Published in final edited form as:

FEMS Microbiol Lett. 2010 January ; 302(2): 106–113. doi:10.1111/j.1574-6968.2009.01827.x.

Biogenesis of *Yersinia pestis* PsaA in Recombinant Attenuated *Salmonella* Typhimurium Vaccine (RASV) Strain

Ascención Torres-Escobar, María Dolores Juárez-Rodríguez, and Roy Curtiss III

Center for Infectious Diseases and Vaccinology at Biodesign Institute and School of Life Sciences, Arizona State University. Tempe, AZ, 85287-5401, USA

Abstract

Yersinia pestis PsaA is an adhesin, important for the establishment of bacterial infection. PsaA synthesis requires the products of the *psaEFABC* genes. Here, by prediction analysis, we identified a PsaA signal sequence with two signal peptidase (SPase) cleavage sites, type-I and type-II (SPase-I and SPase-II). By Edman degradation and site-directed mutagenesis, the precise site for one of these SPase-I PsaA cleavage sites was located between alanine and serine at positions 31 and 32, respectively. *Y. pestis* *psaA* expression and the role of the PsaB and PsaC proteins were evaluated in RASV strains. PsaA was detected in total extracts as a major 15 kDa (mature) and 18 kDa (unprocessed) protein bands. PsaA synthesis was not altered by a $\Delta A31-\Delta S32$ double deletion mutation. In contrast, the synthesis of PsaA ($\Delta A31-\Delta S32$) in *Y. pestis* and delivery to the supernatant was decreased. Otherwise, substitution of the amino acid cysteine at position 26 by valine involved in the SPase-II cleavage site did not show any effect on the secretion of PsaA in *Salmonella* and *Yersinia*. These results help clarify the secretion pathway of PsaA for the possible development of vaccines against *Y. pestis*.

Keywords

Signal peptidase; Chaperone protein; Usher protein; *Yersinia pseudotuberculosis*; *Yersinia enterocolitica*

Introduction

The *Yersiniae* are Gram-negative bacteria with 11 species including the gastrointestinal pathogens *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, and the systemic pathogen *Y. pestis*, which is typically fatal without treatment. Genetic and whole-genome studies indicate that *Y. pestis* is closely related to *Y. pseudotuberculosis*. In contrast, *Y. enterocolitica* is distantly related to *Y. pestis* and *Y. pseudotuberculosis* displaying a more variable genomic arrangement (Achtman *et al.*, 1999). *Y. pestis* is the etiological agent of plague in humans (Perry & Fetherston, 1997) and a recently recognized re-emerging disease. The wide-spread aerosol dissemination combined with high mortality rates make *Y. pestis* a deadly pathogen (Inglesby *et al.*, 2000).

PsaA fimbriar protein serves as an important adhesin in the establishment of *Y. pestis* infections in the three known clinical forms, *i.e.*, bubonic, septicemic or pneumonic development (Cathelyn *et al.*, 2006; Chauvaux *et al.*, 2007; Liu *et al.*, 2009). PsaA forms fimbria-like structures on the bacterial surface when grown in acidic culture medium at 35°

to 41° C (Ben Efraim *et al.*, 1961; Lindler *et al.*, 1990). The lack of synthesis of PsaA in the KIM5 strain causes virulence reduction and an increase in the LD₅₀ of at least 100 fold in mice after retroorbital injection (Lindler *et al.*, 1990). The *psaA* gene is transcribed in the *psaEFABC* operon of *Y. pestis* and *Y. pseudotuberculosis*, with *psaEF* encoding the activator/sensor proteins, while *psaBC* encodes the chaperone/usher proteins (Lindler and Tall, 1993; Yang and Isberg, 1997). This operon is homologous to the *myfEFABC* locus of *Y. enterocolitica* (Iriarte *et al.*, 1993).

The signal peptide of *Y. enterocolitica* MyfA was identified (Iriarte *et al.*, 1993) and needs to be determined for PsaA in both *Y. pseudotuberculosis* and *Y. pestis*. In bacteria, a signal peptide present on proteins that are destined either to be secreted or to be membrane components, it is usually at the amino-terminal and normally absent from the mature protein. The signal peptide is removed by SPases as a SPase-I or a SPase-II (processing of prolipoproteins) (Yamaguchi *et al.*, 1998; Tuteja, 2005).

