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The *psa* Locus Is Responsible for Thermoinducible Binding of *Yersinia pseudotuberculosis* to Cultured Cells

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***Yersinia pseudotuberculosis inv* mutant strains cured of the virulence plasmid exhibit thermoinducible adhesion to cultured mammalian cells. To identify the genes responsible for this phenotype, *Y. pseudotuberculosis* homologs of the *Y. enterocolitica ail* and the *Y. pestis psa* loci were identified. Mutations in the *Y. pseudotuberculosis ail* and *psa* loci were constructed and tested for thermoinducible binding. Results of cellular binding assays indicated that only mutations in *psa*, not in *ail*, resulted in defects for thermoinducible binding, with *inv yadA psa* strains showing no detectable cell adhesion. In addition, an *inv psa* strain was defective for hemagglutination of sheep erythrocytes, in contrast to an *inv psa*$^+$ strain which was fully competent for hemagglutination. The introduction of a plasmid containing a 6.7-kb *Kpn*I-*Cla*I fragment of *Y. pseudotuberculosis* encompassing the *psa* locus was sufficient to complement both the cell adhesion and hemagglutination defects of the *psa* mutant. Results from subcloning and transposon mutagenesis indicated that the complete 6.7-kb region was required for thermoinducible binding and hemagglutination.**

The interaction of enteropathogenic yersiniae with host cells is an important determinant of pathogenicity of these microorganisms. Infection of mammalian hosts by yersiniae is initiated by penetration through intestinal mucosal cells (8, 9, 10, 12). Furthermore, the efficient deposition within target cells of virulence proteins requires binding to host cells (2). Genetic and biochemical studies of the interaction of bacteria with cultured mammalian cells have allowed identification of many of the factors involved in adhesion.

For *Yersinia pseudotuberculosis* grown at ambient temperature, invasin contributes to adhesion of bacteria to cultured mammalian cells and results in their efficient uptake by mammalian cells (11). In addition, the *Y. pseudotuberculosis* virulence plasmid encodes an adhesive protein, YadA, that promotes efficient adhesion and a residual level of penetration (25). A third adhesion factor has been shown to exist in strains lacking YadA and invasin, with binding occurring best when such strains are grown at 37°C. This phenotype was originally designated thermoinducible binding (7). The significance of thermoinducible adhesion in terms of virulence of *Yersinia* infection is unknown, although specific attachment to mammalian cells is required for the function of virulence factors. For example, YopE and YopH, responsible for cytotoxicity and antiphagocytosis, require bacterial adhesion to mammalian cells for their proper deposition within target cells (2, 22).

There are several candidates for loci that could express the thermoinducible binding activity. In *Y. enterocolitica*, the Ail protein has been shown to promote tissue culture adherence and cell line-specific penetration. Expression of Ail is greater when bacteria are cultured at 37°C as opposed to ambient temperature (21). In *Y. pestis*, it has been shown that a pilus-like surface structure called pH 6 antigen, which is expressed at highest levels during growth at 37°C in acidic medium, promotes agglutination of many species of erythrocytes (RBC). The locus encoding pH 6 antigen in *Y. pestis* has been characterized, and at least three genes, *psaE*, *psaA*, and *psaB*, are involved in the synthesis of pH 6 antigen. The antigenic protein in this complex is the product of *psaA*, which apparently encodes a 16-kDa adhesin molecule with sequence similarity to pilus subunits (16, 17). Recently, a homolog of pH 6 antigen in *Y. enterocolitica*, called Myf (mucoid *Yersinia* factor), was identified (4, 5). The genetic structure of the *myf* locus is similar to that of *psa* and apparently includes five open reading frames (ORFs) required for expression and assembly. Myf is not able to mediate hemagglutination, even though its synthesis is regulated in a temperature- and pH-dependent fashion similar to that for *psa* (4, 5).

To investigate the factor(s) mediating thermoinducible binding in *Y. pseudotuberculosis*, we have carried out a series of genetic studies on the homologs of *ail* and *psa* in *Y. pseudotuberculosis*. In this report, we demonstrate that pH 6 antigen is responsible for thermoinducible binding in *Y. pseudotuberculosis* and that functional Ail protein expressed by this organism has no adhesive activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains, plasmids, and bacteriophage used in this study are described in Table 1. Growth media and antibiotics were as previously described (25). Routine growth temperatures were 37°C for *Escherichia coli* and 28°C for *Y. pseudotuberculosis*. For induction of pH 6 antigen of *Y. pseudotuberculosis*, bacteria were grown in L broth adjusted to pH 6.0 (acidic L broth) as described previously (16).

Cellular binding and uptake assays. Binding to cultured mammalian cells was assayed as described previously (25) by quantitating the number of bacteria bound per cell, using light microscopy of Giemsa-stained preparation. For thermoinducible binding assays, freshly diluted overnight cultures of bacteria were allowed to grow in four different conditions for approximately 4 h before they were subjected to cellular binding assays. The four conditions were 28°C in neutral L broth; 28°C in acidic L broth; 37°C in neutral L broth; 37°C in acidic L broth. HEp-2 cells were cultured overnight on 12-mm-diameter circular coverslips in microdilution wells of 24-well tissue culture plates (Falcon). After 90 min of incubation of bacteria on the HEp-2 monolayer, coverslips were washed, stained, dried, and mounted prior to microscopic observation (25). Binding was scored as the number of bacteria associated per cell after counting of approximately 100 cells per coverslip for each of the three coverslips, using at least 50 fields per slide.

The cellular uptake assay was as described previously (25).

