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

ZSUZSA KIENLE,^{1,2} LEVENTE EMÖDY,² CATHARINA SVANBORG³ and PAUL W. O'TOOLE^{1*}

¹Department of Medical Microbiology, University of Lund, Sölvegatan 23, S-223 62 Lund, Sweden

²Department of Microbiology, University Medical School, Pécs, Hungary

³Department of Clinical Immunology, University of Lund, Sölvegatan 23, S-223 62 Lund, Sweden

(Received 9 December 1991; revised 9 March 1992; accepted 13 April 1992)

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 YAD A (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

* Author for correspondence. Present address: Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada V8W 3P6. Tel. (604) 721 7086; fax (604) 721 8855.

Abbreviation: Pla, plasminogen activator protein.

into guinea pigs or intraperitoneal injection into mice (Brubaker *et al.*, 1965). The 9.5 kb plasmid of *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 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 *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 *Escherichia coli*, conferred the ability to bind to type IV collagen. Additionally, it allowed laboratory strains of *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 globoside 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 *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. *Yersinia pseudotuberculosis* strain YPIII was described by Bölin *et al.* (1982). *E. coli* strains TB1 and HB101 were employed as hosts for recombinant plasmids (Yanisch-Perron *et al.*, 1985; Bachmann, 1972). Culture conditions for *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 *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 λ 467 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 respect 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, radioiodinated 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 *Y. pestis* collagen receptor. A gene library was constructed in the cosmid vector pJB8 by ligating EV76c DNA which had been partially digested with *Sau*3A to vector DNA linearized with *Bam*HI. 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, radioiodinated 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

Fig. 1. Restriction maps of the inserts in recombinant plasmids pC4003, pC4004 and pC4006. Arrowheads under pC4004 indicate the site of insertion of transposon Tn5, to produce the phenotypes indicated. The restriction site in parentheses originated in a plasmid vector.

recombinant. The plasmid pC4003 in this series had a 3.6 kb *EcoRI* fragment. A set of deletions was created from each end of the insert using controlled exonuclease digestion by exonuclease BAL31. Among the smaller plasmids retaining the collagen-binding phenotype was pC4004, which had an insert of 2.4 kb. This plasmid was subjected to mutagenesis by Tn5. Mapping of the site of insertions giving rise to loss of collagen binding implicated a region of approximately 1500 bp in expression of the collagen receptor, centered around a unique *Bam*HI restriction site (Fig. 1). This region could be conveniently subcloned on a 1.2 kb *Hind*III-*Pst*I fragment in pUC19, generating the recombinant plasmid pC4006 (Fig. 1).

Membrane profile analysis. Outer membranes of transposon insertion mutants and parental strains were prepared according to the procedure of Filip *et al.* (1973). Membrane fractions were then analysed by conventional SDS-PAGE (Laemmli, 1970). When required, Western immunoblotting was performed upon proteins separated by SDS-PAGE and transferred to nitrocellulose according to standard methodologies (Sambrook *et al.*, 1989).

Fibrinolysis assay. Fibrinolytic activity was detected by the method of Beesley *et al.* (1967). In brief, samples to be tested were spotted onto the surface of a fibrin plate and incubated overnight at 37 °C. Zones of fibrinolysis were visualized by clearing of the opacity in the plate, and could be compared relatively by measurement of zone diameter.

Eukaryotic cells and cell attachment assay. The McCoy cell line used was from Flow Laboratories (UK), and is derived from synovial fluid. Caco-2 intestinal epithelial cells were described by Finlay & Falkow (1990). The A498 cell line was from the American Type Culture Collection (ATCC HTB 44). Cells were cultured in MEM-glutamine medium (Flow Laboratories; Eagle's minimum essential medium supplemented with 2 mM-glutamine) incorporating 10% (v/v) foetal calf serum. A total of 3×10^5 cells, in 3 ml of culture medium, were added to 35 mm tissue-culture dishes and incubated in a CO₂ incubator for 24 h. The dishes were then washed three times in Dulbecco's PBS (DPBS; Flow Laboratories), and 2 ml of a bacterial suspension (5×10^8 cells ml⁻¹ in DPBS) was added. Incubation was continued for a further 3 h in the CO₂ incubator, the cells were washed three times with PBS, after which the cells were fixed in methanol and stained with Giemsa stain. Attachment was quantitatively evaluated by microscopy, counting bacteria per eukaryotic cell for at least 100 cells. For rapid qualitative analysis, counting with a cut-off value for positivity could be used (i.e. 10 bacterial cells per eukaryotic cell).