Recently, a new generation of improved Recombinant Attenuated *Salmonella* Vaccine (RASV) strains, such as, *Salmonella enterica* serovar Typhimurium χ 9558 have been developed and tested using heterologous antigens (Li *et al.*, 2009). These RASV strains will facilitate investigations into the role of selected amino acids in the biogenesis of *Y. pestis* PsaA. The focus of this present study is a better understanding of the PsaA translocation process and improvement of its secretion, with the eventual goal of developing a subunit vaccine against *Y. pestis*.

Materials and Methods

Bacterial strains and media

Escherichia coli, *Salmonella*, *Y. pestis* strains and plasmids used in this study are listed in Table 1. *E. coli* and *Salmonella* strains were grown in Luria-Bertani (LB) medium (1% bacto tryptone, 1% NaCl, 0.5% yeast extract) or on 1.5% LB agar or on McConkey (Difco), when required, the medium was supplemented with 50 μ g mL⁻¹ ampicillin, 10 μ g mL⁻¹ nalidixic acid, 0.2% mannose or 50 μ g mL⁻¹ diaminopimelic acid for growing the strain with Δ *asdA* mutation.

DNA procedures

DNA manipulations were carried out as described by Sambrook *et al.*, 2001. All primers (Integrated DNA Technology) were flanked with restriction enzymes, (uppercase in the primer sequences) as shown in Table S2. The *psaEFABC* genes were amplified by PCR from *Y. pestis* KIM6⁺ strain chromosome, and constructions were verified by DNA sequencing (Arizona State University Facilities). Fifteen codons from *Y. pestis* *psaA* were substituted with the most frequently used codons found in *Salmonella* genes for optimization of *Y. pestis* *psaA* expression in RASV strains. All amino acid substitutions and deletions in *Y. pestis* *psaA* were performed using a Quick-Change site-directed mutagenesis kit (Stratagene). The presence of a desired mutation was verified by DNA sequencing (Figure 1A, Table 1).

Expression of *psaABC* in *E. coli* and *Y. pestis*

The recombinant PsaA-AU1-6XHis protein was overexpressed in *E. coli* strain LMG194, transformed with the pYA3883 (Table 1) and grown in 1X minimal salts media (Curtiss, 1965), supplemented with 0.2% (v/v) glycerol, 2% casamino acids, 50 μ g mL⁻¹ thiamine, and 100 μ g mL⁻¹ ampicillin at 37°C to an optical density at 600nm (OD₆₀₀) of 0.5, and then induced with arabinose at 0.05% for 5 hours (h). *Y. pestis* harboring a different *psaA* expression pYA4787 plasmid derivative was grown in 3 mL of heart infusion broth at 28°C

until an OD₆₀₀ of 0.5, and then centrifuged. The pellet was then resuspended in 100 µL of brain heart infusion broth and inoculated into 3 mL of brain heart infusion broth with 0.5% yeast extract pH6 and grown at 37°C for 8 h.

PsaA-AU1-6XHis purification

The recombinant PsaA-AU1-6XHis protein was purified by nickel-nitrilotriacetic acid agarose chromatography under denaturing conditions. Protein concentration was determined using the Bradford assay with bovine serum albumin as the standard. The PsaA-AU1-6XHis purified protein was used to immunize rabbits for production of PsaA polyclonal antibody.

Cell fractionation and Immunoblots

Cell fractions were prepared from 1 mL of culture by using the PeriPreps periplasting Kit (Epicentre) following the manufacturer's instruction. For isolating proteins released into the culture supernatants, 1 mL of each sample was filtered (0.22-mm pore size, Corning), and precipitated with 10% TCA, then pelleted by centrifugation and resuspended in 100 µL of LDS sample buffer. Each 10 µL fraction was separated on a 4-12% SDS-PAGE (NuPAGE Bis-Tris, Invitrogen) and transferred to nitrocellulose sheets (Bio-Rad). The recombinant protein was immunolocalized using rabbit anti-PsaA serum (1:15000) followed by alkaline phosphatase-conjugated anti-rabbit IgG (Sigma). The rabbit anti-AU1 epitope tag (1:5000) (Bethyl) was used to monitor the eluted fractions during the purification procedure of PsaA-AU1-6XHis (Jenson *et al.*, 1997) (data not shown). All experiments were performed in triplicate.

