INFECTION AND IMMUNITY, Apr. 1995, p. 1340–1348 Vol. 63, No. 4
0019-9567/95/$04.00+0

Pasteurella haemolytica Serotype 2 Contains the Gene for a Noncapsular Serotype 1-Specific Antigen

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Received 8 September 1994/Returned for modification 4 November 1994/Accepted 18 January 1995

An *ssa1*-homologous genomic fragment cloned from *Pasteurella haemolytica* serotype 2 (ST2) enabled transformation of *Escherichia coli* DH5α to a serotype 1 (ST1) phenotype through expression of the ST1-specific antigen (Ssa1). The Ssa1 protein expressed by *ssa1*-transformed *E. coli* was susceptible to heat and protease treatment and was distinct from *P. haemolytica* ST1-specific capsular polysaccharide. Electrophoretic analysis of in vitro-translated proteins, as well as the predicted amino acid sequence, demonstrated that Ssa1 proteins encoded from either ST1- or ST2-derived *ssa1* genes were essentially identical. A comparison of the nucleotide sequences of *ssa1* genes derived from *P. haemolytica* ST1 and ST2 revealed greater than 99% homology. Amino acid sequence homology of the predicted products of ST1- and ST2-derived *ssa1* genes was greater than 98%. Northern (RNA) blot studies revealed that the presence of an increased level of *ssa1* transcript in *P. haemolytica* ST1 grown as surface-adherent cultures on solid medium was correlated with a serologically detectable Ssa1 protein. Expression of the *ssa1* transcript in ST1 was similarly upregulated by a high iron concentration in the growth medium.

Bovine pneumonic pasteurellosis is the major important respiratory disease of cattle in Western Europe and North America, both clinically and economically (1, 29). The disease is multifactorial in nature, involving infection by a variety of microorganisms in conjunction with stressful management practices and environmental factors. *Pasteurella haemolytica* serotype 1 (ST1) has been established as the primary agent responsible for the clinical disease and pathophysiologic events characterized by acute lobar fibrinonecrotizing pleuropneumonia (26).

P. haemolytica ST1 is rarely found in the upper respiratory tracts (URT) of healthy cattle (3, 28). In contrast, *P. haemolytica* serotype 2 (ST2) is easily isolated and comprises the major proportion of the *P. haemolytica* population in the URT of unstressed, healthy cattle (8, 14). Exposure of cattle to stress factors such as viral infection, marketing, shipping, processing at feedlots, and abrupt changes in climate leads to an explosive proliferation of *P. haemolytica* ST1 and its colonization in all areas of the URT of affected animals (9, 13). The greatly increased number of ST1 cells in the URT subsequently results in the accumulation of the organisms in the lung, via aspiration of nasopharyngeal secretions, where they initiate a series of pathophysiologic events which culminate in the severe fibrinonecrotic pleuropneumonia characteristic of this disease (14, 20). The phenomenon of commensal-to-pathogen conversion in the microfloral population, from prevalence of ST2 to an explosive growth of ST1, is consistently observed during the genesis of pneumonic pasteurellosis (10). Hence, colonization of the URT with *P. haemolytica* ST1 appears to be an initial event and an important prerequisite to the development of the disease (20). Intervention in the colonization process might interdict this series of events. Indeed, the presence of antibodies against *P. haemolytica* ST1-specific antigens has been shown to inhibit nasal colonization by *P. haemolytica* under field conditions (11).

Since there appears to be a relationship between *P. haemolytica* ST1 URT colonization and disease, we hypothesized that serotype-specific surface antigens unique to ST1 could be serving as adhesins to facilitate selective colonization of the bovine URT and could be the key to pathogenetic events that lead to pneumonic pasteurellosis. To date, little is known about the surface determinants of *P. haemolytica* that are unique to ST1 which favor colonization or about the molecular basis for the expression of such determinants. For many years, the capsular polysaccharide of *P. haemolytica* ST1 has been shown to confer serotype specificity, perhaps explaining the association of ST1 with disease.

Recently, a cell surface ST1-specific protein antigen designated Ssa1 was identified, and the *ssa1* gene encoding Ssa1 has been cloned and characterized (19, 24). Subsequently, genomic fragments homologous to the *ssa1* gene were detected by Southern blotting in 6 (ST2, ST5, ST6, ST7, ST9, and ST12) of 12 representative serotype strains of *P. haemolytica* (18). Both the *ssa1* gene and the homologous genomic fragment recovered from ST2 were able to transform *Escherichia coli* recipient cells to an ST1 phenotype through expression of the Ssa1 protein (18), although capsular antigen was not detected in the transformants. In this communication, we present data which suggest that *ssa1* genes recovered from *P. haemolytica* ST1 and ST2 similarly encode the Ssa1 protein and that regulation of expression of this gene in *P. haemolytica* occurs primarily at the transcriptional level.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *P. haemolytica* reference strains representing ST1, ST2, ST7, and ST8 and typing antisera against ST1, ST2, and ST7 produced in rabbits were provided by Glynn Frank from the USDA National Animal Disease Center, Ames, Iowa. The typing antisera contained polyclonal antibodies against the serotype-specific capsular polysaccharide antigens and other surface antigens of the bacteria (15). Cultivation of *P. haemolytica* for routine agglutination testing and for the initial Northern (RNA) hybridization experiment was done on blood agar plates with

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TABLE 1. Plasmids and primers used to derive the nucleotide sequence of the *P. haemolytica* ST2-derived *ssa1* gene[a]

Plasmid and primer	Primer sequence	Nucleotide sequence read
pPGA2		
M13 forward	5′-GTAAAACGACGGCCAGT-3′	−25 to 507
Z2285	5′-GAATCTACGATGCAAGTTACCCTC-3′	295 to 820
Z2286	5′-AATGCAAAGAAATGACTTATTAGC-3′	555 to 1015
Z2287	5′-CCGAGCTTGAGAAAAATTTAATT-3′	887 to 1375
Z2288	5′-GTCGATAATGTTTATGGCTGG-3′	1204 to 1513
Z2289	5′-TTCGTCTGGACAAAGCCAATTAG-3′	1508 to 1824
Z2290	5′-TACAAACGGGCAAGCCGGTTTAG-3′	1803 to 2115
Z2291	5′-CGATGTTAAACACCACCAATCACA-3′	2107 to 2422
Z2292	5′-TCAATTTATGCCAAACCAATGGA-3′	2405 to 2839
Z2293	5′-TCGCCAATTAAACGGCGAAGTCG-3′	2703 to 3019
Z2294	5′-CCTCCACTTTCTGGACTTCAATC-3′	3007 to 3315
M13 reverse	3′-AACAGCGATGACCATG-5′	3549 to 3085
pPGA2.6		
M13 forward	As above	1715 to 2115
M13 reverse	As above	2332 to 1962

[a] The nucleotide sequence of pPGA2 containing the *ssa1* insert was determined by reading overlapping segments, using internal primers synthesized at intervals of 300 to 350 bp, according to the ST1 homolog insert (24). Plasmid pPGA2 contained the entire ST2-derived *ssa1* insert. Plasmid pPGA2.6 contained a subcloned 0.6-kb (*Bgl*II/*Pst*I) insert from pPGA2.