An alternative method employed radiolabelled bacterial cells, grown

overnight in LB broth incorporating appropriate antibiotic and [*methyl*-³H]thymidine [$20 \mu\text{Ci ml}^{-1}$ (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 TB1 harbouring 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 Protean 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 homogeneous 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).

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-CaCl₂ and $50 \mu\text{Ci}$ (1.85 MBq) [³⁵S]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. β -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 β -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 β -galactosidase, or 10 mU 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.

Results

Cloning and localization of a determinant encoding a collagen binding protein

A gene library constructed in the cosmid vector pJB8 was screened for collagen binding clones by colony blotting

Fig. 2. SDS-PAGE of outer-membrane preparations, and corresponding Western immunoblot, of *Y. pestis*, *E. coli* cells harbouring the cloned *pla* gene, and deletion and transposon mutants in the cloned locus. The left-hand panel shows the gel after staining with Coomassie brilliant blue; the right-hand panel shows the result of transferring an identical gel to nitrocellulose and immunoblotting with specific antiserum against the *Y. pestis* plasminogen activator (Pla). The Pla polypeptide visible in lanes 1 and 3 is arrowed. Lanes: S, molecular mass standards of indicated size; 1, *Y. pestis* EV76c; 2, *Y. pestis* EV76 lacking the Pla plasmid; 3, *E. coli* TB1/pC4006; 4, representative BAL31-generated deletion mutant of pC4003 which did not bind collagen; 5, *E. coli* TB1/pC4004::Tn5.

with radiolabelled type IV collagen. The *Sau*3A-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 *Bam*HI site in the cloned fragment. This area could be conveniently subcloned

into pUC19 on a 1200 bp fragment generated by digestion with the enzymes *Pst*I and *Hind*III, 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-

Fig. 3. Analysis of the binding of radio-iodinated *Y. pestis* plasminogen activator to immobilized type IV collagen. Inset: Scatchard plot of the binding data.

gen activator (*pla*; Sodeinde & Goguen, 1988). Limited DNA sequence analysis (about 300 residues from both 5' and 3' ends of the coding sequence) of the termini of the cloned fragment in pC4006 confirmed identity with the published sequence for the *pla* locus. Assay of whole cells of *E. coli* harbouring pC4006 (and parental plasmids) for fibrinolytic activity confirmed the presence of plasminogen-activating activity. The insert in pC4006 was used as a probe in Southern hybridization experiments. When digested genomic DNA was probed, only the plasmid-derived *Eco*RI fragment of approximately 7.0 kb present in EV76c hybridized (data not shown).

Quantification of collagen binding by solid-phase assay

Using a standardized coating system, 43 ng of type IV collagen was bound per well of the microtitre strips employed. Addition of increasing amounts of radio-iodinated Pla to these wells demonstrated that binding was saturable (Fig. 3). Scatchard analysis of the data (Scatchard, 1949) yielded a linear plot, indicating a single type of interaction. A dissociation constant of 1 μ M was derived from this data.

Adhesive properties conferred by the plasminogen activator

Laboratory *E. coli* strains did not exhibit significant binding to cultured cells of the McCoy cell line, but introduction of the cloned *pla* gene was sufficient to allow such adhesion (Fig. 4). Analysis of parental *Y. pestis* strains and *E. coli* harbouring the series of recombinant plasmids generated in this study showed that there was an absolute correlation between positivity in the colla-

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 effect in either assay.

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

Inhibition values are percentage reduction in binding after treatment with indicated substance relative to binding in the absence of any treatment. The indicator strain was *E. coli* TB1/pC4006.

Enzyme	Percentage inhibition of binding by Pla to:	
	McCoy cells	Type IV collagen
Endoglycosidase F	15	10
Endoglycosidase H	17	0
β -Galactosidase	46	58
Sialidase	17	0

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.