Isolation of sequences homologous to *ail* of *Y. enterocolitica* in *Y. pseudotuberculosis*. Nine- to 23-kb fragments generated by partial *Sau*3AI digestion were

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TABLE 1. Strains, plasmids, and bacteriophage

Strain, plasmid, or bacteriophage	Genotype or features	Reference or source
E. coli		
MC1061	F$^-$ Δ(*ara leu*) Δ(*lac*) X74 *galE galK hsdR rpsL*	25
SY327λ*pir*	F$^-$ *araD* Δ(*pro lac*) XIII *argE*(Am) *rpoB gyrA recA56* (λ*pir*)	25
SM10λ*pir*	RP4 *kan*^{r} *tet*::Mu *thr leu supF* (λ*pir*) *recA*	25
DH5α	F$^-$ *endA1 hsdR supE* thi *recA1 gyrA relA1* Δ(*lacZYA argF*) *U169 deoR* [ϕ80d *lac*Δ (*lacZ*) M15]	Laboratory collection
LE392	F$^-$ *mcrA hsdR514 supE44 supF58 lacY galK2 galT22 metB1 trpR55*	Laboratory collection
Y. pseudotuberculosis		
YpIII(P$^-$)	*inv*$^+$; pIB1 cured	25
Yp332	*inv*::*tet*; pIB1 cured	25
YY060	*inv*$^+$ *ail*::*cam*; pIB1 cured	This study
YY115	*inv*::*tet ail*::*cam*; pIB1 cured	This study
YY033	*inv*::*tet psa*::*kan*; pIB1 cured	This study
Plasmids		
pRI122	*kan*	9
pJJM1	pGEM-1 *ail*$^+$ (*Y. enterocolitica*)	This study
pVM103	pBR322 *ail*$^+$ (*Y. enterocolitica*)	1
pMA4	pBluescript KS *ail*$^+$ (*Y. pseudotuberculosis*)	This study
pAY09	pACYC184 *psaEFA*	This study
pAY43	pBR322 *ail*$^+$ (*Y. pseudotuberculosis*)	This study
pAY01	pGP81*sacB*	25
pAY33	pAY01 *ail*::*cam* (*Y. pseudotuberculosis*)	This study
pAY15-4A	pAY01 Δ*psaEA*::*kan*	This study
pAY48	pACYC177 *psaEFABC*	This study
pAY50	pACYC177 *psaEFABC*	This study
Bacteriophage λ*b*221*PamOam*:Tn*5*-B21	Tn*5 tet lacZ*	R. Simon

purified from a 10 to 40% sucrose gradient. Fragments were then ligated with λGem12 that was predigested with *Xho*I and partially filled with Klenow enzyme and deoxynucleoside triphosphate mix (New England Biolabs, Beverly, Mass.). The ligation mixture was packaged (Stratagene, Calif.) and introduced into *E. coli* LE392. This λ phage library was then probed with α-^{32}P-labeled restriction fragments internal to the *Y. enterocolitica ail* gene (pVM103) (19), and plaques giving a positive signal were purified. Further work was performed on a single clone designated λ9.

Sequencing of *ail* in *Y. pseudotuberculosis*. Fragments generated by *Sau*3AI-*Eco*RI digestion of λ9 were cloned into M13mp18 and M13mp19 and sequenced by using universal primers. New primers were then designed from these newly obtained sequences for PCR amplification. They correspond to the sequences at nucleotides (nt) 321 to 341 (5′-CAATCTGGATGGTTTTTATG-3′) and nt 1516 to 1497 (5′-CCGAGCAAAATACCGATG-3′) on the *Y. pseudotuberculosis ail* gene. A 1.1-kb PCR fragment was obtained and cloned into M13mp18 and M13mp19 for complete sequencing.

Isolation of sequences homologous to *psaEAB* of *Y. pestis* in *Y. pseudotuberculosis*. Primers homologous to sequences at nt 20 to 41 (5′-GCTTGTATGCCATGCTGAAACG-3′) and nt 3589 to 3571 (5′-GAAGTGCTCATCAATAGAG-3′) in the published *Y. pestis psa* sequence (GenBank accession number M86713 [17]) were synthesized, and a 3.6-kb fragment was obtained by PCR amplification using these two primers and *Y. pseudotuberculosis* chromosomal DNA as a template.

Isolation of mutants containing *ail*::*cam* and *psa*::*kan* mutations in *Y. pseudotuberculosis* strains. *Y. pseudotuberculosis* strains containing *ail*::*cam* mutations were isolated by homologous recombination with the suicide vector pAY33 (Table 1). This plasmid contains the replication origin of R6K, *sacB*, *bla*, *mob* of RP4, and a 1.5-kb fragment of *Y. pseudotuberculosis* DNA containing an insertion of a *cam* cassette within the *ail* ORF. A 1.4-kb *Bsa*A1 fragment containing the *cam* gene from pACYC184 was inserted into the 1.5-kb PCR product containing the *ail* gene of *Y. pseudotuberculosis* at the *Bst*EII site, made blunt with T4 DNA polymerase. By selecting for sucrose and chloramphenicol resistance and screening for ampicillin sensitivity, *Y. pseudotuberculosis* mutants carrying the *ail*::*cam* mutation on the chromosome were obtained.

Isolation of the *psa*::*kan* mutation was performed as described above except that the suicide vector pAY15-4A was used and kanamycin resistance was selected instead of chloramphenicol resistance. This plasmid, pAY15-4A, has the same structure as pAY33, with the *psa*::*kan* region replacing the *ail*::*cam* region. In a 3.0-kb PCR fragment containing *psaEFA* and partial *psaB* sequences, the 1.3-kb region between *Bcl*I sites in *psaE* and *psaA* was replaced by a 1.3-kb *Bam*HI fragment containing *kan* gene from pRI122 (6). This mutation is referred to as Δ*psaEA*::*kan.*

Strains harboring the proper mutation as a result of the above-described recombination events were confirmed by Southern hybridization analyses (data not shown).