5% sheep blood, at 37°C. For the Northern hybridization test comparing surface-adherent and broth-suspended cultures, *P. haemolytica* was grown on brain heart infusion agar or brain heart infusion broth (BHIB) (Difco Laboratories, Detroit, Mich.). The plasmid pPHA1 contained *P. haemolytica* ST1-derived *ssa1* in pBR322 (19). Plasmid pPGA2 contained ST2-derived *ssa1* in pGEM3Zf(+) (18). *E. coli* DH5α cultures containing various plasmids were grown on Luria-Bertani (LB) agar supplemented with ampicillin (100 mg/liter) where appropriate.

Immunofluorescence assay. *P. haemolytica* ST1 and ST2 reference strains were grown at 37°C in a 10% CO_2 atmosphere, on freshly prepared modified Sawata's medium (25) to enhance expression of capsular material. *E. coli* transformed with native plasmid vector pGEM3Zf(+), pPHA1, or pPGA2 was grown likewise for comparison, on LB agar containing ampicillin. Hybridoma culture supernatant containing a monoclonal antibody (MAb IIB6) specific to the capsular polysaccharide of ST1 was used as the primary antibody at a 1:400 dilution. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin M (IgM) (Organon Technika Corp., Durham, N.C.) was used as the secondary antibody at a 1:200 dilution. Specific cellular fluorescence was detected, using the protocol described by Penaredondo et al. (25). The controls included were preimmune mouse serum, an unrelated monoclonal antibody of identical isotype (IgM) directed against an irrelevant epitope of sheep lens fiber cell MP18 membrane protein (from C. F. Louis, University of Minnesota, St. Paul, Minn.), and buffer control.

Dot blot immunoassay with total membrane fractions. *P. haemolytica* ST1 and ST2 reference strains were grown in BHIB, and *E. coli* transformed with pGEM3Zf(+), pPHA1, or pPGA2 was grown in LB broth overnight (37°C, with shaking). The cells were pelleted, washed in phosphate-buffered saline (PBS) (pH 7.4), and sonicated intermittently for 5 min at 100 W. Cell debris were removed by centrifugation (5,000 × *g*, for 5 min, at 4°C), and the supernatant was centrifuged at 100,000 × *g* at 4°C for 1 h. The pelleted total membrane fraction (2) was washed once with PBS and resuspended in Tris-HCl (pH 7.5) at a protein concentration of 1 μg/ml. Two microliters of this membrane extract was applied in triplicate onto a nitrocellulose membrane (Bio-Rad, Hercules, Calif.) and allowed to dry. Blocking of nonspecific antibody binding sites was accomplished by soaking the nitrocellulose blot in Tris-buffered saline (TBS) containing 10 mM Tris-HCl (pH 7.4), 0.9% NaCl, plus 5% bovine serum albumin (BSA). To remove antibodies that cross-reacted with *E. coli* proteins, typing antisera were preabsorbed with sonicated, boiled *E. coli* cell extracts overnight (4°C), and the precipitated antigen-antibody complexes were removed by centrifugation. The blot was then hybridized overnight in ST1 typing antiserum diluted 1:100 in TBS containing 1% BSA and 0.05% Tween 20. Controls included identical blots hybridized with either preimmune rabbit serum or *P. haemolytica* ST2 or ST7 typing antiserum. The blots were then washed three to four times with TBS containing 1% BSA and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Organon Technika) diluted in TBS (1:100) containing 1% BSA and 0.05% Tween 20 for 1 h. After three to five washes in TBS containing 0.1% BSA, detection of antibody-specific reactions was done by immersing the membranes in a solution containing 32 mg of 4-chloro-1-naphthol (Sigma, St. Louis, Mo.) dissolved in 12 ml of methanol–48 ml of TBS–20 μl of 30% hydrogen peroxide. Color development was terminated by repeatedly washing the blots in distilled water.

In vitro transcription-translation of pPHA1- and pPGA2-encoded proteins. In vitro transcription-translation studies using 2.5 μg of closed circular pBR322, pGEM3Zf(+), pPHA1, or pPGA2 plasmid were performed to detect insert-encoded protein products. The cell-free Prokaryotic DNA-Directed Transcription-Translation Kit (Amersham, Arlington Height, Ill.) was used according to manufacturer's directions (30). Reaction mixtures containing L-[^{35}S]methionine were incubated at 37°C for 30 min and chased with nonradiolabelled methionine for 5 min, and then isotope incorporation was determined after trichloroacetic acid precipitation of synthesized proteins. Aliquots containing proteins with specific activities of 4×10^5 to 6×10^5 cpm were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 10% separating gel and a 4% stacking gel (23). After electrophoresis, the gels were fixed, stained with Coomassie blue, photographed, and then impregnated with diphenyloxazole dissolved in dimethyl sulfoxide, prior to drying and fluorographic analysis (4). Fluorographic exposure was done at −70°C.

Sequencing and prediction of gene product structure. The sequence of the ST2-derived *ssa1* gene was determined from a double-stranded pPGA2 plasmid template, with universal M13 primers, when possible, and internal primers synthesized according to known sequences from the ST1-derived *ssa1* gene (24). The sequencing strategy and primers used are summarized in Table 1. With the sequence, a hydropathy plot of the predicted amino acids of Ssa1 was constructed, using a 9-amino-acid reading window (6, 7). The locations of potential membrane-spanning regions of Ssa1 were also deduced from this plot. Finally, a four-state prediction of locations of secondary structures was made (16).

RNA isolation and Northern hybridization. Total bacterial RNA was extracted from *P. haemolytica* ST1, ST2, ST7, and ST8 reference strains grown on blood agar, as well as from *E. coli* transformed with pPHA1 or pPGA2 and untransformed *E. coli* grown on LB agar. Confirmation that the *P. haemolytica* reference strains belonged to the corresponding serotypes was done by the serotyping test (15) before RNA extraction. In the experiment comparing the effects of surface-adherent and broth-suspended cultures on expression of the *ssa1*-specific transcript, *P. haemolytica* was grown to reach mid-logarithmic phase on brain heart infusion agar or in BHIB. To determine the effect of iron levels on *ssa1*-specific transcript expression, bacteria were grown to mid-logarithmic phase in BHIB or LB broth with ampicillin, supplemented with 2,2′-dipyridyl (DP) (Sigma) to a final concentration of 200 μM to achieve iron-restricted growth. Iron-rich growth was achieved by growing the bacteria in BHIB-DP or LB-ampicillin-DP broth supplemented with $FeCl_3$ to a final concentration of 100 μM. Bacteria obtained under different conditions were harvested with sterile PBS, washed once, suspended in PBS to the same optical density, and pelleted by centrifugation at 5,000 × *g* for 10 min at 4°C. Total RNA was then isolated as described by Chomczynski and Sacchi (5).

