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Transcriptional Analysis of the *Yersinia pestis* pH 6 Antigen Gene

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The pH 6 antigen of *Yersinia pestis* is a virulence protein whose gene, *psaA*, is positively regulated at the transcriptional level by low pH, mammalian temperature, and an upstream locus, *psaE*. Low pH appears to be required for initial *psaA* transcription, although increased temperature is necessary for full expression of the gene. In addition, *psaA* is monocistronic and its transcript has a relatively long 5′ nontranslated region.

Yersinia pestis is the etiologic agent of plague, a rodent disease that also occurs in human populations. Several virulence factors in *Y. pestis* have been identified. A common feature of these determinants is their responsiveness to environmental cues. One of these determinants, the pH 6 antigen (PsaA), was first characterized in 1961 by Ben-Efraim and coworkers (1). These researchers found that the protein was expressed in all strains of *Y. pestis*, but only at temperatures above 34°C and pH below 6.7. They named the antigen the pH 6 antigen because of its characteristic acidic pH requirement for expression (1).

In 1990, Lindler and coworkers described two loci, *psaA* and *psaE*, necessary for expression of the pH 6 antigen (8). *psaA* was shown to be the structural gene of the antigen. *psaE*, located upstream from *psaA* in a distinct transcriptional unit, was required for maximal expression of the antigen. Transposon insertions in *psaA* or *psaE* resulted in no expression or greatly diminished expression, respectively, of PsaA and a concomitant decrease in the virulence of *Y. pestis* (8). From the resulting transcriptional fusions, these investigators were able to establish the direction of transcription of *psaA* and *psaE*. In addition, they found that, despite the much-reduced production of PsaA by the PsaE$^-$ mutant, expression of PsaA remained regulated by pH and temperature, indicating that *psaE* was not itself involved in the environmental regulation of *psaA* expression. Interestingly, transcription of *psaE* itself was not influenced by pH or temperature (8).

In 1993, Lindler and Tall described the molecular analysis of the *psa* loci (9). They concluded that the pH 6 antigen is the monomer unit of a yersinial fibrilla and that other *psa* genes were involved in the expression of this fimbria-like structure. A question that arose from the work of Lindler and coworkers was whether expression of *psaA* was controlled at the transcriptional or posttranscriptional level. The goal of the present work was to examine transcription of the pH 6 antigen gene.

RNA was isolated from two strains of *Y. pestis* by a previously described procedure (7). The parent strain, *Y. pestis* KIM5, produces PsaA normally, while *Y. pestis* KIM5-3005.1 is phenotypically PsaA$^-$ because of a Tn*10lacZ* insertion in the positive regulatory locus, *psaE* (8). Cultures of these strains were grown under PsaA-inducing conditions (pH 6, 37°C) or noninducing conditions (pH 7.4, 26°C) as described by Ben-Efraim et al. (1) in the defined medium TMH (12). RNA was separated on formaldehyde-agarose gels by a procedure outlined by Brown (3) and transferred by capillary action onto nitrocellulose membranes. A 1.7-kb fragment of DNA containing the *psaA* gene was labeled with α-^{32}P and used to probe Northern (RNA) blots under stringent hybridization conditions as described by Sambrook et al. (11). Duplicate RNA blot experiments were performed with a γ-^{32}P-labeled *psaA*-specific DNA oligonucleotide probe derived from the published *psaA* sequence (9) by the protocol described by Wahl et al. (14). Results were identical to those seen with the DNA fragment (data not shown). Thus, although the 1.7-kb fragment contains portions of two other open reading frames according to sequence analysis (9), only the *psaA* transcript was detected (Fig. 1). Perhaps the transcripts for these adjacent genes are short-lived or in low abundance.

As shown in Fig. 1A, only the parent strain grown under inducing conditions expressed a transcript that hybridized to the probe DNA. The message size was calculated to be 740 bases. The lack of expression of the *psaA* transcript from noninduced cultures, or from *Y. pestis* KIM5-3005.1 (PsaE$^-$) grown under inducing conditions, suggests that expression of *psaA* is regulated at the level of transcription by pH, temperature, and *psaE*.

