Structural basis for the specific recognition of dual receptors by the homopolymeric pH 6 antigen (Psa) fimbriae of *Yersinia pestis*

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The pH 6 antigen (Psa) of *Yersinia pestis* consists of fimbriae that bind to two receptors: β 1-linked galactosyl residues in glycosphingolipids and the phosphocholine group in phospholipids. Despite the ubiquitous presence of either moiety on the surface of many mammalian cells, *Y. pestis* appears to prefer interacting with certain types of human cells, such as macrophages and alveolar epithelial cells of the lung. The molecular mechanism of this apparent selectivity is not clear. Site-directed mutagenesis of the consensus choline-binding motif in the sequence of PsaA, the subunit of the Psa fimbrial homopolymer, identified residues that abolish galactosylceramide binding, phosphatidylcholine binding, or both. The crystal structure of PsaA in complex with both galactose and phosphocholine reveals separate receptor binding sites that share a common structural motif, thus suggesting a potential interaction between the two sites. Mutagenesis of this shared structural motif identified Tyr126, which is part of the choline-binding consensus sequence but is found in direct contact with the galactose in the structure of PsaA, important for both receptor binding. Thus, this structure depicts a fimbrial subunit that forms a polymeric adhesin with a unique arrangement of dual receptor binding sites. These findings move the field forward by providing insights into unique types of multiple receptor–ligand interactions and should steer research into the synthesis of dual receptor inhibitor molecules to slow down the rapid progression of plague.

adhesin–receptor complex | adhesion | ligand binding | sugar binding | phosphocholine binding

Owing to a well-documented history of deadly pandemics that nearly disrupted human civilization over the last 1,500 years, plague is often mistaken as a disease of antiquity. Contrary to this view, plague is still a major public health concern with widespread epidemics and isolated cases occurring in many countries of the world (1). *Yersinia pestis* is the etiological agent of plague and thus classified as a category A bioterrorism agent. Several major virulence factors of *Y. pestis* are harbored on a 70-kb plasmid, pCD1, which encodes the *Yersinia* type III secretion system (T3SS) and the Yop effector proteins (Yops) (2). Adhesin-mediated binding of *Y. pestis* to host cells facilitates the insertion of the T3SS apparatus on to the host cells and subsequent Yops injection, which is critical to plague pathogenesis. The pH 6 antigen (Psa) of *Y. pestis* has been shown to be a key adhesin that facilitate Yops delivery, besides other adhesins such as Ail and Pla (3).

The Psa of *Y. pestis* is a surface homopolymer made of the structural subunit PsaA that is assembled via the chaperone–usher pathway (4). Psa belongs to the class of FGL (long F1–G1 loop) fimbriae and is a bacterial homopolymeric adhesin or polyadhesin (5). By contrast, the Afa/Dr and Saf polyadhesins of *Escherichia coli* and *Salmonella enterica*, respectively, all consist of a major multimeric adhesin and an additional tip-located invasin (4). In addition to Psa, the surface of *Y. pestis* also displays a homopolymeric structure known as the F1 capsule that is grouped with the FGL surface proteins and is assembled by the

chaperone–usher pathway (4). However, the F1 polymer does not act as a polyadhesin but effectively inhibits phagocytosis (6, 7).

Psa is expressed under the conditions of low pH (5.0–6.7) and temperatures of 35–41 °C (8). Psa has been shown to agglutinate red blood cells (9) and, like F1, to inhibit phagocytosis by macrophages (10). A number of studies have also highlighted that Psa is expressed *in vivo* in infected tissues (11) and plays a key role in virulence of *Y. pestis* (12, 13). Recent studies have shown that the Psa antigen induces a robust immune response and induces significant albeit incomplete protection in a murine model of pneumonic plague (11). Psa has also been shown to mediate binding to cells, including human respiratory tract epithelial cells (7, 9), as well as to pulmonary surfactant (5), which coats the alveolar surface of lungs. Two major receptors that interact with Psa have been identified: β 1-linked galactosyl residues in glycosphingolipids (14) and phosphatidylcholine (PC) present on alveolar epithelial cells (5).