The 10× formaldehyde gel running buffer and RNA sample loading buffer were purchased from 5 Prime-3 Prime, Inc. (Boulder, Colo.) and used as directed by the supplier. RQ1 DNase and rRNasin were obtained from Promega Corp. (Madison, Wis.). Rapid hybridization buffer, the probe labelling kit (Multiprime DNA Labelling), and the nitrocellulose (Hybond-C extra supported; pore size, 0.45 μm) membranes used for Northern blot hybridization were obtained from Amersham. Before separation by electrophoresis, an aliquot of each of the RNA extracts to be used in Northern hybridization analysis was routinely DNase-treated to eliminate nonspecific probe hybridization. A 50-μl reaction mixture containing 39.5 μl (up to 20 μg of RNA) of RNA extract, 1 U of rRNasin, 6 U of RQ1 (RNase-free) DNase, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.5) was incubated at 37°C for 20 min, after which the volume was

TABLE 2. Results of agglutination of ST1-specific antiserum and MAb IIB6[a] with *P. haemolyticus* ST1 and ST2 and *E. coli* DH5α clones harboring different plasmids

Bacteria	Titer of:	
	Antiserum[b]	MAb IIB6
P. haemolytica ST1	16	256
P. haemolytica ST2	NA[c]	NA
E. coli/pPHA1	512	NA
E. coli/pPGA2	2,048	NA
E. coli/pGEM3Zf(+)	NA	NA

[a] MAb IIB6 is a monoclonal antibody directed against the capsular polysaccharide.

[b] Antiserum raised against surface antigens of *P. haemolytica* ST1 in rabbits. Values are reciprocals of the highest dilution showing positive agglutination and the means of three experiments.

[c] NA, no agglutination.

adjusted to 200 μl with the addition of distilled H_2O. The RNA was then recovered by CTAB precipitation (18), vacuum dried, and resuspended in RNase-free distilled water at a concentration of 500 ng/μl. The RNA extracts (5 μg per lane) were electrophoretically resolved in 1% agarose formaldehyde gels and blotted onto nitrocellulose membranes by capillary transfer. Prehybridization was performed at 65°C for 25 min in hybridization buffer (Amersham). Hybridization was done at 65°C for 2 h, using as probe the *ssa1* insert from pPHA1, which was labelled with [α-^{32}P]dCTP (18) to a specific activity of 1×10^7 to 3×10^7 cpm/mg of DNA. The membranes were washed twice at room temperature, for 10 min each time, with 2× SSC (1× SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0)–0.1% SDS; this was followed by one 15-min wash at 65°C and two washes for 15 min each at 65°C with 0.7× SSC–0.1% SDS. The membranes were air dried and autoradiographed at −70°C for at least 24 h.

Nucleotide sequence accession number. The GenBank accession number for the ST2-derived *ssa1* gene sequence is U07788.

RESULTS

Demonstration that Ssa1 is distinct from capsular polysaccharide. *P. haemolytica* ST1 and ST2 were grown in modified Sawata's medium to enhance production of capsular polysaccharide. *E. coli* transformed with pPHA1 or pPGA2 was cultured similarly or on LB agar containing ampicillin. The bacterial cultures were then tested by slide agglutination with *P. haemolytica* ST1 typing antiserum or with a monoclonal antibody (MAb IIB6) to ST1 capsular polysaccharide. Our results showed that *P. haemolytica* ST1 cells agglutinated with both MAb IIB6 and ST1 typing antiserum. By contrast, the pPHA1- and pPGA2-transformed *E. coli* cells agglutinated only with the ST1 typing antiserum, while *P. haemolytica* ST2 showed no agglutination with MAb IIB6 or ST1 typing antiserum (Table 2). These results suggested that the Ssa1 protein was not expressed by *P. haemolytica* ST2, whereas it was expressed by *E. coli* clones transformed with either pPGA2 or pPHA1 containing *ssa1*.

We performed immunofluorescence studies to obtain additional evidence that Ssa1 is distinct from capsular polysaccharide antigen. *E. coli* transformants harboring either pPHA1 or pPGA2 were compared with *P. haemolytica* ST1 and ST2. Thin smears of the bacterial cells were made and fixed on fluorescent microscope slides and were tested for the presence of ST1 capsular antigen with MAb IIB6, directed against ST1 capsular polysaccharide, as the primary antibody. Only *P. haemolytica* ST1 displayed specific staining, shown by fluorescence of the bipolar cells in Fig. 1A. As controls, we used normal mouse serum and an unrelated IgM monoclonal antibody against the MP18 protein of sheep lens fiber; no staining was observed. *E. coli* transformed with pPHA1 or pPGA2 showed no fluorescence (data not shown), although sufficient cell numbers were detected in the smears when slides were viewed by bright-field microscopy. Likewise, *P. haemolytica* ST2 also showed no fluorescent staining (Fig. 1B). These results suggest that Ssa1 is distinct from the capsular polysaccharide antigen.