Isolation of molecular clones complementing *psa*::*kan* mutation in *Y. pseudotuberculosis*. *Y. pseudotuberculosis* chromosomal DNA was completely digested by *Cla*I prior to separation by electrophoresis on low-melting-temperature agarose. Fragments of 7 to 12 kb were purified, ligated with *Cla*I-linearized pACYC177, and introduced into *E. coli* MC1061, selecting for ampicillin resistance. Kanamycin-sensitive clones were identified and then probed with an α-^{32}P-labeled PCR fragment homologous to the sequences of *psa*, corresponding to nt 2840 to 3380 in the published sequence of *Y. pestis psaEAB* (17). Positive clones were tested in *E. coli* for the ability to confer hemagglutination and thermoinducible binding as well as in *Y. pseudotuberculosis* for the ability to complement the *psa* insertion-deletion mutation in YY033 (Δ*psaEA*::*kan*).

Transposon mutagenesis. Transposon mutagenesis was performed as described previously (25). pAY50, harbored in *E. coli* MC1061, was subjected to mutagenesis by transposition of Tn*5*-B21 from the replication-deficient phage λ*b*221*PamOam* (24). Twelve independent pools of mutagenized plasmids were generated. Four to five Amp^r Tet^r colonies were randomly picked from each pool and were tested for hemagglutination or thermoinducible binding.

Sequencing of the *psa* locus in *Y. pseudotuberculosis*. The 6.7-kb *Kpn*I-*Cla*I region of *Y. pseudotuberculosis* was sequenced by subcloning and double-stranded DNA sequencing, using appropriate synthetic oligonucleotide primers.

Preparation of antibody against Ail of *Y. pseudotuberculosis*. The *Y. pseudotuberculosis* Ail protein was purified from *E. coli* HB101(pMA4) as previously described (18) and eluted from a 4 to 20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel. Rabbit antisera were obtained by subcutaneous immunization with ~100 μg of Ail protein over a period of 8 weeks by standard techniques (14).

Western blot (immunoblot) analyses. Bacterial pellets of equal numbers of cells determined by optical density measurement were resuspended in protein loading buffer containing β-mercaptoethanol prior to boiling and loading on SDS–15% polyacrylamide gels (23). Proteins were transferred to Immobilon-P (Millipore, Bedford, Mass.) and probed with antibodies after blocking (23). To detect Ail, the primary antibody was used at a dilution of 1:1,000. For pH 6 antigen, a dilution of 1:1,500 of rabbit antiserum directed against *Y. pestis* pH 6 antigen (kindly provided by S. Straley) was used.

Serum resistance assays. Serum resistance assays were performed as described previously (21).

Hemagglutination assays. Hemagglutination assays were performed as described previously (17). Fresh overnight cultures of bacteria in L broth with appropriate antibiotics at 28°C were diluted 1:50 in fresh medium prior to growth of the bacteria under inducible conditions (37°C in acidic medium) or noninducible conditions (28°C in L broth) for 3 to 4 h. Bacterial pellets were resus-

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YEPail:     4 MNKILLVSSLIACLSIASVNVYAEGESSISIGYAQSRVKDDGYKLDKNPRGFNLKYRYEF 63
              M K LL SSLIACLSIASVNVYA  ESSISIGYAQS VK++GY LD +P+GFNLKYRYE
ENTail:     1 MKKTLLASSLIACLSIASVNVYAASESSISIGYAQSHVKENGYTLDNDPKGFNLKYRYEL 60
                                                  Surface

YEPail:    64 NNDWGVIGSFAQTRRGFEESVDGFKLIDGDFKYYSVTAGPVFRINEYVSLYGLLGAGHGK 123
              +++WGVIGSFA T +G++      K   GD  YYSVT GP FRINEYVSLYGLLGA HGK
ENTail:    61 DDNWGVIGSFAYTHQGYDFFYGSNKFGHGDVDYYSVTMGPSFRINEYVSLYGLLGAAHGK 120
                          Surface

YEPail:   124 AKVSIFGQSESRSKTSLAYGAGLQFNPHPNFVIDASYEYSKLDDVKVGTWMLGAGYRF 86
               K+S+F +S S SKTS+AYGAG+QFNP PNFVIDASYEYSKLD +KVGTWMLGAGYRF
ENTail:   121 VKASVFDESISASKTSMAYGAGVQFNPLPNFVIDASYEYSKLDSIKVGTWMLGAGYRF 178
              Surface                                  Surface
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FIG. 1. Sequence similarity of predicted proteins from *Y. enterocolitica* 0:8 and *Y. pseudotuberculosis ail* genes (YEPail and ENTail, respectively). Shown are the translations predicted from the nucleotide sequences of the two genes. Sequence divergences between these two proteins are located primarily in contiguous regions predicted to be exposed on the surface of the bacterial outer membrane according to the model of Beer and Miller (1).

pended in 0.5 volume of normal saline (0.9% NaCl-H_2O), serially diluted in the same buffer at a dilution ratio of 1:2 in 96-well test plates, and then mixed with an equal volume of normal saline-washed 0.3% sheep RBC (SRBC) (Remel). Hemagglutination was expressed as the highest dilution of bacteria that gave visible hemagglutination after a 2-h incubation with SRBC at 37°C, adjusted for viable counts added to each well.