Potassium thiocyanate-extracted proteins isolated from the cultures described above were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blot (immunoblot) with adsorbed anti-PsaA serum, as described previously (2, 6, 8). The results were similar to those reported previously (8): PsaA was detected when the parent (PsaE$^+$) was grown under inducing conditions (Fig. 1B). However, unlike the situation with earlier work, no PsaA was detected from cultures of the PsaE$^-$ mutant grown under inducing conditions (Fig. 1B). The reason for this discrepancy is unknown. The PsaA$^-$ phenotype of *Y. pestis* KIM5-3005.1 correlates with results of our RNA blot experiment, in which no *psaA* transcript was detected from this strain grown under inducing conditions (Fig. 1A).

Cultures of *Y. pestis* KIM5 were grown under mixed inducing and noninducing conditions to determine which condition, pH or temperature, was the initial environmental signal regulating transcription of *psaA*. The *psaA* transcript was expressed by cultures grown in pH 6 medium but not by cultures grown in pH 7.4 medium (Fig. 2A, lanes 1 and 2), although the amount of message produced at pH 6, 26°C, was reproducibly much less than that produced at pH 6, 37°C (Fig. 1A, lane 2). These findings imply that pH is the initial environmental stimulus for expression of *psaA* but that increased temperature is required for full expression of the pH 6 antigen gene. No reactive bands were visible in Western blots in either of the mixed-condition cultures (Fig. 2B, lanes 1 and 2). The absence of PsaA in cultures producing small amounts of *psaA* transcript could be due to posttranscriptional control of PsaA expression.

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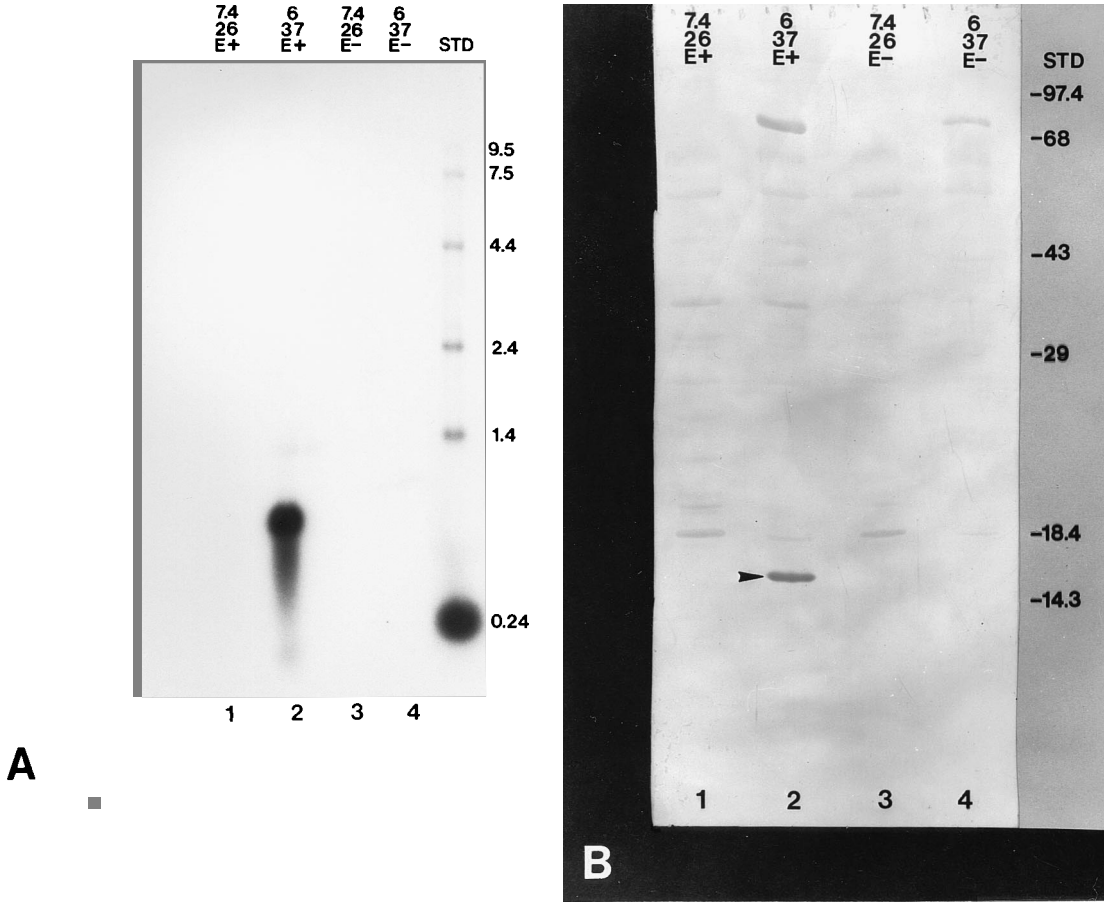