Interestingly, many of the FGL-assembled structures are either not detectable by negative-stain electron microscopy (thus designated afimbrial or nonfimbrial adhesins) or appear as featureless electron-dense masses around the bacteria (4, 7). Only in some studies were a few of these structures visualized as bacterial extensions (15, 16) or as linear thin fimbriae (of ~2–4 nm wide) that are mostly clumped together in small bundles (17, 18). However, a structural view at atomic resolution on the subunit PsaA, its assembly into a polyadhesin, and how the two receptors interact with the Psa fimbriae were absent. In fact, the lack of structural information on Psa and its low sequence similarity to known structures of FGL family members further aggravate the problem in defining the role of these two receptors in the infection process by *Y. pestis*. By using structural techniques, site-directed mutagenesis, and ligand binding assays, we identified amino acid residues that are involved in Psa binding to the two receptors. We mapped these residues to the structure of PsaA determined by X-ray crystallography in the presence of receptors. The role of these residues in the context of Psa binding to human cells is further demonstrated.

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Results

Psa Shares a Consensus Motif with a Family of Bacterial Choline-Binding Proteins. Previous studies carried out in our laboratory have identified that Psa binds in a dose–response fashion to PC, which is inhibited by choline or phosphocholine (PCho), indicating that Psa interacts with the choline head group in PC (5). PC is ubiquitously present as a major component of most eukaryotic membrane, whereas it is rarely present in prokaryotes (19). Choline, on the other hand, is encountered in the cell wall as a component of teichoic and lipoteichoic acids of a number of bacteria including many species of streptococci (20). A family of bacterial proteins, broadly called choline-binding proteins (CBPs), use choline binding as a strategy to get noncovalently anchored to the choline-containing gram-positive cell wall after being exported to the bacterial surface. Each choline-binding module consists of multiple tandem repeats of choline-binding motif made of 20-amino acid residues with a consensus sequence of W-6X-WY (21). Sequence comparison and alignment of PsaA with choline-binding repeats of CBP of pneumococci (Fig. 1) revealed that PsaA harbors a single copy of the same motif. Because each Psa fimbria is a bacterial surface organelle consisting of hundreds of polymerized PsaA subunits (17), it is conceivable that Psa could interact with choline molecules in a manner not too different from bacterial CBPs.

To demonstrate experimentally that this choline-binding motif in PsaA is indeed involved in binding PC, we substituted key amino acids in and around the consensus W-6X-WY sequence of PsaA with alanine or other amino acids in some cases. Substituted variants of PsaA were expressed in the nonfimbriated *E. coli* SE5000 strain. The bacteria were then checked for total expression of PsaA, for fimbriation by heat extraction of exported and surface assembled Psa, and by agglutination with anti-Psa serum (Table 1). The bacteria were also evaluated for receptor binding by agglutination with liposomes containing PC or galactosylcerebroside (GC; Table 1). Substitution of the highly conserved tryptophan residue W118 or W125 in the putative choline-binding motif with alanine reduced PsaA detection and interfered with Psa fimbriation, suggesting protein misfolding that leads to cellular degradation and/or defective fimbrial biogenesis. Even though substitution with another hydrophobic residue (isoleucine) gave a comparable result, change to another aromatic residue (phenylalanine) restored fimbrial expression and binding to both receptors, suggesting that aromatic residues in these positions are essential not only for structural stability of PsaA and subsequent fimbrial biogenesis but also for receptor binding. Alanine substitutions of four residues between the two conserved tryptophan residues (V119, G120, D122, and N123) resulted in no detectable difference from the WT phenotype. By contrast, replacement of isoleucine I121 with alanine resulted in defective binding to PC. Surprisingly, two other substitutions (N124A and Y126A) affected GC, but not PC, recognition. The latter result indicated that the canonical choline-binding motif of PsaA includes residues that are exclusively required for recognition of the second receptor, GC. This finding was unanticipated, because previous biochemical studies suggested independent binding pockets for the two receptors (5). As expected, a mutant with double mutations I121A and N124A lost both binding properties. Mutants D122A and Y126A expressed Psa antigen on the bacterial surface that recognized at least one receptor, but the isolation of mutant Psa by

heat extraction was less efficient or not possible, suggesting that the mutated proteins created new and stronger interactions with an anchored bacterial surface component.