Nucleotide sequence accession number. The GenBank accession number for the *Y. pseudotuberculosis ail* sequence is L49439. The GenBank accession number for the complete sequence of *psa* in *Y. pseudotuberculosis* is L76301.

RESULTS

Identification of a locus in *Y. pseudotuberculosis* homologous to the *ail* gene of *Y. enterocolitica*. To determine whether there exists in *Y. pseudotuberculosis* a homolog of the *Y. enterocolitica ail* gene that has the ability to promote adhesion, a phage λ library of *Y. pseudotuberculosis* chromosomal fragments was probed with a restriction fragment internal to the *ail* gene of *Y. enterocolitica*. A 1.6-kb probe-positive fragment found in all candidate phages gave two predicted ORFs. One of them was predicted to encode a protein of 183 amino acids that possessed high sequence similarity to Ail in *Y. enterocolitica* (Fig. 1). Consistent with previous analysis of *ail* variants in *Y. enterocolitica*, the regions of highest sequence similarity are predicted to span the outer membrane, according to the model predicted by Miller and coworkers (1). Western blot analyses using antibody directed against the overproduced product of this ORF indicated that Ail in *Y. pseudotuberculosis* has an apparent molecular mass of 17 kDa on SDS-polyacrylamide gels (Fig. 2).

Cellular uptake and adhesion are unaffected in a *Y. pseudotuberculosis ail* mutant strain. When introduced into *E. coli*, the DNA region containing the *ail* ORF did not confer any cellular adhesion or uptake, in contrast to the results with the *Y. enterocolitica* homolog (data not shown). One possibility is that other *Yersinia* factors are required for its function. To test for this possibility, *Y. pseudotuberculosis ail* mutants were isolated to determine if they were defective for cellular adhesion and uptake.

Y. pseudotuberculosis strains containing an *ail*::*cam* mutation were isolated and analyzed for cell adhesion (Materials and Methods). The 17-kDa Ail protein was not found in strains of *Y. pseudotuberculosis* containing an insertion mutation in *ail* (Fig. 2; compare lanes 1 and 2 with lanes 3 and 4), whereas introduction into this mutant of a plasmid containing the putative *Y. pseudotuberculosis ail* ORF allowed expression of the 17-kDa protein (Fig. 2, lanes 5 and 6), as determined by immunoprobing.

One of the *ail* isolates, YY115, was tested for cellular adhesion and compared with the isogenic strain Yp332, containing the *ail* wild-type allele. Both of these strains are *inv* mutant and have been cured of the virulence plasmid pIB1 so that no YadA or invasin proteins are expressed. As shown in Table 2, when tested for cellular adhesion under four different growth conditions, YY115 and Yp332 exhibited indistinguishable adhesive abilities. Both strains showed a high level of cellular adhesion when grown at 37°C in medium adjusted to pH 6.0. Therefore, the thermoinducible cell adhesion activity present in a *Y. pseudotuberculosis inv* mutant is not due to Ail protein.

A series of strains having each possible combination of *inv* and *ail* alleles was tested for cellular uptake. As shown in Table 3, YY060 (*inv*$^+$ *ail*) was indistinguishable from the *inv*$^+$ *ail*$^+$ strain [YPIII(P$^-$)] with respect to uptake, whereas the *inv ail* double mutant (YY115) was no more defective for uptake than an *inv ail*$^+$ strain (Yp332). Invasin, therefore, was the primary determinant that mediated cellular uptake, and *Y. pseudotuberculosis* uptake was reduced when bacteria were grown at 37°C prior to infection relative to 28°C for the *inv*$^+$ strains (Table 3; compare YpIII and YY060 at 37 and 28°C). This was the expected result, given that invasin expression is less at 37°C than at 28°C (13).

In *Y. enterocolitica*, Ail appears to mediate cell line-specific cellular uptake, with more efficient uptake into CHO cells than into the HEp-2 cell line (19, 20). When a *Y. pseudotuberculosis inv ail* mutant strain was compared with an isogenic *inv ail*$^+$

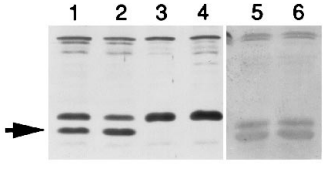

FIG. 2. Western blot analysis of *Y. pseudotuberculosis ail* mutation and demonstration of complementation. Whole cell lysates of equal numbers of bacteria were separated on SDS–15% polyacrylamide gels and transferred to Immobilon filters. The filters were probed with rabbit antiserum directed against the *Y. pseudotuberculosis* Ail protein followed by alkaline phosphatase-conjugated goat anti-rabbit antibody. Lanes: 1, Yp332 (*inv ail*$^+$) grown at 28°C; 2, Yp332 grown at 37°C; 3, YY115 (*inv ail*) grown at 28°C; 4, YY115 grown at 37°C; 5, YY115/pAY43 (*inv ail*/*ail*$^+$) grown at 28°C; 6, YY115/pAY43 grown at 37°C. The arrow indicates a unique band found in *Y. pseudotuberculosis ail*$^+$ strains but not found in *ail* mutants. Bands other than Ail are cross-reactive proteins found in both *E. coli* and *Y. pseudotuberculosis*. Growth conditions were as described in Materials and Methods.

