to bind basement membrane components could be important in plague, when colonization of the lungs facilitates dissemination of the disease.

One conclusion of this study is that a protein with a function established in the literature as being plasminogen activation has the additional ability to mediate binding to the basement membrane component collagen type IV. We could not detect significant homology between the sequences of plasminogen and collagen which might have suggested recognition by the Pla polypeptide of shared primary amino acid sequences. We have preliminary evidence showing that the two bindings are biochemically distinct (Z. Kienle & P. W. O'Toole, unpublished). There are, however, precedents for such dual functionality, namely the binding to plasminogen and laminin by S-fimbriae of E. coli (Parkkinnen & Korhonen, 1989; Virkola et al., 1991), and plasminogen activation by fimbriated Salmonella enteritidis (Lahteenmaki & Korhonen, 1991). In the former example, R. Virkola and co-workers used a reconstituted basement membrane (Matrigel) to demonstrate increased invasiveness by the S-fimbriated strain in the presence of added plasminogen. The authors propose a novel mechanism for basement membrane penetration, involving fimбриae-mediated adhesion and localized proteolytic activity on bacterial cells. More work will be required to extrapolate from these model systems to microbial pathogenesis. We are currently analysing the possible interaction between Pla, plasminogen and basement membrane components other than collagen.
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References


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