Amino acid sequence of the N-terminal region of *Y. pestis* PsaA protein

The PsaA protein from *Y. pestis* P325 transformed with pYA4788 (Table 1) was isolated from the periplasmic fraction using the PeriPreps periplasting Kit (Epicentre) by cutting the identified band from a PVDF membrane (Invitrogen) after separation and transfer from an SDS-PAGE gel. The Edman degradation method (Edman, 1960) was used to determine the amino-terminus sequence of the mature PsaA in two independent experiments (Arizona State University Facilities).

Results

Determination of the cleavage site of *Y. pestis* PsaA

PsaA is predicted to be a 163 amino acid protein with an estimated molecular mass of 17.93 kDa. Sequence analysis of PsaA with the computer algorithm SignalP 3.0, LipoP 1.0 and DOLOP (Bendtsen *et al.*, 2004; Babu *et al.*, 2006) predicted a prokaryotic signal sequence at its amino-terminal region with a potential SPase-I cleavage site between alanine at position 31 and serine at position 32 (ANA[▼]S⁺¹T⁺²) with an expected mature form of 14.6 kDa. In addition, a putative SPase-II cleavage site was identified between the alanine at position 25 and the cysteine at position 26 (IAA[▼]C⁺¹G⁺²) with an expected PsaA mature form of 15.1 kDa. To identify the cleavage site in PsaA, the first 10 amino acids of the mature PsaA form were determined by Edman degradation and matched exactly from serine at position 32 to serine at position 41 of *Y. pestis* PsaA, indicating that the cleavage site is located between alanine 31 and serine 32 and it is recognized by the SPase-I (Fig. 1A).

Validation of the amino acid residues involved in the SPase sites of *Y. pestis* PsaA

The substitution of amino acid residues involved in the SPase-I and SPase-II sites of *Y. pestis* PsaA was evaluated in the *Y. pestis* P325 strain, which does not express the PsaA unless the strain is complemented with the pYA4788 harboring the *psaEFABC* locus (Fig. 2A). When the PsaA amino acid alanine 31 (pYA4792) or serine 32 (pYA4793) involved in

SPase-I cleavage site was deleted, the PsaA was observed in all subcellular fractions (data not shown). However, secretion of the PsaA $\Delta A31\text{-}\Delta S32$ double deletion (pYA4794) was drastically decreased in the supernatant fraction (Fig. 2B, lane 4). In contrast, when the cysteine 26 involved in the SPase-II recognition site was changed by valine, PsaA synthesis and secretion were not affected (data not shown); similarly, substitution of cysteine at position 10 by valine (pYA4789) or C10V-C26V double substitution (pYA4791) (Fig. 2B, lane 3) did not affect PsaA synthesis and its secretion. These data confirm that the predicted SPase-I cleavage site is located between the alanine 31 and serine 32.

PsaA synthesis and secretion to the extracellular medium in RASV strain

The RASV $\chi 9558$ strain was described in detail by Li *et al.*, 2009. This *Salmonella* strain contains the deletion-insertion mutation $\Delta relA198::araC P_{BAD} lacI$ TT, that encodes for arabinose regulated LacI synthesis, which regulates the expression from P_{trc} . The $\chi 9558$ strain was transformed with the plasmid pYA3705 and the expression of *psaA* under P_{trc} was analyzed in LB with 0.2% or without arabinose at 37°C until an OD₆₀₀ of 0.8. The PsaA protein had an unprocessed form of 18 kDa and a mature form of 15 kDa in total cell extracts of both growth conditions, with an expected reduction in synthesis with arabinose due to the *lacI* repressor gene expression. In the periplasmic fraction and in the culture supernatant PsaA was detected as a mature 15 kDa protein (Fig. 3A). The detection of PsaA in the supernatant was a result of its secretion, since the detection of the cytoplasmic protein σ^{70} was only observed in the total extract and not in the supernatant (Fig. 3B). The PsaA synthesis profile with *psaA*-optimized (pYA3705) was similar to the *psaA* wild type (pYA3704) (Fig. 4A, lane 7 and 8). To analyze the synthesis and secretion of *Y. pestis* PsaA in $\chi 9558$ strain, growth was compared using 0.2 %, 0.02% and 0.0% arabinose in the culture medium. Synthesis was found to be proportional at these different concentrations and we report the results without arabinose.