TABLE 2. The *psa* locus, but not the *ail* gene, is responsible for thermoinducible binding in *Y. pseudotuberculosis*[a]

Strain	Genotype	Mean no. of bacteria/HEp-2 cell ± SD			
		pH6		pH7	
		37°C	28°C	37°C	28°C
Yp332	*inv ail*$^+$ *psa*$^+$	4.9 ± 0.91	0.33 ± 0.44	0.97 ± 0.21	0.06 ± 0.02
YY115	*inv ail psa*$^+$	5.6 ± 0.98	0.91 ± 0.38	0.28 ± 0.13	0.06 ± 0.04
YY033	*inv ail*$^+$ *psa*	0.22 ± 0.11	<0.02	<0.02	<0.02
YY033/pAY48	*inv psa/psa*$^+$	25.4 ± 3.4	14.8 ± 12.8	19.8 ± 9.6	6.0 ± 2.0
YY033/pAY50	*inv psa/psa*$^+$	21.0 ± 0.4	33.4 ± 9.1	18.6 ± 1.56	9.7 ± 6.8
YY033/pACYC177	*inv psa*	0.02 ± 0.01	<0.02	<0.02	<0.02

[a] Test strains were cultured under the four conditions listed and introduced onto HEp-2 cell monolayers spread on sterile coverslips. Cell-associated bacteria were fixed after exhaustive washing and stained prior to counting (Materials and Methods). Numbers are the means from three coverslips. The bacterium/HEp-2 cell ratio was approximately 100:1.

strain in regard to cellular uptake by these two cell lines, no effect of the *ail* mutation on uptake was seen (data not shown).

From these mutation analyses, therefore, we conclude that the product of the *ail* locus in *Y. pseudotuberculosis* does not mediate cellular uptake or adhesion.

***Y. pseudotuberculosis* Ail protein confers serum resistance.** Ail protein in *Y. enterocolitica* confers serum resistance, as does a *Salmonella* homolog, Rck (3, 21). To test whether the Ail protein in *Y. pseudotuberculosis* has the same function, one of the *ail*::*cam* mutant strains, YY060, was tested for serum resistance and compared with its isogenic *ail*$^+$ parent. As shown in Table 4, YY060 was highly sensitive to normal human serum, with viability after exposure to serum reduced more than 10^3 relative to an isogenic *ail* wild-type strain [Table 4; compare YpIII(P−) with YY060]. The serum sensitivity was due solely to the absence of Ail. When a plasmid containing only the *ail* ORF was introduced into YY060 (*inv*$^+$ *ail*), serum resistance was increased to the level of *ail* wild-type strains [Table 4; compare YY060/pACYC184 with YY060/pAY43 and YpIII(P−)]. It is clear that, like its homologs in *Y. enterocolitica* and *Salmonella* species, *Y. pseudotuberculosis* Ail confers serum resistance, although it does not mediate cellular adhesion or uptake.

Identification of the *Y. pseudotuberculosis* locus encoding pH 6 antigen. When a *Y. pseudotuberculosis inv* strain, cured of virulence plasmid, was tested for cellular binding, it exhibited maximal binding when grown at 37°C in acidic medium (Table 2). The fact that a *Y. pseudotuberculosis* strain defective for production of invasin, YadA, and Ail was still capable of promoting adhesion to mammalian cells indicated the presence of another locus involved in tissue culture adhesion. As adhesion was induced by growing bacteria at 37°C in acidic medium, we decided to determine if pH 6 antigen was the source of this adhesion.

Sequences homologous to *psaEAB* of *Y. pestis* were identified by PCR amplification of *Y. pseudotuberculosis* chromosomal DNA. Sequencing of the 6.7-kb region encompassing and flanking this PCR product confirmed that *Y. pseudotuberculosis* has a chromosomal locus with high sequence identity to the *Y. pestis psa* locus (see below). The fact that an antibody that recognizes the product of the *Y. pestis psaA* gene also recognizes the 16-kDa protein in *Y. pseudotuberculosis* strongly argues that this locus expresses functional pH 6 antigen (Fig. 3A, lanes 1 and 2).

Thermoinducible binding and hemagglutination are defective in *Y. pseudotuberculosis psa* mutant strains. To test whether pH 6 antigen is involved in thermoinducible binding to cultured cells, *psa* mutations in *Y. pseudotuberculosis* were constructed. A 1.3-kb region between *psaE* and *psaA* was replaced with a gene fragment encoding kanamycin resistance (Materials and Methods; Fig. 4). Mutants constructed in this fashion produced no detectable cross-reacting protein in Western blot analysis when probed with antiserum raised against *Y. pestis* pH 6 antigen (Fig. 3A, lanes 3 and 4).

One of the mutant isolates, YY033 (*inv psa*), was then compared with an isogenic *inv psa*$^+$ strain (Yp332) in regards to thermoinducible binding and hemagglutination. As shown in Table 2, when grown under inducible condition (37°C in acidic medium), Yp332 exhibited efficient cellular binding, whereas YY033 (*inv psa*) showed very low adhesion to HEp-2 cells under any assay condition. Furthermore, YY033 was also unable to promote hemagglutination of SRBC, although the *inv psa*$^+$ strain Yp332 showed efficient hemagglutination under conditions of maximal induction (Table 5). These results indicate that *psa* mutants are defective for cellular binding and hemagglutination in *Y. pseudotuberculosis*. The *psa* locus in *Y. pseudotuberculosis* is functional, and there is no evidence that *ail* contributes to cellular adhesion, as no adhesion could be detected in the *inv ail*$^+$ *psa yadA* strain (Table 2). As expected from these results, an *inv ail psa yadA* derivative was no more defective for binding mammalian cells than the isogenic *ail*$^+$ strain (data not shown).