Crystal Structure of the *in cis* Donor-Strand Complemented Fimbrial Subunit PsaA in Complex with Galactose. The crystal structure of donor-strand complemented fimbrial subunit PsaA (dscPsaA) in complex with galactose was determined and refined (Table S1). Similar to subunit structures of other fimbriae that are assembled by the usher–chaperone pathway (4) the structure of dscPsaA has a typical Ig fold of a seven-stranded β -sandwich (Fig. 2*A* and *B*). During fimbria assembly, the last β -strand or G strand is provided by the N-terminal donor strand of an adjacent subunit in a mechanism dubbed the donor-strand complementation (DSC) (4). In dscPsaA, DSC is achieved by self-complementation through the C-terminally *in cis*-engineered donor strand (Fig. S1). Prominent features perhaps unique to PsaA are the structural motifs between β -strands C' and E including β -strands C'', D', D'', and D''', as well as associated loops between the β -strands, which deviates significantly from other major fimbrial subunits with known structures (Fig. 2*A* and *B*).

Calculation of the difference Fourier revealed a prominent feature that was unambiguously identified as the bound galactose molecule (Fig. 2*C*). In all of the structures reported here, the galactose moiety is found identically residing in a shallow surface niche bound by residues from β -strands C', D'', and D''' and associated loops (Figs. 2*C* and S2). The structure of the complex reveals that the galactose-binding niche bears similarity to that of type C carbohydrate-binding module (CBM), which is classified as CBMs binding optimally to mono, di-, or trisaccharides (22). Like all CBMs, the side chain of the aromatic residue Y126 from the strand D''' plays a critical role as a hydrophobic platform to “mirror” the pyranose ring of galactose through a carbohydrate– π interaction (Fig. 2*C*), which is commonly observed in structures of carbohydrate–CBM complexes (22, 23). Despite a low IC_{50} (~40 mM) of galactose toward PsaA, the binding site was shown to be specific for the galactose moiety (5). The bound galactose is surrounded by side chains from residues R89, N124, and N128, likely forming hydrogen bonds to the sugar (Fig. 2*C*). In particular, residue N128 provides its amide group to form a hydrogen bond (2.7 Å) with the hydroxyl oxygen of the galactose at C6 position and presumably determines the binding preference for hexoses over pentoses in this pocket. Discrimination against other hexoses such as glucose and mannose is provided by R89 and N124, whose side chains interact with hydroxyl groups from carbon atoms C2, C3, and C4 of the bound galactose (Fig. 2*C*).

Interestingly, the difference Fourier map also revealed a large piece of electron density located in a hydrophobic surface depression near the sugar binding niche, which is delimited at one end by the twin tryptophan residues of the consensus choline-binding motif W-6X-WY. This density can be best fitted with the protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) that was used during protein purification (Fig. S3). Unlike PCho that has a quaternary amine group, the AEBSF possesses a terminal amino group that is pointing toward the twin tryptophan residues. In the absence of the protease inhibitor, the purified dscPsaA crystallized into a different crystal form with the

CbpF	GWYKEGSTWYLLDAENGDMRT	GWQNLGNKWWYLRSSGAMAT	GWYQESSTWYLLNASNGDMKT	GWQVNGWYAYDSGALAV
CbpG		GWKKINGSWYHYRQHDQKT	GWQINDTWYLLDSS	GWKMLT
CbpC	GWYVRGKWWYLLDNKNGDMKT	GWQYLGKWWYLRSSGAMVT	GWYQDGTWYLLDPSNGDMKI	GWTKVNGKWWYLLNSGAMVT
CbpJ	GWYQEGTWYLLDHPNGDMKT	GWQNLGNKWWYLRSSGAMAT	GWYQDGTWYLLNAGNGDMKT	GWQVNGWYAYSSGALAV
CbpI	GVFKESGSWYLLSNS	GAMFT	GWGTDGSRWYFDGSGAMKT	GWYKENGWYLLDEA
CbpD	GWKKINGSWYHFKSN	GSKST	GWLDKDGWYLLKLSGEMQT	GWLKENGWYLLGSS
PcpA	GWVKDKGLWYLLNES	GSMAT	GWVKDKGLWYLLNESGSMAT	GWVKDKGLWYLLNES
PcpA	GWAKVNGSWYLLNAN	GAMAT	GWVKDGTWYLLNANGSMAT	GWVKDGTWYLLNANGSMAT
CbpA	GWLQYNGSWYLLNAN	GSMAT	GWVKDGTWYLLNANGSMAT	GWVKDGTWYLLNANGSMAT
PcpC		WKQENGWYFYNTDGSMT	GWQVNSWYLLNSN	GSMKV
				NQWFKVGGKWWYVNTSGELAV
PsaA (Y.p)	86	GGWRLIPTGDS	KGGYMSADGDYVGLYSYMM	SWVGIDNNWYINDSPDKID
MyfA (Y.e)	85	KGNVNRPTGAS	EGGQMSADGTRVDLHTNEL	SW...DNDEWWDGDSER
				VEA
				TFFLAAGDEVKAGEYQFTGRV
				154