Function of *Y. pestis* PsaB and PsaC in RASV strain

The synthesis of the PsaB chaperone protein was required for export of PsaA from the cytoplasm to the periplasmic space (pYA4798), but the secretion of PsaA to the supernatant was almost undetectable (Fig. 4A, lane 5). The detection of PsaA co-expressed with PsaC (pYA4799) (Fig. 4A lane 6) was sparsely localized to the membrane fraction. Conversely, when a set of 16 amino acid residues was added in frame at the carboxy-terminal of PsaA (pYA4797), the product of *psaA* minus *psaBC* was detected as a ~19 kDa protein band in total extracts (Fig. 4A lane 4), barely delivered in the supernatant and was in an insoluble form in the membrane fractions. When a set of 8 amino acid residues was added in frame at the carboxy-terminal end of PsaA without PsaBC (pYA4796), PsaA was not detected (data not shown). These results indicate that PsaB and PsaC are essential for the processing and translocation of PsaA from the cytoplasm to the cell surface in *Salmonella*.

Role of the amino and carboxy-terminal regions of *Y. pestis* PsaA in the RASV strain

When the first 26 amino acids of PsaA amino-terminal region (pYA3711) were deleted, it prevented the translocation of PsaA from the cytoplasm to the cell surface, and the unprocessed PsaA form was not observed and the mature 15 kDa protein was decreased in the total extract, cytoplasm and membrane fractions (Fig. 4B lane 1). Deletion of the last 9 amino acids of PsaA at the carboxy-terminal region (pYA4800), from threonine at position 155 to phenylalanine at position 163, drastically decreased its expression and was barely detectable as a ~13.5 kDa product in supernatant and membrane fraction (Fig. 4A lane 9). These results indicate that the amino-terminal region is necessary to secrete PsaA and the carboxy-terminal region is required for its stability.

Substitution and deletion on PsaA of the amino acids involved in the SPase cleavage sites in the RASV strain

Deletion of the PsaA A31 (pYA4374) or S32 (pYA4375), which form part of the SPase-I cleavage site, did not affect the synthesis or secretion of PsaA as observed in all subcellular fractions (Fig. 4B, lane 7 and 8), but with the Δ A31- Δ S32 double-deletion (pYA4376), the unprocessed 18kDa product was not detected in the total extract and barely observed in the membrane fraction (Fig. 4B lane 9). In contrast, when the amino acids involved in the PsaA predicted SPase-II cleavage site, cysteine at position 26 changed to valine (pYA3708) and the glycine at position 27 by serine (pYA3709), the PsaA synthesis was not affected (Fig. 4B, lane 5 and 2). To determine if the cysteine residues at position 10 and 26 play a role in the PsaA biogenesis and stability, the cysteine10 (pYA3707) was replaced with valine and either cysteine were changed to valine (pYA3706). None of these mutations affected PsaA synthesis or secretion (Fig. 4B, lane 4 and 6). We observed the same expression profile, when the RASV strain containing each one of the previously described plasmids was grown with either 0.2% or 0.02% arabinose in the culture medium (data not shown). The amino acid substitution of the putative glycosylation site, asparagine 30 to leucine (pYA3710) produced a shorter unprocessed ~17 kDa PsaA (Fig. 4B lane 3). These results indicate that in the absence of either A31 or S32, other amino acids flanking this SPase-I cleavage site can generate alternative cleavage sites, but deletion of both A31-S32 produces a new cleavage site, which is processed more efficiently than the original. On the other hand, the C10V and C26V or double-substitution (C10V-C26V) and G27S had no effect on the translocation of PsaA to the cell surface.

Discussion

In this study, we elucidated the role in secretion and biogenesis of the *Y. pestis* PsaA amino-terminal and carboxy-terminal regions.