Complementation of the *psa* mutation in *Y. pseudotuberculosis*. To demonstrate that the defect in the *psa* mutation strain was due to the absence of synthesis of pH 6 antigen, complementation of the *psa* mutation was attempted. Plasmid pAY09 containing a region from *psaE* to *psaB* was unable to complement the *psa* mutation in YY033 (Fig. 3), and no PsaA could be detected by immunoprobing (Table 5; Fig. 3B, lanes 1 and 2). The failure to complement may be a result of poor expression of PsaA or because the *psaEA* deletion has a polar effect on downstream genes, such as *psaB*.

TABLE 3. *ail* mutation has no effect on cellular uptake of *Y. pseudotuberculosis*[a]

Strain	Genotype	Mean % survival ± SD	
		28°C	37°C
YpIII(P$^-$)	*inv*$^+$ *ail*$^+$	4.9 ± 1.6	3.2 ± 0.79
YY060	*inv*$^+$ *ail*	6.9 ± 4.4	2.4 ± 0.62
Yp332	*inv ail*$^+$	0.015 ± 0.005	0.024 ± 0.001
YY115	*inv ail*	0.019 ± 0.012	0.10 ± 0.032

[a] Individual *Y. pseudotuberculosis* strains were grown at the indicated temperatures and then incubated with HEp-2 cells, and uptake was allowed to proceed for 90 min. Numbers represent the mean percentage of viable counts that survived gentamicin treatment at this time point relative to the input (Materials and Methods).

TABLE 4. Ail in *Y. pseudotuberculosis* confers serum resistance[a]

Strain[b]	Genotype	Log_{10} killing ± SD[c]
YpIII	*ail*$^+$	0.18 ± 0.087
YY060	*ail*	3.53 ± 0.100
YY060/pAY43	*ail*/*ail*$^+$	−0.32 ± 0.057
YY060/pBR322	*ail*	4.54 ± 0.13

[a] Fresh cultures of test strains were incubated separately with equal volumes of heat-inactivated and untreated normal human serum at 37°C for 60 min. Viable counts were then determined for each condition.

[b] All strains tested were *inv*$^+$. Results from the *inv* strains were similar to those presented (data not shown).

[c] Log_{10} killing = log_{10} CFU per milliliter surviving in heat-inactivated serum − log_{10} CFU per milliliter surviving in untreated serum.

To attempt to complement the mutation, a *Y. pseudotuberculosis Cla*I library was constructed on pACYC177 in *E. coli* MC1061, and clones containing a 9.0-kb *Cla*I fragment encompassing *psa* were isolated (Materials and Methods). When the *E. coli* strains harboring the clones isolated in this fashion were tested for hemagglutination, all were positive, and surprisingly all showed hemagglutination regulated by temperature and pH, as in *Y. pseudotuberculosis* (data not shown). Introduction of one of the molecular clones containing the 9.0-kb *Cla*I region (pAY48) in YY033 resulted in restoration of thermoinducible binding and hemagglutination to the *psa* deletion strain (Tables 2 and 5; Fig. 3B, lanes 3 to 6), although the expression of plasmid-encoded pH 6 antigen did not appear to be as tightly regulated in *Y. pseudotuberculosis* as in *E. coli* (compare Fig. 3B, lanes 3 to 6, with Fig. 3C, lanes 1 to 4).

DNA regions required for thermoinducible binding include *psaEFABC*. The foregoing analyses indicated that thermoinducible binding in *Y. pseudotuberculosis* is mediated by pH 6 antigen and that expression in this organism requires a cluster of genes. To characterize the chromosomal region required for complementation of the binding defects seen in the Δ*psaEA* mutant, molecular subcloning and transposon mutagenesis were performed. Subcloning indicated that a 6.7-kb region was sufficient for the expression and function of pH 6 antigen (pAY50 [Fig. 3B, lane 7 to 10; Fig. 3C, lanes 5 to 8; Tables 2 and 5]).

Mutagenesis using the *lacZ* transcriptional fusion transposon Tn*5*-B21 was performed on plasmid pAY50 containing the 6.7-kb region. Among 50 analyzed insertions, 34 resulted in simultaneous defects in hemagglutination and thermoinducible binding. Most of the hemagglutination-defective clones had the transposon inserted in the ORF of *psaE*, *psaA*, or *psaB* (predicted by the *Y. pestis* sequence), and a few were located in the region between *psaE* and *psaA* (Fig. 4D). Two of the hemagglutination-defective insertions contained Tn*5*-B21 in the 3.0-kb region downstream from the ORF4 sequences in *Y. pestis*. Loss of function in these transposon mutagenized strains indicated that the whole 6.7-kb region is required for pH 6 antigen synthesis and function and that there is a region in *Y. pseudotuberculosis* required for hemagglutination not accounted for in the *Y. pestis* sequences (17).

As described in Materials and Methods, the 6.7-kb *Kpn*I-*Cla*I fragment was sequenced. Five ORFs were predicted within this region by computer analyses. Among them, the *psaEAB* genes were highly homologous to *psaEAB* in *Y. pestis* (Fig. 4A and B). The ORF located between *psaE* and *psaA* was highly homologous to *myfF*, the gene which is proposed to be a transcriptional regulator in *Y. enterocolitica*. It was hence named *psaF*. The product encoded by the ORF downstream of *psaB* is highly homologous to PapC as well as MyfC, putative outer membrane usher proteins. This ORF, which when disrupted resulted in loss of hemagglutination, was named *psaC* to account for the sequence similarity to the two other genes.

DISCUSSION

Previous studies indicated that a *Y. pseudotuberculosis inv* mutant cured of the *Yersinia* virulence plasmid promoted efficient cellular adhesion when grown at 37°C (7). In this work, we identified a *Y. pseudotuberculosis* homolog of the *Y. enterocolitica ail* gene and showed that it apparently did not contribute to cellular uptake or adhesion. Although we have no evidence for other homologs of *ail* within *Y. pseudotuberculosis*, even if such homologs exist, they could not contribute to tissue culture adhesion under our assay conditions. *Y. pseudotuberculosis inv psa yadA* mutant strains showed no detectable cellular adhesion.