Fig. 1. Sequence motif of dscPsaA. Alignment of the amino acid sequences of PsaA of *Y. pestis* and MyfA of *Y. enterocolitica* with those of the choline-binding motifs of *Streptococcus pneumoniae* TIGR4 CBPs (21). Highly conserved residues of the motif are shown in bold. PCho and β 1-linked galactose-binding residues of Psa are indicated by a period and a colon above the PsaA sequence, respectively.

Table 1. Phenotypes and characterization of engineered *psaA* mutants*

PsaA mutation	PsaA expression [†]	Psa on bacteria		Binding [‡]		HA titer	Suggested roles by structure/function analyses
		Isolation [‡]	Antigen [§]	PC	GC		
WT**	+	+	+	+	+	128	
Δ psaA	—	—	—	—	—	<1	
R89A ^{††}	+	+	+	+	—	128	Gal binding (2.9 Å) ^{‡‡}
Y100A	+	+	+	—	+	<1	PC binding
L111A	—	—	—	—	—	ND	Structural
Y112A	+	+	+	—	+	8	PC binding
W118A	+/-	—	—	—	—	ND	PC binding, structural
W118I	+/-	—	—	—	—	ND	PC binding, structural
I121A	+	+	+	—	+	32	Structural
D122A	+	—	+	+	+	128	In contact with Gal (2.5 Å) ^{‡‡}
N124A	+	+	+	+	—	128	Gal binding
W125A	—	—	—	—	—	ND	PC binding, structural
W125I	—	—	—	—	—	ND	PC binding, structural
Y126A	+	+/-	+	+	—	<1	Gal binding, two sites communication
N128A	+	+	+	+	+	ND	In contact with Gal (2.7 Å) ^{‡‡}
L139A	—	—	—	—	—	ND	Structural
Y161A	ND	ND	+	+	+	ND	In contact with Gal (4.2 Å) ^{‡‡}
F163A	ND	ND	+	+	+	ND	None
I121A,N124A ^{§§}	+	+	+	—	—	<1	
R89A,N124A	+	+	+	+	—	128	

ND, not determined.

**E. coli* SE5000 expressing the *Y. pestis* *psaABC* from a constitutive *tetA* promoter.

[†]PsaA expression in whole cell lysates (Western blot) using an antiserum to Psa (7).

[†]Fimbriae were isolated from bacterial surfaces by heat extraction (7).

[§]Bacterial surface-exposed Psa antigen was detected bacterial seroagglutination (7).

*Bacterial binding to liposomes carrying PC or GC (5).

^{||}Hemagglutination by *E. coli* SE 5000 expressing WT and mutant variants of Psa (9).

**WT and mutants with the same phenotypes: Y100F, Y112F, M115A, M116A, W118F, V119A, G120A, N123A, and W125F.

^{††}Arginine was replaced with an alanine at position 89 of PsaA.

^{††}Contact distance in Å.

^{§§}Mutant with same phenotypes: $\Delta V119-N124::DNDH$ (V119-N124 substituted with the corresponding sequence of MyfA [DNDH]).

symmetry of space group $P6_5$ (Table S1). Unexpectedly, the AEBSF site remained occupied by the imidazole ring of a histidine residue from the hexahistidine tag of a neighboring molecule.