Using different computer analyses we identified two putative signal peptidase cleavage sites in the PsaA signal sequence, with their tripartite consensus regions: n-, a positively charged amino-terminus; h- a hydrophobic core; and c-, terminal cleavage site. In Gram-negative bacteria the lipoproteins are anchored to either the inner or the outer membrane and an aspartic acid residue at position +2 (D^{+2}) is proposed to determine the final destination of the lipoproteins (Yamaguchi *et al.*, 1998). The D^{+2} substitution to amino acid residues as phenylalanine, tryptophan, tyrosine, glycine and proline maintains the retention of the lipoprotein to the periplasmic face of the cytoplasmic membrane (Seydel *et al.*, 1999). The glycine at position 27 is the amino acid +2 in the *Y. pestis* PsaA putative SPase-II cleavage site and substitution of the amino acids from this cleavage site, such as C26V (pYA3708) and G27S (pYA3709) did not show any effect in the translocation process of PsaA, neither did the substitution C10V (pYA3707) or double substitution C10V-C26V (pYA3706). Further studies using electron microscopy will be required to determine whether the PsaA structure and its assembly into multisubunit protein polymers are affected by the mutations on PsaA cysteine residues. Surprisingly, the substitution of the hydrophilic asparagine at position 30 to the hydrophobic leucine generated a shorter unprocessed PsaA form, but the mature PsaA form did not change. The asparagine at position 30 forms part of the putative glycosylation consensus sequence, N-X-S/T, where X can be any amino acid except proline (Fig. 1A) (Gavel & von Heijne). However, to date no N-glycosylation system has been reported in *Salmonella* or *Yersinia* (Upreti *et al.*, 2003). In our analysis, the mechanism by which the substitution of N30L that generates the shorter unprocessed form of PsaA remains to be clarified. With the deletion of either A31 or S32 or both, alternative cleavage sites could be generated among the flanking amino acid residues as asparagine, serine and threonine with similar properties (polar, hydrophilic and neutral). Surprisingly, the PsaA with the SPase-I cleavage site derived by the Δ A31- Δ S32 double deletion mutations was

more efficiently secreted in *Salmonella*, but in *Yersinia* impaired the secretion of PsaA to the supernatant, indicating a different affinity for the SPase-I cleavage site between *Salmonella* and *Yersinia*.

Two highly conserved regions were observed between the amino acid sequence of PsaA and its counterpart MyfA in *Y. enterocolitica*, one at the amino-terminal region and the second at the carboxy-terminal region (Fig 1B). Similar regions play a role in the interaction with the chaperone and are therefore essential for the biogenesis of the protein (Kuehn *et al.*, 1993; Soto *et al.*, 1998). Here we show that when nine alternating hydrophobic/hydrophilic residues are removed from the carboxy-terminal end of PsaA, the PsaA synthesis is drastically affected even with the co-expression of the chaperone and usher protein PsaB/PsaC. Although additional experiments are required to validate this result, this suggests that this PsaA region is essential for its biogenesis.

The co-expression of PsaA with the PsaB chaperone protein allowed the detection of PsaA in the cytoplasm, periplasm and membrane fraction. The role of PsaB in the cytoplasm possibly to avoid the degradation of PsaA and the cytoplasmic interactions between PsaA/PsaB still needs to be established. In contrast, the co-expression of PsaA with only PsaC resulted in a lack of detection of PsaA, confirming that the interaction of PsaABC proteins is essential in the secretion process of PsaA.

These results will help to provide new design strategies for delivery of PsaA in RASV strains using their own secretion pathway, this combined with a new more efficient SPase-I cleavage site should aid in improving RASV for the effective delivery *Y. pestis* antigens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are thankful to Dr. J.D. Fetherston (University of Kentucky, Lexington) who generously provided the *Y. pestis* P325 strain. We also thank Dr. Clara Espitia (UNAM, México) for her critically reading of the manuscript and Dr. David S. Reiner (Burnham Institute for Medical Research, USA) and Isabel Perez Montfort (UNAM, México) for correction of the English version of this manuscript. This research was supported by the National Institutes of Health, grant AI057885.

References

- Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, Carniel E. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A*. 1999; 96:14043–8. [PubMed: 10570195]
- Babu MM, Priya ML, Selvan AT, Madera M, Gough J, Aravind L, Sankaran K. A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. *J Bacteriol*. 2006; 188:2761–73. [PubMed: 16585737]
- Ben-Efraim S, Aronson M, Bichowsky-Slomnicki L. New antigenic component of *Pasteurella pestis* formed under specified conditions of pH and temperature. *J Bacteriol*. 1961; 81:704–14. [PubMed: 16561898]
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol*. 2004; 340:783–95. [PubMed: 15223320]
- Cathelyn JS, Crosby SD, Lathem WW, Goldman WE, Miller VL. RovA, a global regulator of *Yersinia pestis*, specifically required for bubonic plague. *Proc Natl Acad Sci U S A*. 2006; 103:13514–9. [PubMed: 16938880]

