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An *Aeromonas salmonicida* Gene Which Influences A-Protein Expression in *Escherichia coli* Encodes a Protein Containing an ATP-Binding Cassette and Maps beside the Surface Array Protein Gene

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A conserved *Aeromonas salmonicida* gene (*abcA*) affecting expression of the surface array protein gene (*vapA*) in *Escherichia coli* was identified. The 924-bp gene starts 205 bp after *vapA* and codes for a protein with a deduced molecular weight (M_r) of 34,015 containing an N-terminal P-loop and significant homology to the ATP-binding cassette transport protein superfamily. AbcA was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using T7 polymerase expression and DNA-directed translation and was copurified with the sarkosyl-soluble cytoplasmic membrane fraction. The protein displayed aberrant migration during SDS-PAGE. A *lacZ* fusion containing 128 bp of upstream sequence and 387 bases in the 5′ end of *abcA* was constructed, and the β-galactosidase activity of the *abcA-lacZ* fusion gene was shown to be similar in *E. coli* and *A. salmonicida*. The 130,000-M_r AbcA-LacZ fusion protein was purified, and by using an ATP affinity column, the 129 AbcA N-terminal P-loop-containing residues were shown to bind ATP.

S-layer proteins are an important class of secreted proteins. These paracrystalline surface protein arrays cover the cell envelopes of a wide variety of bacteria (27, 38). The secreted protein subunits which comprise the S-layer self assemble into a supramolecular structure with precise ultrastructural morphology which encloses the bacterial cell. However, while much is known concerning the morphology of S-layers (5), and while various S-layer proteins have been identified and characterized (27, 38) and several S-protein genes have been cloned and sequenced (reviewed in reference 38), there is virtually no information available concerning other genes which might influence the expression of S-layer protein genes, the export of S-layer proteins or, indeed, the identity of the genes flanking S-protein structural genes. Because the S-layer protein subunits must be produced in high copy number and, in the case of gram-negative bacteria, the subunits must be translocated across the inner membrane, periplasm, and the outer membrane in a presumed energy-dependent process before assembling into an array and becoming anchored to the cell surface, it is likely that a number of genes and gene products are involved in S-layer production.

The best described S-layer in terms of structure, function, biochemistry, export, surface anchoring, and molecular genetics is the so-called A-layer of the fish pathogenic bacterium *Aeromonas salmonicida*. This tetragonally arranged paracrystalline array contributes to the ability of *A. salmonicida* to colonize and produce disease in fish (25) and is composed of a single species of protein (A-protein) (10), the gene for which (*vapA*) has recently been cloned and sequenced (6, 10). The cloned gene was carried on a 4.6-kb insert of *A. salmonicida* DNA, and in vector pTZ this insert expressed A-protein in *Escherichia coli*. Sequence analysis of DNA 3′ to *vapA* showed that the insert also carried a portion of a second open reading frame (ORF). Deletion and complementation analysis further indicated that this ORF influenced the expression of *vapA* in *E. coli*. We have now sequenced this downstream gene in its entirety, and here we report that this species-specific gene encodes a protein which exhibits ATP-binding activity and which in *E. coli* copurifies with the membrane fraction. Sequence analysis further indicates that the protein carries an ATP-binding cassette (ABC) and similarity to a superfamily of ATP-driven membrane transporters (22, 24, 30). Sequence analysis also shows that the *A. salmonicida* gene contains a potential leucine zipper basic region. Here we report on the ABC-containing protein gene (*abcA*) of *A. salmonicida*.