We also determined the structure of dscPsaA cocrystallized with the disaccharide lactose (Table S1). Not surprisingly, the galactosyl moiety of the lactose was found binding to the dscPsaA in exactly the same manner as it was found in the galactose bound structures, whereas the glucose moiety stayed outside the binding niche, making no apparent contacts with the protein (Fig. S2). This structure further confirmed the specificity of PsaA toward binding of the galactosyl moiety. More importantly, this result suggests that PsaA most probably only recognize the galactosyl moiety located at the tip of a polysaccharide.

Crystal Structure of the Ternary Complex of dscPsaA, Galactose, and Phosphocholine. The presence of the AEBSF binding site nearby the carbohydrate-binding niche inspired us to crystallize the dscPsaA, galactose, and PCho ternary complex. The complex crystal, obtained in the presence of 10 mM PCho, diffracted X-rays to 2.3 Å. As expected, the difference Fourier map gave excellent density for the bound galactose. Most importantly, the difference map also revealed a piece of extra density with a shape resembling that of PCho in the same site previously occupied by AEBSF.

The PCho site and the galactose-binding niche are separated by the D''-D''' hairpin. In other words, the D''-D''' hairpin contributes to both galactose and PCho binding (Figs. 2B and 3A). Compared with the galactose-binding niche, the PCho binding site is considerably more hydrophobic, and it is surrounded, in addition to the twin tryptophan residues W118 and W125, by predominately hydrophobic residues such as G99, Y100, Y112, and G110 (Fig. 2D). Similar to the binding of various choline to

their respective receptors, both natural and synthetic (24, 25), the binding of PCho to PsaA is mediated by the “aromatic guidance” mechanism, in which the positively charged quaternary ammonium group from PCho is trapped by an aromatic ring system made of side chains from residues W118, W125, and Y112 via the characteristic cation- π interaction (Fig. 2D). Additionally, one of the oxygen atoms of the phosphate group is likely hydrogen bonded to the amide nitrogen of G110 (3 Å), providing additional interaction. Thus, the cation- π interaction, although relatively weak, forms the structural basis for the specificity of Psa toward PC (26, 27).

Interactions Between Galactose and Phosphocholine Binding Sites Revealed by Site-Directed Mutagenesis. The consensus choline-binding motif in PsaA (Fig. 1) includes a conserved aromatic residue Y126, which, in the structure of CBP from bacteria such as *Streptococcus pneumoniae* (PDB: 2XBM), contributes to the choline-binding site of the preceding motif. The crystal structure of dscPsaA reveals instead that Y126 is an integral constituent of the galactose-binding site, providing direct contact with the bound galactose via the pyranose ring- π interaction (Fig. 2C). Interestingly, *E. coli* expressing mutant Y126A Psa fimbriae lost binding not only to GC, but also to PC immobilized on a solid surface (Table 1; Fig. 4A and B). This mutation also abrogated hemagglutination activity of Psa fimbriae (Table 1). More importantly, the same observation extends to binding of the Y126A mutant to both lung epithelial-derived A549 and murine macrophage line RAW264.7 cells (Fig. 4C and D). Thus, there exists structural and biochemical evidence to suggest a functional linkage between the sugar and choline binding sites in PsaA. Consistent with this view, residues R89 and N124 also play a role in GC binding as

mutations of these two residues reduce binding of GC to varying degrees (Fig. 4 *A* and *B*). Expectedly, the reduction of galactose binding in these mutants also affected their binding to PC, which was indeed observed in the binding assay to A549 and RAW264.7 cells (Fig. 4 *C* and *D*).

Because the choline-binding motif is a large part of the D"-D''' hairpin, it is conceivable that mutations introduced to residues in this hairpin would affect binding of either receptor. This effect was precisely observed for residue I121 that is located at the very tip of the D"-D''' hairpin and has no contacts with either bound Gal or PCho, yet its mutation led to the loss of binding to PC (Fig. 4A). The variant with double mutations of I121A and N124A resulted in the loss of binding to both GC

and PC, further supporting the importance of D''-D''' hairpin for receptor binding.