- Chauvaux S, Rosso ML, Frangeul L, et al. Transcriptome analysis of *Yersinia pestis* in human plasma: an approach for discovering bacterial genes involved in septicemic plague. *Microbiology*. 2007; 153:3112–23. [PubMed: 17768254]
- Curtiss R III. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. *J Bacteriol*. 1965; 89:28–40. [PubMed: 14255678]
- Curtiss R III. Regulation of attenuation of live vaccines to enhance cross protective immunity. International patent. 2003 WO 03/096812.
- Edman P. Phenylthiohydantoin in protein analysis. *Ann NY Acad Sci*. 1960; 88:602–610. [PubMed: 13725736]
- Gavel Y, von Heijne G. Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. *Protein Eng*. 1990; 3:433–42. [PubMed: 2349213]
- Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Koerner JF. Plague as a biological weapon: medical and public health management. *JAMA*. 2000; 283:2281–90. [PubMed: 10807389]
- Iriarte M, Vanooteghem JC, Delor I, Díaz R, Knutton S, Cornelis GR. The Myf fibrillae of *Yersinia enterocolitica*. *Mol Microbiol*. 1993; 9:507–20. [PubMed: 8105362]
- Jenson AB, Jenson MC, Cowser L, Ghim SJ, Sundberg JP. Multiplicity of uses of monoclonal antibodies that define papillomavirus linear immunodominant epitopes. *Immunol Res*. 1997; 16:115–119. [PubMed: 9048212]
- Kang HY, Srinivasan J, Curtiss R III. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar typhimurium vaccine. *Infect Immun*. 2002; 70:1739–49. [PubMed: 11895935]
- Kuehn MJ, Ogg DJ, Kihlberg J, Slonim LN, Flemmer K, Bergfors T, Hultgren SJ. Structural basis of pilus subunit recognition by the PapD chaperone. *Science*. 1993; 262:1234–41. [PubMed: 7901913]
- Li Y, Wang S, Scarpellini G, Gunn B, Xin W, Wanda SY, Roland KL, Curtiss R III. Evaluation of new generation *Salmonella enterica* serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against. *PspA Proc Natl Acad Sci U S A*. 2009; 106:593–8.
- Lindler LE, Klempner MS, Straley SC. *Yersinia pestis* pH 6 antigen: genetic, biochemical, and virulence characterization of a protein involved in the pathogenesis of bubonic plague. *Infect Immun*. 1990; 58:2569–77. [PubMed: 2164509]
- Lindler LE, Tall BD. *Yersinia pestis* pH 6 antigen forms fimbriae and is induced by intracellular association with macrophages. *Mol Microbiol*. 1993; 8:311–24. [PubMed: 8100346]
- Liu H, Wang H, Qiu J, et al. Transcriptional profiling of a mice plague model: insights into interaction between *Yersinia pestis* and its host. *J Basic Microbiol*. 2009; 49:92–99. [PubMed: 18759226]
- Nakayama K, Kelly SM, Curtiss R III. Construction of an ASD+ Expression-Cloning Vector: Stable Maintenance and High Level Expression of Cloned Genes in a *Salmonella* Vaccine Strain. *Bio/Technology*. 1988; 6:693–697.
- Perry RD, Fetherston JD. *Yersinia pestis*--etiologic agent of plague. *Clin Microbiol Rev*. 1997; 10:35–66. [PubMed: 8993858]
- Sambrook, J.; Russell, DW. *Molecular cloning: a laboratory manual*. 3rd. Cold Spring Harbor Laboratory Press, Cold Spring Harbor; NY: 2001.
- Seydel A, Gounon P, Pugsley AP. Testing the '+2 rule' for lipoprotein sorting in the *Escherichia coli* cell envelope with a new genetic selection. *Mol Microbiol*. 1999; 34:810–21. [PubMed: 10564520]
- Soto GE, Dodson KW, Ogg D, Liu C, Heuser J, Knight S, Kihlberg J, Jones CH, Hultgren SJ. Periplasmic chaperone recognition motif of subunits mediates quaternary interactions in the pilus. *EMBO J*. 1998; 17:6155–67. [PubMed: 9799225]
- Tuteja R. Type I signal peptidase: an overview. *Arch Biochem Biophys*. 2005; 441:107–11. [PubMed: 16126156]
- Upreti RK, Kumar M, Shankar V. Bacterial glycoproteins: functions, biosynthesis and applications. *Proteomics*. 2003; 3:363–79. [PubMed: 12687605]

- Yamaguchi K, Yu F, Inouye M. A single amino acid determinant of the membrane localization of lipoproteins in *E. coli*. *Cell*. 1988; 53:423–32. [PubMed: 3284654]
- Yang Y, Isberg RR. Transcriptional regulation of the *Yersinia pseudotuberculosis* pH6 antigen adhesin by two envelope-associated components. *Mol Microbiol*. 1997; 24:499–510. [PubMed: 9179844]

Fig. 1.