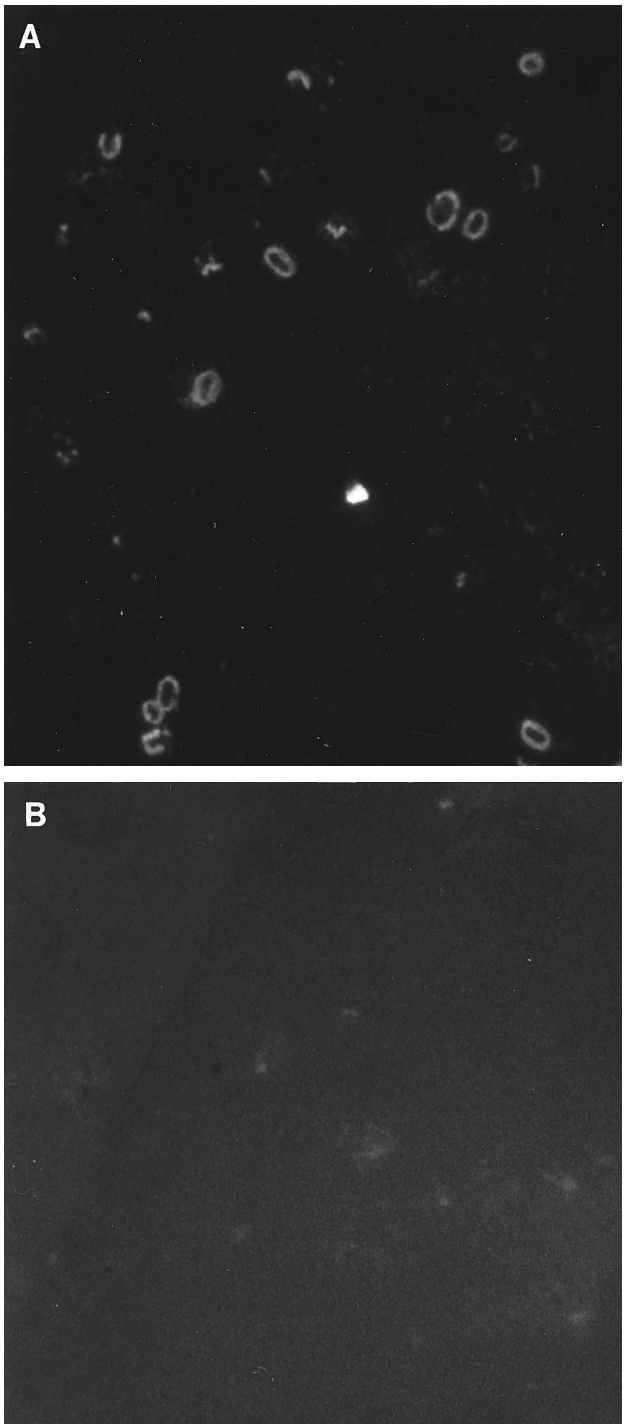


FIG. 1. Ssa1 is distinct from ST1-specific capsular polysaccharide. (A) Specific immunofluorescence of the capsular polysaccharide of *P. haemolytica* ST1 cells reacted with MAb IIB6, directed against capsular polysaccharide, and stained with fluorescein isothiocyanate-conjugated anti-mouse IgM. (B) No immunofluorescence with *P. haemolytica* ST2 cells. Magnification, ×500.

Expression of cloned Ssa1 protein in transformed *E. coli* cells. Total membrane fractions were extracted from *P. hae-*

TABLE 3. Effect of various treatments on reactivity of Ssa1 in total membranes from *P. haemolytica* ST1 and ST2 and *E. coli* clones carrying plasmids pPHA1, pPGA2, or pGEM3Zf(+), as determined by dot blot immunoassay

Bacterial extract	Effect[a] with indicated treatment		
	None	Proteinase K	Heat
P. haemolytica ST1	++	+	+
P. haemolytica ST2	−	−	−
E. coli/pPHA1	++	−	−
E. coli/pPGA2	++	−	−
E. coli/pGEM3Zf(+)	−	−	−

[a] ++, strongly positive reaction against ST1 antiserum. Results verified in three experiments; +, diminished ST1 reactivity after the treatments. Results verified in three experiments; −, no reactivity.

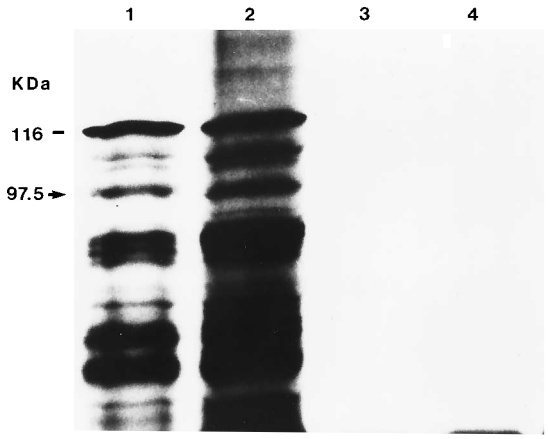


FIG. 2. In vitro transcription-translation products of *P. haemolytica* ST1- and ST2-derived *ssa1* genes. Fluorograph of SDS-PAGE profiles of [^{35}S]methionine-labelled protein products from pPHA1 (lane 1), which contained the *P. haemolytica* ST1-derived *ssa1* insert; pPGA2 (lane 2), with ST2-derived *ssa1* insert; native plasmid vector pBR322 (lane 3); and pGEM3Zf(+) (lane 4). The 116-kDa protein band is the predicted size of the *ssa1* gene product (Ssa1), and the 97.5 kDa protein band corresponds to membrane-bound mature Ssa1, described previously by Lo et al. (24).

molytica ST1, ST2, and *E. coli* transformed with recombinant plasmids (pPHA1 or pPGA2) or native plasmid vector pGEM3Zf(+). The extracts were tested by dot blot immunoassay with preabsorbed typing antisera against *P. haemolytica* ST1, ST2, or ST7. Heat-treated (56°C, 30 min) or proteinase K-treated (100 μg/ml; 37°C, 30 min) membrane extracts were tested likewise.

The dot blot immunoassay showed reactivity of total membrane fractions from *E. coli* clones carrying pPHA1 or pPGA2 with *P. haemolytica* ST1 typing antiserum (Table 3). The degree of reactivity approximated that of the positive control, which was a membrane fraction from *P. haemolytica* ST1. The membrane fraction from *P. haemolytica* ST2 showed no reactivity with ST1 typing antiserum. No cross-reactions with ST2 or ST7 typing antisera were seen (data not shown). Complete loss of reactivity with ST1 antiserum was observed after the *E. coli* membrane fractions were treated with heat or proteinase K. Membrane fractions from *E. coli* transformed with the cloning vector showed no reactivity with any of the typing antisera used (data not shown). By contrast, membrane fractions from *P. haemolytica* ST1 showed only partial loss of reactivity with ST1 typing antiserum after treatment with heat or proteinase K. This residual reactivity can be attributed to the reaction between other antigens present in this membrane fraction which are heat stable and proteinase K resistant and to their antibodies which are present in the ST1 typing antiserum.