Sequences downstream of the *ail* ORF in *Y. pseudotubercu-*

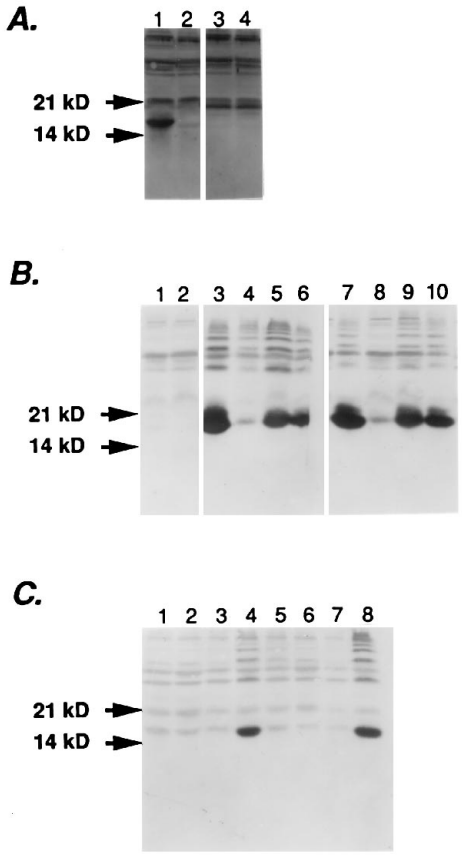

FIG. 3. Western blot analysis of *psa* mutation and its complementation. Whole cell lysates of equal numbers of bacteria were separated on SDS–15% polyacrylamide gels and transferred to Immobilon filters. The filters were probed with rabbit antiserum directed against *Y. pestis* pH 6 antigen (Materials and Methods). (A) Lanes: 1 and 2, Yp332 (*inv psa*$^+$) grown at pH 6 and 37°C (lane 1) and pH 7 and 28°C (lane 2); 3 and 4, YY033 (*inv* Δ*psaEA*::*kan*) grown at pH 6 and 37°C (lane 3) and pH 7 and 28°C (lane 4). (B) Lanes: 1 and 2, YY033/pAY09 (*inv psa*/*psaEFA*) grown at pH 6 and 37°C (lane 1) and pH 7 and 28°C (lane 2); 3 to 6, YY033/pAY48 (*inv psa*/*psaEFABC*) grown at pH 6 and 37°C (lane 3), pH 7 and 28°C (lane 4), pH 6 and 28°C (lane 5), and pH 7 and 37°C (lane 6); 7 to 10, YY033/pAY50 (*inv psa*/*psaEFABC*) grown at pH 6 and 37°C (lane 7), pH 7 and 28°C (lane 8), pH 6 and 28°C (lane 9), and pH 7 and 37°C (lane 10). (C) Lanes: 1 to 4, *E. coli* MC1061/pAY48 grown at pH 7 and 28°C (lane 1), pH 6 and 28°C (lane 2), pH 7 and 37°C (lane 3), and pH 6 and 37°C (lane 4); 5 to 8, *E. coli* MC1061/pAY50 grown at pH 7 and 28°C (lane 5), pH 6 and 28°C (lane 6), pH 7 and 37°C (lane 7), and pH 6 and 37°C (lane 8).