Compound	Binding to <i>E. coli</i> :	
	HB101	HB101/pC4006 (Pla ⁺)
Total glycolipid extract (A498 cells)	—	+
Total glycolipid extract (Caco-2 cells)	—	+
Total glycolipid extract, meconium	—	—
Neutral glycolipid (pig erythrocyte)	+	+
Acidic glycolipid (pig erythrocyte)	—	—
Lactosylceramide	—	+
Total glycolipid, uroepithelial cells	—	+
Total neutral extract dog mucosum	+	+
Buccal extract	+	+
Globoside	—	+

Fig. 4. Adhesion to cultured eukaryotic cells mediated by the cloned *pla* locus. Monolayers of McCoy cells were incubated with cells of *E. coli* harbouring the vector plasmid pUC19 (a) or the recombinant plasmid pC4006 which has the cloned *pla* gene (b). *E. coli* cells were stained with Giemsa stain. Bars, 20 µm.

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

cloned DNA spanning about 1500 bp, which was shown to encode the plasminogen activator, Pla. The cloned collagen-binding protein had the fibrinolytic activity expected, and limited DNA sequence determination revealed absolute identity with the published data.

Sodeinde & Goguen (1989) also reported the existence of an open reading frame overlapping *pla* on the complementary strand, which could encode a protein 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 GalNAc β 1-3Gal α 1-4Gal β 1-4Glc β 1-1Ceramide (Leffler & Svanborg-Edén, 1981) while the hydroxylysine-linked carbohydrate of collagen type IV has the structure Glc α 1-2Gal β 1-5Hydroxylysine (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 μ M. 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* (Parkkinen & 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 fimbriae-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.

This work was supported by grants to P.O.T. from the A. Österlund Foundation, the Swedish Society against Rheumatism, the Kock Foundation and the Medical Faculty of Lund University. We are grateful to H. Wolf-Watz for supplying strains, Janos Erdei for helpful advice, to Ingrid Nilsson for cell culture, and to Ragnar Linstedt and Majlis Svensson for *in vitro* binding assays. We are grateful to Professor T. Wadström for providing a stipend for Dr Kienle (from grant 16X-04723 from the Swedish Medical Research Council), and for fruitful discussions.