FIG. 1. (A) Northern blot analysis of *psaA* expression in PsaE$^+$ and PsaE$^-$ strains. RNA was probed with the 1.7-kb *psaA*-containing DNA probe. Lane 1, RNA isolated from *Y. pestis* KIM5 (E$^+$) grown under noninducing conditions for pH 6 antigen expression (pH 7.4, 26°C); lane 2, RNA isolated from *Y. pestis* KIM5 grown under inducing conditions (pH 6, 37°C); lane 3, RNA isolated from *Y. pestis* KIM5-3005.1 (E$^-$) grown under noninducing conditions; lane 4, RNA isolated from *Y. pestis* KIM5-3005.1 grown under inducing conditions. Blot exposure time, 42 h. The far right lane depicts RNA size standards (STD) in kilobases. The size standard sample was electrophoresed with the bacterial samples and transferred to nitrocellulose but was probed separately with ^{32}P-labeled *Hin*dIII-digested lambda bacteriophage DNA. (B) Immunoblot analysis for PsaA expression of potassium thiocyanate protein extracts of cultures described in the legend for panel A. Proteins were screened with adsorbed anti-PsaA serum. Lane 1, potassium thiocyanate extracts of total proteins isolated from *Y. pestis* KIM5 grown under noninducing conditions (pH 7.4, 26°C) for expression of the pH 6 antigen; lane 2, potassium thiocyanate extracts of total proteins isolated from *Y. pestis* KIM5 grown under inducing conditions (pH 6, 37°C); lane 3, potassium thiocyanate extracts of total proteins isolated from *Y. pestis* KIM5-3005.1 (E$^-$) grown under noninducing conditions; lane 4, potassium thiocyanate extracts of total proteins isolated from *Y. pestis* KIM5-3005.1 grown under inducing conditions. The arrowhead indicates the 15-kDa pH 6 antigen band. The numbers on the right denote migration positions of protein molecular mass standards (STD), measured in kilodaltons.