Discussion

Phospholipid Binding in Psa. The Psa polyadhesin has two known cellular receptors; both are abundantly present in mammalian cell membranes (5). Variable binding properties for different cells are most likely modulated by relative receptor densities, length and hydroxylation of the fatty acid chains, and types and densities of neighboring membrane lipids and glycoproteins (28). The importance of an intact choline-binding motif to preserve

Fig. 3. Structural analysis of PsaA, its alignment with Cfa1 pilin, and functional implications. (A) Electrostatic potential surface of dscPsaA. The red and blue surfaces represent negative and positive potentials, respectively. White surface indicates a hydrophobic surface. Bound receptors are shown as stick models with the same color codes as in Fig. 2. (B) Structural alignment of dscPsaA and Cfa1 pilin in the FGL family, showing α tracing in green and yellow, respectively. (C) Model of Psa polyadhesin based on the structure of the F1 fimbriae (32). Seven subunits are shown, in which residues interacting with galactose are drawn as sphere models in light green and those interacting with PCho are colored in pink. Magnified portion shows the linkage between two neighboring subunits.

variable D strands of the adhesin subunits, which are consensus receptor-binding sites in poly-adhesion fimbriae (4, 29). The D''-D''' hairpin motif presents a feature unique to Psa fimbriae, a longer D''-D''' hairpin that can be seen clearly by aligning PsaA with other known structures among the FGL family fimbriae such as Caf1 and DraE. Caf1 and DraE are more similar to each other than to PsaA; PsaA has a longer D''-D''' hairpin, whereas Caf1 and DraE each has a longer C''-D' loop (Fig. 3B; Fig. S5).

Model for Polyadhesin Binding to Host Cells. Based on the dscPsaA structure of the ternary complex, a putative model for Psa fimbriae can be built (Fig. 3C). Unlike rod-shaped fimbriae such as CFA/I, where the connection between the two major subunits CfaB is a single proline residue (30), the intersubunit connection for a Psa fimbria has four residues (48-KGQL-51) that makes it very flexible and allows simultaneous binding by a number of subunits to available receptors, as previously described with polymeric inhibitors (5). Additive fimbrial binding to the two cell receptors (5) suggested simultaneous binding to both receptors, consistent with the subunit crystal structure with its two receptors. That different fimbrial filaments bind different receptors is unlikely, because of the 10-fold higher affinity of Psa for PC than for GC (5).

The two binding sites of Psa share a common character of very weak binding to their respective receptors, implying a selective mechanism when subunits are polymerized. Conceivably, the binding avidity depends on the presence and distribution of both receptors on cell membranes and on the average size of the Psa fimbriae, as well as the number of fimbriae per cell for bacterial binding. Psa might first encounter and bind to galactose residues that decorate glycolipid or glycoprotein on cell membranes from a greater distance than PCho before binding

with greater avidity to PC. Whether shear forces enhance the binding of Psa, as described for some other fimbriae, remains to be determined (4).

Materials and Methods

Psa-Expressing Bacteria and *psaA* Mutagenesis. Recombinant Psa was expressed on the nonfimbriated *E. coli* host strain SE5000 (MC4100 *recA56* Fim[−]) from plasmid pGS1, and *psaA* site-directed mutants were constructed by fusion PCR (Table S2) as described in *SI Materials and Methods*.

Isolation of Psa Fimbriae: Binding and Agglutination Assays. The isolation of Psa fimbriae, binding, and agglutination assays were described previously (5, 7, 9), with slight modifications given as part of *SI Materials and Methods*.

Cloning, Expression, and Purification of *in cis* dscPsaA for Structure Analysis. An *in cis* donor-strand complemented monomeric *psaA* (*dscpsaA*) construct with a His-tag was created by PCR (Table S2) using pGS1 as a template to create pET-22b::*dsc18psaA(his)*₆ for the expression and purification of a stable dscPsaA protein, using a previously described strategy (31), as detailed in *SI Materials and Methods*.

Crystallization, Structure Determination, Refinement for dscPsaA Structures, and ITC. Details of crystallization and ITC procedures are given in *SI Materials and Methods*.

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