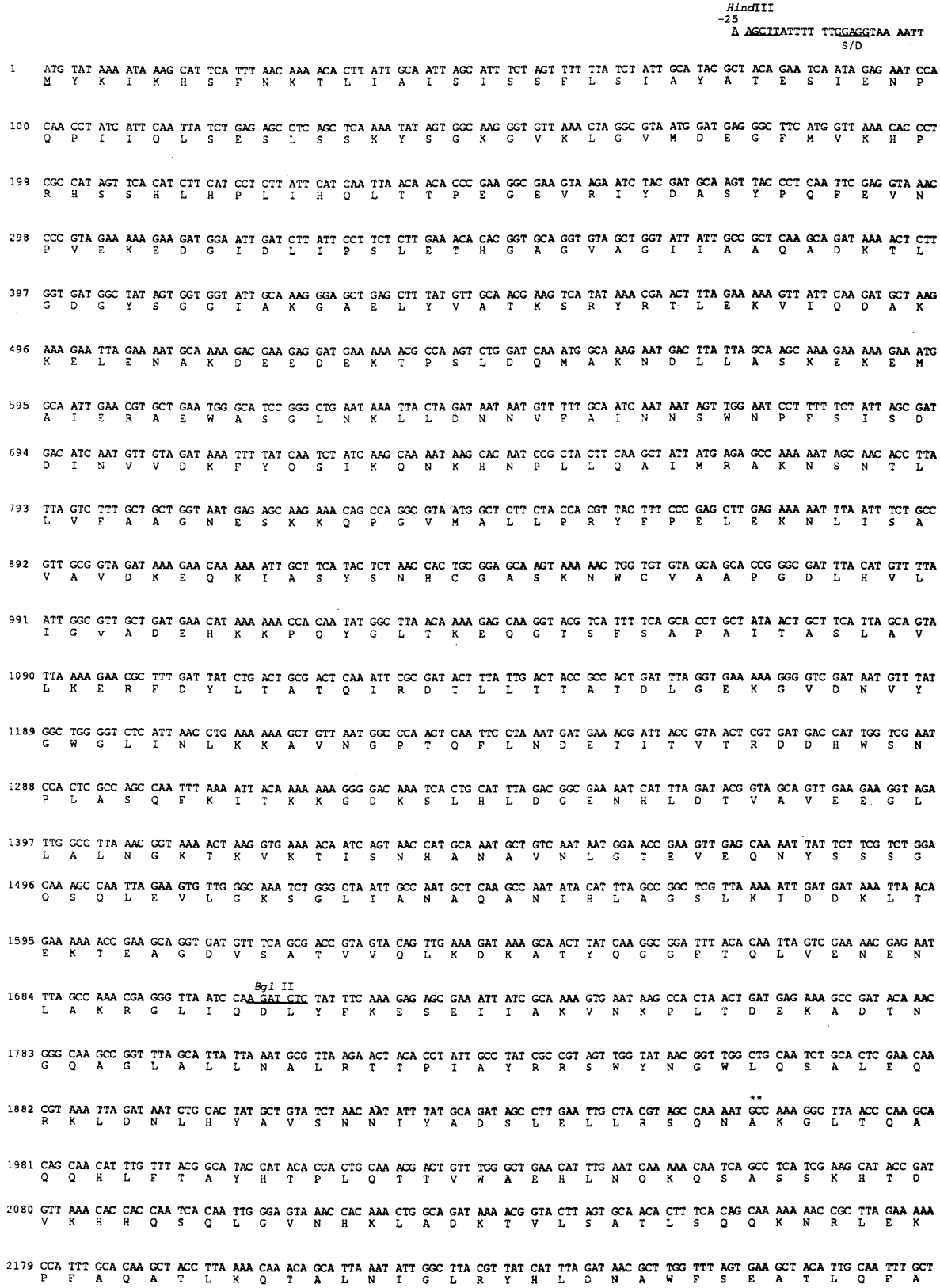
Analysis of in vitro transcription-translation products of *P. haemolytica* ST1(pPHA1)- and ST2(pPGA2)-derived *ssa1* genes. To identify the protein products expressed by pPHA1 and pPGA2, we performed in vitro transcription-translation studies with purified plasmid templates. The ST1-derived *ssa1* insert contained in pPHA1 and the ST2-derived *ssa1* insert in pPGA2 expressed identical protein products from the in vitro synthesis reactions (Fig. 2, lanes 1 and 2, respectively). The 116-kDa protein is approximately the size of the predicted gene product encoded by the ST1-derived *ssa1* single open reading frame (24). The arrow shows a 97.5-kDa protein band that corresponds to the molecular mass of mature Ssa1, described in the same study (24). The presence of numerous protein bands in these two lanes may be due to protease cleavage of the main gene products or premature termination of transcription-translation at various points. None of these proteins were expressed by the native plasmid vectors, pBR322 and pGEM3Zf(+) (Fig. 2, lanes 3 and 4, respectively).

Nucleotide sequence analysis of *ssa1* from *P. haemolytica* ST2. The *ssa1* gene from *P. haemolytica* ST2 consisted of a single open reading frame of 2,802 nucleotides which encoded a protein of 934 amino acid residues (approximately 103.6 kDa). In Fig. 3, asterisks show areas of nonhomology with the *ssa1* gene derived from ST1 (24). At nucleotides 1960 to 1961, a CG-to-GC change resulted in the substitution of an alanine for an arginine at the predicted amino acid 654. The single nucleotide deletion after nucleotide 2759 resulted in substitutions in the last 14 carboxy-terminal residues of the predicted amino acid sequence of the ST1-encoded Ssa1. Two other single nucleotide deletions at nucleotides 3288 and 3307 occurred outside the structural gene. A comparison of the ST2-derived *ssa1* gene with that of its ST1-derived homolog revealed 99.7% nucleotide homology and 98.7% amino acid homology.

Prediction of transmembrane region locations in Ssa1. Eisenberg hydrophobic moment analysis was used to locate possible membrane-spanning regions of the predicted amino acid sequence of Ssa1 encoded by ST2-derived *ssa1* (Fig. 4A). A four-state polypeptide structure analysis, which revealed that most of these predicted transmembrane regions were comprised of β-sheets, with only 2 of the 11 regions being apparently of α-helical structure (Fig. 4B), was also done. The probable transmembrane regions are indicated by horizontal bars (Fig. 4).

Northern blot analysis to detect expression of *ssa1* transcript in *P. haemolytica* and *E. coli* transformant clones. We used Northern blot analysis to determine whether expression of the *ssa1*-specific transcript occurred exclusively in *P. haemolytica* ST1 or whether it could similarly be detected in other serotypes containing the homologous *ssa1* gene. RNA was extracted from mid-logarithmic-growth-phase cultures of *P. haemolytica* ST1, ST2, and ST7 which contained *ssa1*, ST8 which did not contain *ssa1*, *E. coli* transformed with pPHA1 or pPGA2, and untransformed *E. coli*. RNAs from surface-adherent and broth-suspended cultures were also compared, and the effect of iron levels on expression of the *ssa1* transcript was examined likewise.