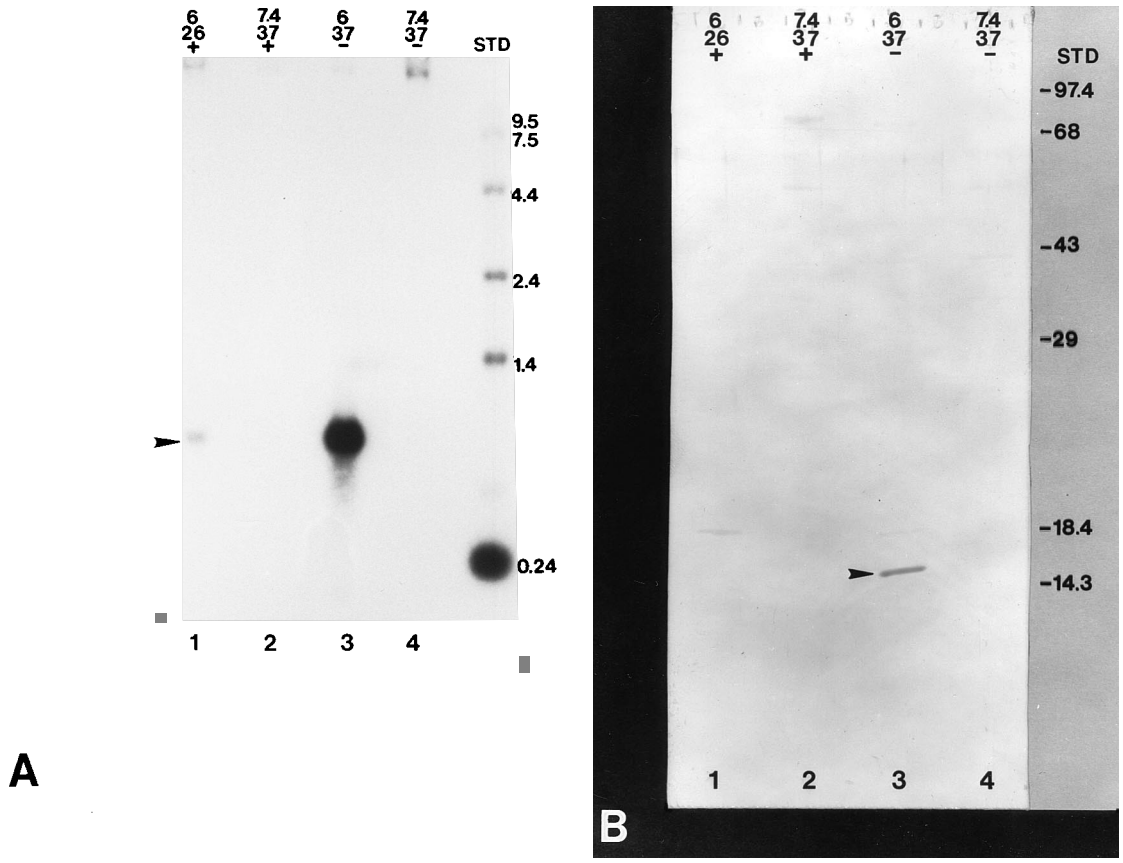

FIG. 2. (A) Northern blot analysis of *psaA* expression in *Y. pestis* KIM5 grown under mixed culture conditions. Lane 1, RNA isolated from KIM5 grown at the inducing pH, pH 6, and the noninducing temperature, 26°C, with Ca^{2+} (+); lane 2, RNA isolated from KIM5 grown at the noninducing pH, pH 7.4, and the inducing temperature, 37°C, with Ca^{2+}; lane 3, RNA isolated from KIM5 grown at the inducing pH, pH 6, in the absence of Ca^{2+} (−) at 37°C; lane 4, RNA isolated from KIM5 grown at the noninducing pH, pH 7.4, in the absence of Ca^{2+} at 37°C. The arrowhead indicates a faint *psaA* transcript and the inducing temperature, 37°C, with Ca^{2+}. Blot exposure time, 30 h. (B) Immunoblot analysis for PsaA expression of potassium thiocyanate protein extracts of cultures described in the legend to panel A with adsorbed anti-PsaA sera. Lane 1, potassium thiocyanate extracts of total proteins isolated from KIM5 grown at the inducing pH, pH 6, and noninducing temperature, 26°C with Ca^{2+} (+); lane 2, potassium thiocyanate extracts of total proteins isolated from KIM5 grown at the noninducing pH, pH 7.4, and inducing temperature, 37°C with Ca^{2+}; lane 3, potassium thiocyanate extracts of total proteins from KIM5 grown in the absence of Ca^{2+} (−) at pH 6, 37°C; lane 4, potassium thiocyanate extracts of total proteins from KIM5 grown in the absence of Ca^{2+} at pH 7.4, 37°C. The arrowhead indicates the 15-kDa pH 6 antigen band. The numbers on the right denote migration positions of protein molecular mass standards (STD), measured in kilodaltons.

Neither the *psaA* transcript nor the protein product was detected in cultures of *Y. pestis* KIM5 grown under growth-restricted conditions inductive for the low-calcium response, i.e., pH 7.4, 37°C, in the absence of calcium (Fig. 2). These data conflict with the findings of Mehigh and Brubaker, who list the pH 6 antigen as a member of a small group of non-low-calcium-response proteins produced when most other yersinial genes are repressed (10). The reason for this discrepancy is unknown. The *psaA* transcript and PsaA were produced in cultures grown in the absence of calcium at pH 6 (Fig. 2), although cultures grown under these conditions were not as tightly growth restricted as are cultures grown under classical growth restriction conditions (data not shown).