References

- BACHMANN, B. J. (1972). Pedigrees of some mutant strains of *Escherichia coli* K12. *Bacteriological Reviews* **36**, 525–557.
- BEESLEY, E. D., BRUBAKER, R. R., JANSSEN, W. A. & SURGALLA, M. J. (1967). Pesticins. III. Expression of coagulase and mechanisms of fibrinolysis. *Journal of Bacteriology* **172**, 4661–4671.
- BERCOVIER, H., MOLLARET, H. H., ALONSO, J. M., BRAULT, J., FANNING, G. R., STIEGERWALT, A. G. & BRENER, D. J. (1980). Intra- and interspecies relatedness of *Yersinia pestis* by DNA hybridization and its relationship to *Yersinia pseudotuberculosis*. *Current Microbiology* **4**, 225–229.
- BÖLIN, I., NORLANDER, L. & WOLF-WATZ, H. (1982). Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. *Infection and Immunity* **37**, 506–512.
- BOYLE, M. D. P. (editor) (1990). *Bacterial Immunoglobulin Binding Proteins*, vol. 1. New York: Academic Press.
- BRUBAKER, R. R. & SURGALLA, M. J. (1962). Pesticins. II. Production of pesticides I and II. *Journal of Bacteriology* **82**, 940–949.
- BRUBAKER, R. R., BEESLEY, E. D. & SURGALLA, M. J. (1965). Role of pesticide I and iron in experimental plague. *Science* **149**, 422–424.
- BRUBAKER, R. R. (1984). Molecular biology of the Dread Black Death. *ASM News* **50**, 240–245.
- BRUBAKER, R. R. (1991). Factors promoting acute and chronic diseases caused by *Yersiniae*. *Clinical Microbiology Reviews* **4**, 309–324.
- COLEMAN, D. C. & FOSTER, T. J. (1981). Analysis of the reduction in expression of tetracycline resistance determined by transposon Tn10 in the multicopy state. *Molecular and General Genetics* **182**, 171–177.
- EMÖDY, L., HEESEMANN, J., WOLF-WATZ, H., SKURNIK, M., KAPPERUD, G., O'TOOLE, P. & WADSTRÖM, T. (1989). Binding to collagen by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*: evidence for *yopA*-mediated and chromosomally encoded mechanisms. *Journal of Bacteriology* **171**, 6674–6679.
- FERBER, D. M. & BRUBAKER, R. R. (1981). Plasmids in *Yersinia pestis*. *Infection and Immunity* **31**, 839–841.
- FILIP, C., FLETCHER, G., WULF, J. L. & EARHART, C. F. (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. *Journal of Bacteriology* **115**, 717–722.
- FINLAY, B. & FALKOW, S. (1990). *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. *Journal of Infectious Diseases* **162**, 1096–1106.
- GLANVILLE, R. W., QIAN, R., SIEBOLD, B., RISTELI, J. & KUHN, K. (1985). Amino acid sequence of the N-terminal aggregation and cross-linking region (7S domain) of the alpha 1(IV) chain of human basement membrane collagen. *European Journal of Biochemistry* **152**, 213–219.
- GLANVILLE, R. W. (1987). Type IV collagen. In *Structure and Function of Collagen Types*, pp. 43–79. Edited by R. Mayne & R. E. Burgeson. London: Academic Press.
- GOGUEN, J. D., SODEINDE, O., QUAN, T. & YOUNG, B. (1990). Role of the *pla* gene in the virulence of *Yersinia pestis*. *Symp. Molec. Biol. Yersiniae: A Model for Gram-negative Pathogenesis*. 19th Annual meeting UCLA Symp. Molec. Cell Biol., Frisco, Color., USA, Feb. 22–27, 1990. *Journal of Cellular Biochemistry (Suppl.)* **14C**, 177.
- HEESEMANN, J., ALGERMISSEN, B. & LAUFS, R. (1984). Genetically manipulated virulence of *Yersinia enterocolitica*. *Infection and Immunity* **46**, 105–110.
- HELFMAN, D. M., FERAMISCO, J. R., FIDDES, J. C., THOMAS, G. P. & HUGHES, S. H. (1983). Identification of clones that encode chicken tropomyosin by direct immunological screening of a cDNA expression library. *Proceeding of the National Academy of Sciences of the United States of America* **80**, 32–35.
- HULL, R. A., GILL, R. E., HSU, P., MINSHAW, B. H. & FALKOW, S. (1981). Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. *Infection and Immunity* **33**, 933–938.
- ISH-HOROWICZ, D. & BURKE, J. F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Research* **9**, 2989–2993.
- KARLSSON, K.-A. (1987). Preparation of total non-acid glycolipids for overlay analysis of receptors for bacteria and viruses and for other studies. *Methods in Enzymology* **138**, 212–220.
- LAEMMLI, U. K. (1970). Cleavage of the structural protein during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAHTENMAKI, K. & KORHONEN, T. K. (1991). *t*-PA-mediated plasminogen activation on fimbriate *Salmonella*. *FEMS Symposium Abstracts, Molecular Recognition in Host-Parasite Interactions: Mechanisms in Viral, Bacterial and Parasite Infections*, 5–7 August 1991, Porvoo, Finland, p. 28.
- LEFFLER, H. & SVANBORG-EDÉN, C. (1981). Glycolipid receptors for uropathogenic *Escherichia coli* on human erythrocytes and uroepithelial cells. *Infection and Immunity* **34**, 920–929.
- LINSTEEDT, R., LARSON, G., FALK, P., JODAL, U., LEFFLER, H. & SVANBORG, C. (1991). The receptor repertoire defines the host range for attaching *Escherichia coli* strains that recognize Globo-A. *Infection and Immunity* **59**, 1086–1092.
- MCDONOUGH, K. A. & FALKOW, S. (1989). A *Y. pestis*-specific DNA fragment encodes temperature-dependent coagulase and fibrinolysin-associated phenotypes. *Molecular Microbiology* **3**, 767–775.
- NOWICKI, B., HOLTHÖFER, H., SARANEVA, T., RHEN, M., VÄISÄNEN-RHEN, V. & KORHONEN, T. K. (1986). Location of adhesion sites for P-fimbriated and for O75X-positive *Escherichia coli* in the human kidney. *Microbial Pathogenesis* **1**, 169–180.
- OFEK, I. & SHARON, N. (1990). Adhesins as lectins: specificity and role in infection. In *Current Topics in Microbiology and Immunology* **151**, *Bacterial Adhesins*, pp. 91–113. Edited by K. Jann & B. Jann. Berlin: Springer-Verlag.
- OWEN, P. (1985). Crossed immunoelectrophoresis in the study of outer membrane antigens. In *Enterobacterial Surface Antigens: Methods for Molecular Characterisation*, pp. 207–242. Edited by T. K. Korhonen, E. A. Dawes & P. H. Makela. Amsterdam: Elsevier.
- PARKKINEN, J. & KORHONEN, T. K. (1989). Binding of plasminogen to *Escherichia coli* adhesion proteins. *FEMS Microbiology Letters* **250**, 437–440.
- PORTNOY, D. A., MOSELEY, S. L. & FALKOW, S. (1981). Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. *Infection and Immunity* **31**, 775–782.
- PORTNOY, D. A., BLANK, H. F., KINGSBURY, D. T. & FALKOW, S. (1983). Genetic analysis of essential plasmid determinants of pathogenicity in *Yersinia pestis*. *Journal of Infectious Diseases* **148**, 297–304.
- PORTNOY, D. A., WOLF-WATZ, H., BÖLIN, I., BREEDER, A. B. & FALKOW, S. (1984). Characterization of common virulence plasmids in *Yersinia* species and their role in expression of outer membrane proteins. *Infection and Immunity* **43**, 108–114.
- PRIDMORE, R. D. (1987). New and versatile cloning vectors with kanamycin resistance marker. *Gene* **56**, 309–312.
- PROTSENKO, O. A., ANISIMOV, P. I., MOZHAROV, O. T., KONNOV, N. P., POPOV, Y. A. & KOKUSHIKIN, A. M. (1983). Detection and characterization of *Yersinia pestis* plasmids determining pesticide I, fraction I antigen and “mouse” toxin synthesis. *Genetika* **19**, 1081–1090.
- ROSQVIST, R., SKURNIK, M. & WOLF-WATZ, H. (1988). Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. *Nature, London* **334**, 522–525.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