Figure 5 shows RNA from uninoculated blood agar plate medium (lane 1), untransformed *E. coli* (lane 2), *E. coli* transformed with pPGA2 (lane 3), *P. haemolytica* ST1 (lane 4), ST2 (lane 5), ST7 (lane 6), and ST8 (lane 7). The 2.7-kb *ssa1*-specific transcript was detected only in *E. coli* transformed with


HindIII
-25
A AGCTTATTTT TTGGAGGTAA AATT
S/D
Bgl II
**

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                                                                         Pst I
2278 CGC CAG AAA TAT CAA CAA AGC CGT CGA TTT GCT AGC CAT CAA CTA GGT ACT GCA GAA ACC AGA GGT TCA ACG CTC GGT GGT GAA ATG CGG ATC GGT TAT
      R   Q   K   Y   Q   Q   S   R   R   F   A   S   H   Q   L   G   T   A   E   T   R   G   S   T   L   G   G   E   M   R   I   G   Y

2377 CAA TTT ATG CCA AAC CAA TGG ATA ATT GAA CCA AGC CTT GGC GTA CAA TGG ATT CAA ACC AAA ATG AAT GGT TTA AAT GAA AGT GGC GAA CTT GCG ACT
      Q   F   M   P   N   Q   W   I   I   E   P   S   L   G   V   Q   W   I   Q   T   K   M   N   G   L   N   E   S   G   E   L   A   T

2476 CAA ACG GCT GCA ATG CGT TAT CGT GAT GTG AAT ATT GTT CCA AGC GTG AAA TTA CAG CGA ACT TTC CAA CTT GAG CAA GGC TCT ATT TCC CCT TAT ATC
      Q   T   A   A   M   R   Y   R   D   V   N   I   V   P   S   V   K   L   Q   R   T   F   Q   L   E   Q   G   S   I   S   P   Y   I

2575 GGG CTA AAT TAC TTG CAT AGA TTA AAT GGA AAA ATA ACC AAA ATT ACC AGT AAC ATT GCA GGA AAA ACA CTG CAT AGT GAA GCA ACG ACG AAA CGT AAT
      G   L   N   Y   L   H   R   L   N   G   K   I   T   K   I   T   S   N   I   A   G   K   T   L   H   S   E   A   T   T   K   R   N

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2674 CGC CAA TTA AAC GGC GAA GTC GGG GTG AAA CTG CAT TAT AAA AAT TGG TTT ACT GCG ATG AAT TTA GAT TAC AGT CGA GTG AAA TCT GCA AAC CTA TTT
      R   Q   L   N   G   E   V   G   V   K   L   H   Y   K   N   W   F   T   A   M   N   L   D   Y   S   R   V   K   S   A   N   L   F

2773 GGT TGG AAA GTA AAT GTT GGC TTT AGT TTC TAA CTTATTATGG TGGGACACAA GCGGTTAATT TAGGGTGAAA ATTTGCAAAT TTCACTCTAT TTTCAACCGC TTGAGAGAAA
      G   W   K   V   N   V   G   F   S   F   STP

2886 AAACCTTGAA CAACTGTTCA AGGTTTTTGT TTAGTGTTCT ATGACTAAAT AAGAAAAAAC TTATTGCTTA CGGACAAATA AGGTAATTCC CTCCACTTTC TGGACTTCAA TCTTATCTGC

                                                     XhoI                                                            Sph I  XhoI
3006 TACTTGTAAA TTATTACCTA TAACACGCCA AGTCGTATCA CCAAATTTTC CTCGAGCGAT GCCATTAGGT AAGATTTCAA CCACAAAGCC TTGAGCTCCT AGCATAGCAT GCTCTCGAGA

3126 GTTAAGGGAA CTGCTCTGAT CCTCTTTCTT GTCTTTTCCA TGCTGATATT TCCACCAAAT AAGACTAAAA ATAGTCGCAA TTATCGCAAA TAACGCAATT TGTAACGCTT GCCCTAATGT

                                             *                     *
3246 AAAAAGTGCA ACGATGAAAG CAAGAATAAG CGAGGCAAAT CCCCACCACA TAATAAAGAC ACCCGGTAAT ACTAATTCTA ATGCAAGTAG TACAAAGCCT AATATGCTCC AGCCAATCCA

3366 ATTAAAAAAC CAGTCCATAC TCCCTCCATA TACTCAATTA ATCCGGTTTT AGGCTATGAG CTTGTTTTAT TGCTTTTTAA AATTTCAGCA ATACCAGCAA CAGAACCAAT CAAATTTCCA