The size of the *psaA* transcript strongly suggests that the *psaA* gene is monocistronic, because it is not long enough to include upstream or downstream genes reported to be adjacent to *psaA* (9). Primer extension analysis was performed on RNA isolated from *Y. pestis* KIM5 grown under pH 6 antigen-inducing conditions (pH 6, 37°C) and noninducing conditions (pH 7.4, 26°C) to address the discrepancy between the 740-base transcript size and the published 474-bp open reading frame size of *psaA* (9). The aforementioned *psaA*-specific oligonucleotide probe, whose sequence was complementary to bases 35 to 51 of *psaA* (sequence, 5′ CGGTTACCACACTAATG 3′), was used to produce DNA-RNA hybrids by a published procedure (13). The extension products were separated by polyacrylamide gel electrophoresis, and their sizes were determined by comparison with standard markers. Five strong transcription initiation signals were detected in RNA isolated from the induced culture. These products initiated approximately 106, 109, 112, 113, and 298 nucleotides upstream from the primer (Fig. 3). No primer extension products were detected in RNA isolated from *Y. pestis* KIM5 cultured under noninducing conditions (Fig. 3). The primer extension results suggest that *psaA* has a relatively long 5′ nontranslated region and may explain the discrepancy between the gene size and transcript length of *psaA*. The function of this 5′ nontranslated region is unknown. It is also unclear why the *psaA* transcript has multiple initiation signals. Perhaps the 5′ ends of these shorter messages denote a region of secondary structure that prematurely terminates primer extension.

Homologs of *psaA* have been found in virulent strains of the other two *Yersinia* species pathogenic for humans, *Y. pseudotuberculosis* (9) and *Y. enterocolitica* (5). The *Y. enterocolitica* homolog, *myfA*, has a predicted amino acid sequence that is 44% homologous to the pH 6 antigen. Like that of *psaA*, *myfA*

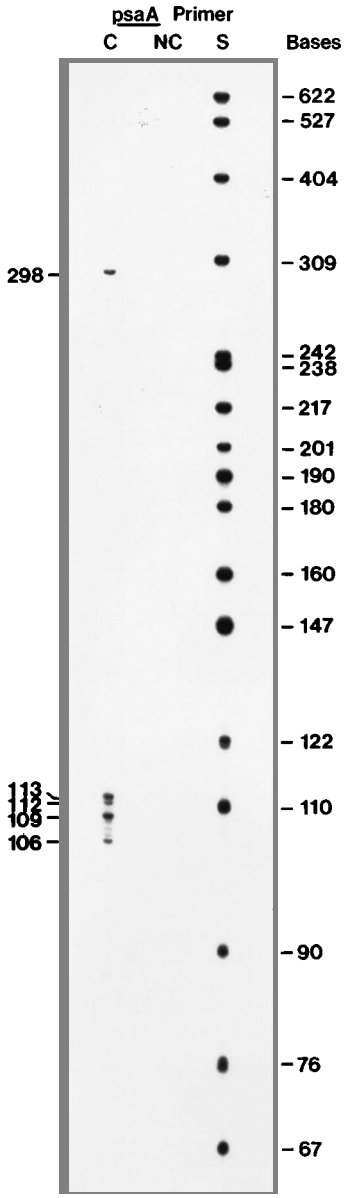

FIG. 3. Primer extension analysis of *psaA*. The transcriptional start sites of *psaA* were determined by using RNA isolated from *Y. pestis* KIM5 grown under inducing (C) and noninducing (NC) conditions for pH 6 antigen expression. The *psaA* primer used in the analysis was an oligonucleotide complementary to bases +35 to +51 of *psaA*. The major primer-extended products are indicated by size in nucleotide bases on the left. 3′-End-labeled marker fragments (S), derived from *Msp*I-digested pBR322, and their sizes in nucleotide bases are indicated on the right.

expression is regulated at the level of transcription by temperature and pH. Iriarte and Cornelis described two other genes, *myfE* and *myfF*, that regulate *myfA* transcription (4). *myfE* appears to be the homolog of *psaE*, which regulates *psaA* transcription (Fig. 1A). *myfF* in *Y. enterocolitica* may correspond to an open reading frame positioned between *psaE* and *psaA* in *Y. pestis* (4, 9).

The *myfA* transcript is 650 nucleotides in length, with initiation of transcription occurring at a position 98 or 99 nucleotides upstream from the translational start site (4). We found the *psaA* transcript to be somewhat larger (740 nucleotides). Transcription of *psaA* may initiate further upstream than does that of *myfA*, although the multiple primer extension products detected for *psaA* confuse this issue (Fig. 3).

It is not known if PsaA and MyfA serve the same function in their respective host yersiniae. Both are virulence factors and appear to be major fimbrial subunits of a temperature- and pH-induced adhesin. Further studies of both systems are required to ascertain if the two play similar roles in pathogenesis.

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