- SCATCHARD, G. (1949). The attraction of proteins for small molecules and ions. *Annals of the New York Academy of Sciences* **51**, 660–672.
- SCHWARZ, U., SCHUPPAN, D., OBERBAUMER, I., GLANVILLE, R. W., TIMPL, R. & KUHN, K. (1986). Structure of mouse type IV collagen. *European Journal of Biochemistry* **157**, 49–56.
- SODEINDE, O. A., SAMPLE, A. K., RUBAKER, R. R. & GOGUEN, J. D. (1988). Plasminogen activator/coagulase gene of *Yersinia pestis* is responsible for degradation of plasmid-encoded outer membrane proteins. *Infection and Immunity* **56**, 2749–2752.
- SODEINDE, O. A. & GOGUEN, J. D. (1988). Genetic analysis of the 9.5-kilobase virulence plasmid of *Yersinia pestis*. *Infection and Immunity* **56**, 2743–2748.
- SODEINDE, O. A. & GOGUEN, J. D. (1989). Nucleotide sequence of the plasminogen activator gene of *Yersinia pestis*: relationship to *ompT* of *Escherichia coli* and Gene *E* of *Salmonella typhimurium*. *Infection and Immunity* **57**, 1517–1523.
- SPIRO, R. G. & SPIRO, M. J. (1971). Studies on the biosynthesis of the hydroxylysine-linked disaccharide unit of basement membranes and collagens. *Journal of Biological Chemistry* **246**, 4899–4909.
- VESIKARI, T., NURMI, T., MAKI, M., SKURNIK, M., SUNDQUIST, C., GRANFORS, K. & GRONROOS, P. (1981). Plasmids in *Yersinia enterocolitica* serotypes O:3 and O:9: correlation with epithelial cell adherence *in vitro*. *Infection and Immunity* **33**, 870–876.
- VIRKOLA, R., RAUNIO, T., PARKKINEN, J. & KORHONEN, T. K. (1991). Basement membrane binding and plasminogen activation by S-fimbriate *Escherichia coli*. *FEMS Symposium Abstracts, Molecular Recognition in Host-Parasite Interactions: Mechanisms in Viral, Bacterial and Parasite Infections*, 5–7 August 1991, Porvoo, Finland, p. 36.
- WOLF-WATZ, H., PORTNOY, D., BÖLIN, I. & FALKOW, S. (1985). Transfer of the virulence plasmid of *Yersinia pestis* to *Yersinia pseudotuberculosis*. *Infection and Immunity* **48**, 241–243.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.