                                                         Hind III
3486 GCTTCTAACG GCATAAGTAC AACTTTACTG TTATCTGAAG AACCTATTTC TTTCAAAGCT T
```

FIG. 3. Nucleotide sequence and the predicted amino acid sequence of *ssa1* from *P. haemolytica* ST2. The sequence consists of 3,571 nucleotide base pairs, numbered according to the +1 methionine initiation codon. The cloned insert does not contain the −35 and −10 promoter sequences but contains the Shine-Dalgarno (S/D) sequence. Cleavage sites for four restriction enzymes are shown. Sequences different from the ST1-derived *ssa1* gene are indicated by asterisks. The last two single nucleotide deletions occur outside the structural gene. The italicized, underlined region between nucleotides 2836 and 2864 contains inverted repeats which may be involved in *rho*-independent termination of transcription. This sequence contains a single open reading frame of 2,802 nucleotides which codes for a protein of 934 amino acids.

pPGA2 and *P. haemolytica* ST1, which also expressed Ssa1, as determined by the slide agglutination test with typing ST1 antiserum. The transcript was not detected in RNA extracts of *P. haemolytica* ST2 and ST7, which contained the *ssa1* gene but did not express detectable Ssa1, nor from *P. haemolytica* ST8, which did not contain the homologous gene. RNA from untransformed *E. coli* likewise did not reveal the presence of the *ssa1* transcript. All lanes except lane 1 were loaded with equal quantities (5 μg) of total RNA. Lane 1 was loaded with a PBS extract from uninoculated blood agar which was processed in the same way as was used for the various bacterial suspensions. This was merely another control used to eliminate a possible

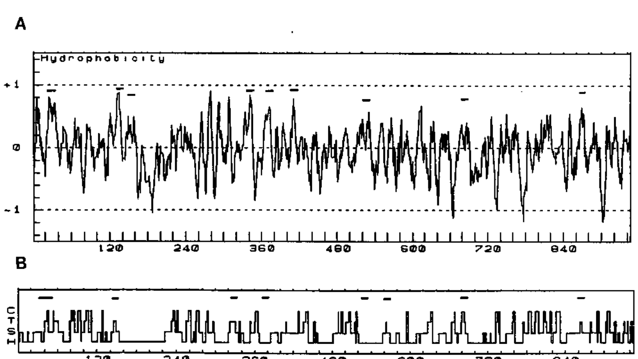

FIG. 4. Prediction of transmembrane region locations in Ssa1 coded by the ST2-derived *ssa1* gene. (A) The Eisenberg hydrophobic moment analysis program was used to locate possible membrane-spanning regions of the predicted amino acids of Ssa1. (B) A four-state polypeptide structure analysis shows that most of these predicted transmembrane regions are composed of β-sheets, with only 2 of the 11 regions being apparently of α-helical structure. The probable transmembrane regions are indicated by horizontal bars. CTSH denotes the predicted locations of random coils (C), reverse turns (T), β-sheets (S), and α-helices (H).

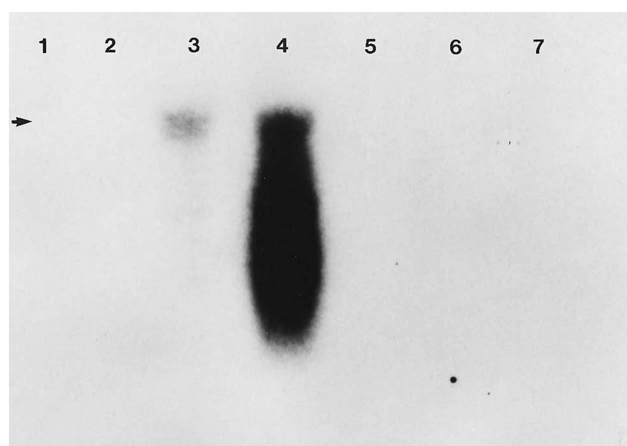

FIG. 5. Northern blot analysis of *ssa1*-specific transcript. Total RNAs extracted from uninoculated blood agar plate (lane 1), untransformed *E. coli* (lane 2), pPGA2-transformed *E. coli* (lane 3), and *P. haemolytica* ST1, ST2, ST7, and ST8 (lanes 4 through 7, respectively) were separated by electrophoresis, transferred to nitrocellulose membranes, and probed with the 3.6-kbp *Hin*dIII fragment from pPHA1, [α-^{32}P]dCTP-labelled to a specific activity of 10^7 cpm/μg. The arrow indicates the presence of a 2.7-kb transcript in lanes 3 and 4.

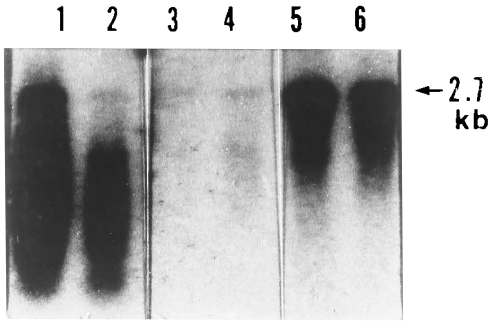

FIG. 6. Comparison of *ssa1* transcript expression in bacteria grown as surface-adherent and broth-suspended cultures. Lanes: 1, *P. haemolytica* ST1 grown as a surface-adherent culture on brain heart infusion agar; 2, ST1 grown as a broth-suspended culture in BHIB; 3, ST2 grown as a surface-adherent culture; 4, ST2 grown as a broth-suspended culture; 5, pPHA1-transformed *E. coli* grown as a surface-adherent culture; 6, pPHA1-transformed *E. coli* grown as a broth-suspended culture.

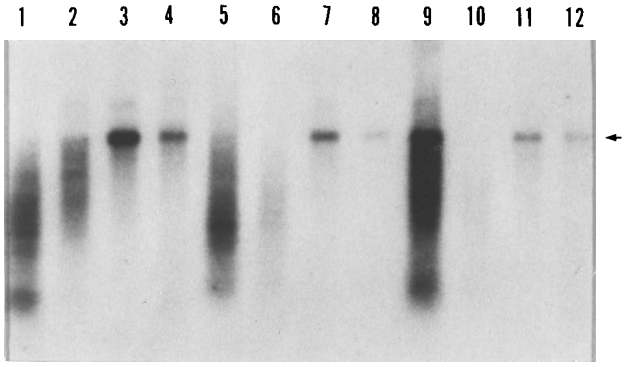

FIG. 7. Effect of iron on *ssa1* transcript expression. Lanes 1 to 4, RNA from bacterial cultures grown in BHIB; lanes 5 through 8, RNA from cultures grown in BHIB supplemented with 200 μM DP; lanes 9 through 12, RNA from cultures grown in BHIB with 200 μM DP and 100 μM ferric chloride ($FeCl_3$). The bacteria used were *P. haemolytica* ST1 (lanes 1, 5, and 9) and ST2 (lanes 2, 6, and 10), *E. coli* transformed with pPHA1 (lanes 3, 7, and 11), and *E. coli* transformed with pPGA2 (lanes 4, 8, and 12). The arrow indicates the position of the 2.7-kb transcript.

source of nonspecific hybridization. On the basis of our preblotted electrophoresis gels, no detectable RNA was present in this extract from uninoculated blood agar.

In a related experiment, a significant increase in the level of the *ssa1*-specific transcript was noted in RNA from *P. haemolytica* ST1 grown as surface-adherent cultures (Fig. 6, lane 1), relative to the transcript level in RNA from broth-suspended cultures (Fig. 6, lane 2). The increase in transcript level was correlated with a significant increase in Ssa1 expression, as shown by an increased agglutination titer with ST1 typing antiserum (Table 4). A comparison of *ssa1*-specific transcript levels in RNA extracts from *P. haemolytica* ST2 showed that insignificant levels of the transcript were detected in surface-adherent cultures (Fig. 6, lane 3), while RNA from broth-suspended cultures had a higher relative level of the transcript (Fig. 6, lane 4). Whether there was any significance in this transcript level difference was uncertain, because none of these ST2 cultures expressed detectable Ssa1 protein (Table 4). RNA from pPHA1-transformed *E. coli* showed similar levels of transcript from both surface-adherent (Fig. 6, lane 5) and broth-suspended cultures (Fig. 6, lane 6).

Figure 7 shows a comparison of *ssa1* transcript levels from cultures grown in BHIB (Fig. 7, lanes 1 to 4), BHIB supplemented with DP to achieve iron-restricted growth (Fig. 7, lanes 5 to 8), and BHIB-DP with added ferric chloride ($FeCl_3$) to provide iron-rich growth (Fig. 7, lanes 9 to 12). The results show that *P. haemolytica* ST1 grown in iron-rich medium contained an increased level of 2.7-kb *ssa1* transcript (Fig. 7, lane 9), relative to that in RNA from ST1 grown in BHIB (Fig. 7, lane 1) or in RNA from ST1 grown in iron-restricted medium (Fig. 7, lane 5). This increased transcript level correlated with a twofold increase in Ssa1 expression (Table 4). Higher levels of *ssa1* transcript were present in RNA from *E. coli* transformed with either pPHA1 or pPGA2 grown in BHIB (Fig. 7, lanes 3 and 4), relative to that in RNA from bacteria grown in iron-restricted medium (Fig. 7, lanes 7 and 8) or iron-rich medium (Fig. 7, lanes 11 and 12). These differences in transcript levels, however, did not exhibit a direct correlation with Ssa1 expression levels, as determined by the agglutination test (Table 4). An unexpectedly high level of the 2.7-kb *ssa1* transcript was detected in RNA from *P. haemolytica* ST2 grown in BHIB (Fig. 7, lane 2), although undetectable levels of the transcript were found in ST2 grown in iron-restricted medium (Fig. 7, lane 6) or iron-rich medium (Fig. 7, lane 10). However, the presence of this transcript in *P. haemolytica* ST2 did not correlate with expression of the Ssa1 protein, as determined by the absence of an agglutination reaction with ST1 typing antiserum (Table 4).