A) *Y. pestis* *psaA* nucleotide and protein sequence. The putative start codons are boxed and indicated by arrows with the expected molecular mass. The amino acid residues shown in bold were changed to the amino acid residues above them. The amino acid residues deleted are indicated by Δ. The changed third base for optimization of the fifteen codons is indicated in bold above the codon. The signal peptidase type I cleavage site is indicated by the scissors, the N-glycosylation sequence is underlined. The amino acid sequence determined by Edman degradation is indicated inside a gray box. B) Protein sequence alignment of *Y. pestis* PsaA (Yp), *Y. pseudotuberculosis* PsaA (Yptb) and *Y. enterocolitica* MyfA (Ye). Residue numbers are shown to the right of each protein sequence and the periplasm chaperone recognition motifs (PCRM) are underlined and in bold. *Y. enterocolitica* MyfA signal peptide is indicated in bold. Cysteine residues are indicated by an asterisk.

Fig. 2.

A) Immunoblot of cell fractions profile of the *Y. pestis* P325 alone or harboring pYA4788. B) Immunoblot of cell fractions profile of the *Y. pestis* P325 strain harboring different PsaA expression plasmids lanes: 1, pYA4787 cloning vector used as control; 2, pYA4788; 3, pYA4791 [PsaEFA(C10V-C26V)BC]; 4, pYA4794 [PsaEFA(Δ A31- Δ S32)BC]. M, molecular mass makers (Invitrogen). Total extract TE, supernatant SN, periplasm P, cytoplasm C. The molecular marked bands (22 kDa, 16 kDa) are indicated by a dash at the side of the blot. The unprocessed PsaA is indicated by the arrow, the mature PsaA is showed by the asterisk, either on the blot and at the side of the blot.

Fig. 3.

A) Immunoblot of cell fractions profile of the *S. enterica* serovar Typhimurium χ 9558 harboring pYA3705. The χ 9558 strain was grown in LB with mannose 0.2% (+) and arabinose 0.2% (+) or without arabinose at 37° C, with shaking to 0.8 units of an OD₆₀₀. B) Immunoblot of an analogous cell fractions profile of χ 9558 harboring pYA3705 with anti- σ^{70} antibody was used as a loading control on total extract and supernatant. M, molecular mass markers (Invitrogen). The molecular marked bands (22 kDa, 16 kDa) are indicated by a dash at the side of the blot. The unprocessed PsaA is indicated by the arrow, the mature PsaA is showed by the asterisk, either on the blot and at the side of the blot.

Fig. 4.

Immunoblot of cell fractions profile of the *S. enterica* serovar Typhimurium χ 9558. A) lanes: 1, χ 9558 strain alone; 2, pYA3342 cloning vector; 3, pYA4795; 4, pYA4797; 5, pYA4798; 6, pYA4799; 7, pYA3704; 8, pYA3705 and 9, pYA4800. B) lanes: 1, pYA3711; 2, pYA3709; 3, pYA3710; 4, pYA3707; 5, pYA3708; 6, pYA3706; 7, pYA4374; 8, pYA4375 and 9, pYA4376. The RASV strain with and without plasmid was grown in LB medium with shaking at 37°C until 0.8 units of OD₆₀₀ was reached. The unprocessed PsaA is indicated by the arrow, the mature PsaA is shown by the asterisk, the truncated size of the PsaA is indicated by a T and the PsaA-AU1-6XHis is indicated by a H. Detection of σ^{70} with anti- σ^{70} antibody was used as a loading control on total extract.