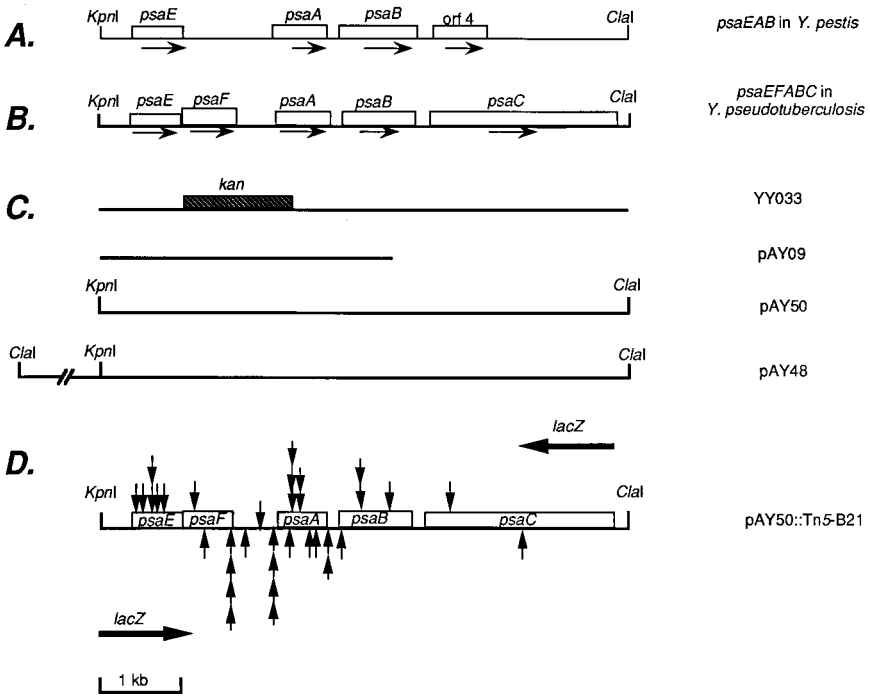

FIG. 4. Physical map of the *psa* locus in *Y. pseudotuberculosis*. (A) Map of *psaEAB* in *Y. pestis* (17). (B) Map of the *psa* locus in *Y. pseudotuberculosis*, summarized from our complete sequencing of the region shown (GenBank accession number L76301). Arrows below the boxes indicate the orientations of the indicated genes. (C) *psa* derivatives used in this study. For YY033, the cross-hatched box represents a 1.3-kb *psa* region replaced by the gene encoding kanamycin resistance. pAY09 is derived from pACYC184 carrying a 3.0-kb PCR fragment containing *psaEFA*. pAY50 is a derivative of pACYC177 carrying a 6.7-kb *Kpn*I-*Cla*I fragment containing *psaEFABC*. pAY48, the parental plasmid of pAY50, contains an additional 2.3-kb *Cla*I-*Kpn*I fragment upstream of the *psa* locus. (D) Vertical arrows point to the sites where Tn*5*-B21 inserted in *psa*, resulting in hemagglutination-defective clones. Insertion mutations represented by vertical arrows below the line have the *lacZ* gene from Tn*5*-B21 reading from left to right (orientation identical to the direction of transcription of the *psa* genes), whereas those represented by arrows above the line have *lacZ* oriented in the opposite direction (counter to the direction of transcription of *psa*).

losis contain an ORF consisting of a series of repeated sequences. In the derivative analyzed, an 18-bp sequence was tandemly repeated 11 times. We found that the number of repeats varies from strain to strain in *Y. pseudotuberculosis* (data not shown). The significance of this repetitive sequence and of the variation in number of repeats is not clear.

TABLE 5. The *psa* locus is responsible for hemagglutination in *Y. pseudotuberculosis*[a]

Strain[b]	Genotype	Hemagglutination[c]			
		pH 6		pH 7	
		37°C	28°C	37°C	28°C
Yp332	*psa*$^+$	1:128	>1:2	>1:2	>1:2
YY033	*psa*	>1:2	ND	ND	>1:2
YY033/pAY48	*psa*/*psa*$^+$	<1:128	<1:128	<1:128	<1:128
YY033/pAY50	*psa*/*psa*$^+$	<1:128	<1:128	<1:128	<1:128
YY033/pAY09	*psa*/*psa*$^+$	>1:2	>1:2	>1:2	>1:2
YY033/pACYC177	*psa*	>1:2	ND	ND	>1:2
MC1061/pAY48		<1:128	1:2	1:8	>1:2
MC1061/pAY50		<1:128	>1:2	1:2	>1:2
MC1061/pACYC177		>1:2	>1:2	>1:2	>1:2

[a] Test strains were allowed to grow under the four conditions listed. Cultures were serially diluted at a ratio of 1:2 in normal saline before the addition of an equal volume of 0.3% SRBC. Hemagglutination was scored after 2 h of incubation at 37°C. In this assay, 1:2 was the lowest dilution and 1:128 was the highest. Samples that exhibited no hemagglutination at the lowest dilution were scored as >1:2, and samples that exhibited positive hemagglutination at the highest dilution were scored as <1:128.

[b] All strains tested were *inv*.

[c] Maximal dilution of bacteria that allows hemagglutination. ND, not determined.

We have demonstrated that the previously described thermoinducible binding factor (7) in *Y. pseudotuberculosis* is pH 6 antigen, which requires the products of the cluster of *psa* genes for its synthesis and assembly. Of the 6,710 bp sequenced, 16 nucleotide differences from the *Y. pestis* sequence were identified. Two single-base insertions relative to the *Y. pestis* sequence at nt 4052 and 4230 in *Y. pseudotuberculosis* resulted in changes in ORF designations (GenBank accession number L76301) (17). These changes resulted in the designation of the newly identified gene *psaC*.

The initial isolation of the locus responsible for pH 6 antigen synthesis in *Y. pestis* demonstrated that at least two genes other than the structural gene *psaA* are involved in its synthesis and assembly on the microbial surface. *psaE* is believed to be a positive regulator, responsible for maximal expression of the PsaA adhesin, since insertion mutations in *psaE* cause reduction of expression of a *psaA-lacZ* transcriptional fusion. The product of *psaB* is proposed to be a chaperone protein because of its high similarity to *papD*, whose product acts as a periplasmic chaperone involved in P-pilus biosynthesis in *E. coli* strains associated with pyelonephritis (15).

Similar to results for *Y. pestis*, we have shown that in *Y. pseudotuberculosis*, *psa* mutations cause defects in hemagglutination. In addition, we showed here that the *psa* locus is responsible for mediating thermoinducible adhesion to tissue culture cells (7). Evidence was presented that full complementation of a Δ*psaEA* mutation required an ORF downstream from *psaB* that we designated *psaC* (Fig. 4). In addition, trans-

poson mutagenesis analyses indicated that mutations in an ORF between *psaE* and *psaA* eliminated synthesis of PsaA. We have called this ORF *psaF* because of its high sequence similarity to the *Y. enterocolitica myfF* gene (4, 5). Although the functions of PsaE and PsaF are not clear, their high homology to MyfE and MyfF, respectively, suggests that their products are regulatory proteins (4, 5). Recent results indicate that they are not required for proper assembly of pH 6 antigen on the bacterial surface but are required for regulating the expression of PsaA (26).

The products of the *Y. enterocolitica myf* cluster, which are highly homologous to the *psa* genes, do not promote hemagglutination, even though the assembly and regulation of *myfA* apparently involves the identical proteins (4, 5). Whether *myfA* is able to promote tissue culture adhesion is not known. Given that *myf* and *psa* are similar with respect to regulation and structure, one might speculate that both promote adherence in vivo. The role of *psa* in an animal infection model is currently under investigation.

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