TABLE 4. Ssa1 expression by *P. haemolytica* ST1 and ST2 and *E. coli* clones carrying pPHA1 or pPGA2 grown under different conditions, as determined by slide agglutination test

Medium	Ssa1 expression[a] by:			
	P. haemolytica		*E. coli* carrying:	
	ST1	ST2	pPHA1	pPGA2
BHIB	2	NA[b]	64	128
BHIB + 200 mM DP	2	NA	64	128
BHIB + DP + 100 mM $FeCl_3$	4	NA	64	128
BHIA[c]	16	NA	512	2048

[a] Values are reciprocals of the highest dilution of ST1 typing antiserum showing agglutination. Results represent means of six experiments.
[b] NA, no agglutination.
[c] BHIA, brain heart infusion agar.

DISCUSSION

P. haemolytica strains have been grouped into 15 serotypes on the basis of a rapid plate agglutination reaction with a battery of standardized typing antisera produced in rabbits (15). Presumably, the capsular polysaccharide antigen is responsible for serotype specificity in this agglutination reaction, since no other serotype-specific surface antigens of *P. haemolytica* have been previously identified. Our earlier data (19) and a more recent study (24) identified an *ssa1* gene encoding a proteinaceous, ST1-specific antigen (Ssa1) from *P. haemolytica* ST1. Results of this present study, using bacterial agglutination and immunofluorescence, confirmed that Ssa1 was an ST1-specific noncapsular polysaccharide of *P. haemolytica*. Moreover, dot blot immunoassay results revealed that, in contrast to known properties of polysaccharide antigens, Ssa1 was susceptible to proteinase treatment and was heat labile. These properties further demonstrated the proteinaceous nature of this antigen.

By in vitro transcription-translation, we showed that the proteins expressed by the ST1- and ST2-derived *ssa1* genes were identical. This concurred with results of the initial gene

cloning experiments and agglutination tests which showed that either the ST1- or ST2-derived *ssa1* encoded the same Ssa1 protein (18). A comparison of nucleotide sequences of *ssa1* genes recovered independently from the two serotypes revealed greater than 99% homology. The amino acid sequences of the predicted products of these *ssa1* genes were essentially identical and differed only in the 14 carboxy-terminal amino acids.

Our results also indicate that *P. haemolytica* ST2 does not express serologically detectable levels of the Ssa1 protein. However, the ST2-derived *ssa1* was functional in expressing Ssa1 when transformed into *E. coli* (Table 4). These observations suggest that the mechanisms regulating expression of Ssa1 in ST1 and ST2 may be quite different. We used Northern blot analysis to investigate the regulation of *ssa1* gene expression at the transcriptional level. Our data showed the presence of a 2.7-kb *ssa1*-specific transcript in the RNA extracts from *P. haemolytica* ST1 and *E. coli* transformed with pPGA2 (Fig. 5), both expressing Ssa1, as revealed by the agglutination test. By contrast, extracts from *P. haemolytica* ST2 and ST7, which contained the homologous gene fragment but did not express Ssa1, showed the absence of the *ssa1* transcript. These data imply that regulation of gene expression in *P. haemolytica* serotypes containing *ssa1* occurs primarily at the transcriptional level. A comparison of RNA extracted from surface-adherent and broth-suspended *P. haemolytica* ST1 cultures indicated that surface culturing induced an increased expression of the *ssa1* transcript (Fig. 6). This increase in transcript level was correlated with a significant increase in Ssa1 expression. However, this does not imply that Ssa1 plays a role in promoting surface adherence of *P. haemolytica* ST1. Further studies must be conducted before a role as an adhesion molecule can be assigned to this protein.

Northern hybridization experiments showed that changes in *ssa1* transcript levels from RNA extracts of *P. haemolytica* ST2 and the *ssa1*-transformed *E. coli* did not show a direct correlation with Ssa1 expression levels, as monitored with the agglutination test (Table 4). The *ssa1* transcripts also were detected in extracts from broth-suspended ST2 cultures, but no Ssa1 agglutination was observed. These results indicate that the level of expression is reduced in non-ST1 bacteria and that the amount of protein is below the level detectable by the agglutination test. Alternatively, it is possible that additional regulatory mechanisms may be involved in the production and/or export of Ssa1 in ST1 or that agglutination is enhanced by other *P. haemolytica* surface antigens not present in *E. coli*. There have been recent indications that this could be the case for *P. haemolytica* leukotoxin (22). It appears that a more sensitive assay for detecting Ssa1 than the bacterial agglutination test needs to be developed for future studies.

A smaller 1.2-kb transcript was also detected in most of the RNA extracts tested. At present, we do not know if the smaller transcript encodes another protein or if it is a degraded form of the major transcript, since transcript degradation in bacteria occurs immediately following expression. Nevertheless, the RNA hybridization data showed that high levels of iron in the culture medium induced elevated levels of *ssa1* transcript in *P. haemolytica* ST1, which correlated with a twofold increase in the Ssa1-specific agglutination titer (Table 4). These findings could have a pathobiological significance, considering that *P. haemolytica* produces a hemolysin that facilitates the release of free iron from blood cells. The increased levels of free iron may then upregulate expression of Ssa1 in vivo. Whether Ssa1 is a determinant factor in the stress-precipitated commensal-to-pathogen conversion in the URT microfloral population of cattle will have to be determined by future studies. It would also be interesting to investigate whether there is a genetic correlation between regulation of *ssa1* and leukotoxin, since leukotoxin transcript expression has likewise been shown to be upregulated by high levels of iron (17, 21, 29).

ACKNOWLEDGMENTS

This work was made possible by USDA-NRIC grant 91-37204-6409, awarded to S.K.M.

We are grateful for the expertise of Sheila St. Cyr and Eric Eccleson from the Microchemical Facility, Institute of Human Genetics, University of Minnesota (Minneapolis), for the sequencing and structure analyses and for helpful advice in interpretation of the data.

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