Table 1

Strains and plasmids used in this work

Strain or plasmid	Relevant genotype ^a or characteristics ^b	Source or reference
<i>E. coli</i>		
LMG194	$\Delta ara714$	Invitrogen
<i>S. enterica</i> serovar Typhimurium		
$\chi 9558$	$\Delta asd27::TTaraC$ P _{BADc2} $\Delta relA198::araC$ P _{BADlacITT}	Li <i>et al.</i> , 2009
<i>Y. pestis</i>		
KIM6 ⁺	Pgm ⁺ , pMT1, pPCP1, cured of pCD1	Perry RD UK, Lexington, KY
P325	$\Delta psaEFAB$, partial deletion of <i>psaC</i>	Fetherston JD UK, Lexington, KY
Plasmid		
pBAD-HA	Amp ^r pBR <i>origin</i> replication	Invitrogen
pBK-CMV	Kam ^r pUC <i>origin</i> replication	Stratagene
pYA3342	Asd ⁺ P _{trc} pBR <i>origin</i> replication	Kang <i>et al.</i> , 2002
pYA3337	Asd ⁺ P _{trc} pSC101 <i>origin</i> replication	Curtiss, 2003
pYA4787	Amp ^r pSC101 <i>origin</i> replication	This study
Derived from pBK-CMV		
pYA3970	<i>psaA</i>	This study
Derived from pBAD-HA		
pYA3883	P _{BAD} <i>psaA</i> -(AU1)-(6XHis) <i>BC</i>	This study
pYA3884	P _{BAD} <i>psaA</i> -(AU1)-(KLGCFGG)	This study
pYA3867	P _{BAD} <i>psaA</i> (ΔSP -I) <i>BC</i>	This study
pYA3952	P _{BAD} <i>psaA</i>	This study
pYA3953	P _{BAD} <i>psaAB</i>	This study
pYA3954	P _{BAD} <i>psaABC</i>	This study
pYA3955	P _{BAD} <i>psaA</i> (codon optimized) <i>BC</i>	This study
pYA3957	P _{BAD} <i>psaA</i> (C10V-C26V) <i>BC</i>	This study
pYA3959	P _{BAD} <i>psaA</i> (C10V) <i>BC</i>	This study
pYA3961	P _{BAD} <i>psaA</i> (C26V) <i>BC</i>	This study
pYA3963	P _{BAD} <i>psaA</i> (G27S) <i>BC</i>	This study
pYA3965	P _{BAD} <i>psaA</i> (N30L) <i>BC</i>	This study
Derived from pYA3342		
pYA4795	P _{trc} <i>psaA</i>	This study
pYA4796	P _{trc} <i>psaA</i> -(AU1)	This study
pYA4797	P _{trc} <i>psaA</i> -(AU1)-(6XHis)	This study
pYA4798	P _{trc} <i>psaAB</i>	This study
pYA4799	P _{trc} <i>psaAC</i>	This study
pYA4800	P _{trc} <i>psaA</i> (Δ carboxy-terminal region) <i>BC</i>	This study
pYA3704	P _{trc} <i>psaABC</i>	This study
pYA3705	P _{trc} <i>psaA</i> (codon optimized) <i>BC</i>	This study

Strain or plasmid	Relevant genotype ^a or characteristics ^b	Source or reference
pYA3707	<i>P_{trc}psaA(C10V)BC</i>	This study
pYA3708	<i>P_{trc}psaA(C26V)BC</i>	This study
pYA3706	<i>P_{trc}psaA(C10V-C26V)BC</i>	This study
pYA3709	<i>P_{trc}psaA(G27S)BC</i>	This study
pYA3710	<i>P_{trc}psaA(N30L)BC</i>	This study
pYA3711	<i>P_{trc}psaA(ΔSP-I)BC</i>	This study
pYA4374	<i>P_{trc}psaA(ΔA31)BC</i>	This study
pYA4375	<i>P_{trc}psaA(ΔS32)BC</i>	This study
pYA4376	<i>P_{trc}psaA(ΔA31-ΔS32)BC</i>	This study
Derived from pYA4787		
pYA4788	<i>psaEFABC</i>	This study
pYA4789	<i>psaEFA(C10V)BC</i>	This study
pYA4790	<i>psaEFA(C26V)BC</i>	This study
pYA4791	<i>psaEFA(C10V-C26V)BC</i>	This study
pYA4792	<i>psaEFA(ΔA31)BC</i>	This study
pYA4793	<i>psaEFA(ΔS32)BC</i>	This study
pYA4794	<i>psaEFA(ΔA31-ΔS32)BC</i>	This study

^a In the descriptions of the genotype, TT is transcription terminator, P stands for promoter, and the subscripted number refers to a composite deletion and insertion of the indicated gene.

^b Amp^r ampicillin resistance, Cam^r chloramphenicol resistance, Kan^r kanamycin resistance, Tet^r tetracycline resistance.