MATERIALS AND METHODS

Strains, vectors, and growth conditions. *Aeromonas* strains used in this study are listed in Table 1. Growth of *A. salmonicida* subsp. *salmonicida* and *masoucida* at 20°C was in L broth or on L agar plates, while growth of *A. salmonicida* subsp. *achromogenes* and *nova* was on medium supplemented with blood. *Aeromonas hydrophila*, *Aeromonas veronii*, and *E. coli* DH5α, KX100, and JM109 were grown at 37°C in L broth or on L agar unless otherwise noted. Plasmid vectors pTZ18R (37), pMMB67 (15), pMC1871 (47), pSU2718 (35), pGP1-2 (43), and cosmid pLA2917 (1) and M13 vectors (52) have been previously described. Recombinant plasmids pSC150 and pTZ521 were constructed in this laboratory (10). Antibiotic supplements to the media, where applicable, were ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). When required, 5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma) was added to media to a concentration of 50 μg/ml.

DNA techniques. Vector DNAs were prepared by using the methods of Sambrook et al. (44). *Aeromonas* chromosomal DNAs were prepared as described previously (7). DNA digestion, ligation, and transformation were done according to the method of Sambrook et al. (44). Restriction enzymes

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TABLE 1. *Aeromonas* strains used and presence of *abcA*

Organism and strain[a]	Source	Presence of *abcA*[b]
A. salmonicida subsp. *salmonicida* ("typical" strains)		
A202	Japan	+
A203	Japan	+
A251	ex-NCMB[c] 1102	+
A362	Atlantic salmon; United Kingdom	+
A395	Scotland	+
A440	ex-ATCC 14174, brook trout; United States	+
A447	Weymouth, United Kingdom	+
A488	Brook trout; West Virginia	+
A449	Brown trout; Eure, France	+
A450	Brown trout; Tarn, France	+
A451	Rainbow trout; Manch, France	+
A488	Brook trout; West Virginia	+
A500	Atlantic salmon	+
A505	Japan	+
A506	Japan	+
A. salmonicida subsp. *masoucida* ("atypical" strains)		
A206	Masou, Japan	+
A400	Goldfish; Boolara, Australia	+
A401	Goldfish; Boolara, Australia	+
A419	Goldfish; Arkansas	+
A430	*Haemophilus piscium* ex-ATCC 10801^T, trout; United States	+
A442	*H. piscium* ex-ATCC 15711	+
A. salmonicida subsp. *achromogenes*		
A461	Atlantic salmon; Nova Scotia, Canada	+
A475	Norway	+
A477	European carp; Europe	+
A480	European carp; The Netherlands	+
A. hydrophila[d]		
TF7	Trout; Quebec, Canada	−
A274	Sloth; Australia	−
A. veronii biotype sobria[d]		
A700	Human diarrhea sample	−
A702	Human diarrhea sample	−

[a] Typical strains belong to *A. salmonicida* subsp. *salmonicida*, while atypical strains belong to *A. salmonicida* subsp. *nova*, *A. salmonicida* subsp. *masoucida*, or *A. salmonicida* subsp. *achromogenes*.

[b] Determined by Southern analysis of *Kpn*I-digested chromosomal DNAs with plasmid pSC162 or PCR analysis with primers TJT21 and TJT35, AP1 and RAA3, RAA2 and RAA3 or AP1 and TJT35.

[c] ex-NCMB, strain originally from the National Collection of Marine Bacteria, Aberdeen, Scotland, but has been subcultured extensively in a number of laboratories over the years.

[d] S-layer-producing strains.

and T4 DNA ligase were purchased from Pharmacia Canada, Inc. (Baie D'Urté, Québec, Canada).

DNA sequencing. A Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) was used according to the manufacturer's instructions. Template DNAs were prepared in pTZ plasmids and M13 vectors, and commercially available universal and custom primers (synthesized at the Regional DNA Synthesis Laboratory, University of Calgary) were used. ^{35}S-ATP was purchased from Du Pont-NEN Research Products (Boston, Mass.).

Southern analysis. pSC162 containing a 1.4-kb *Aeromonas* DNA insert including the intact *acbA* gene was labelled with digoxigen-11-dUTP. The reagents and procedures were provided in a nonradioactive DNA labelling and detection kit (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.). *Aeromonas* DNAs were digested by *Kpn*I and blotted from agarose gel to nitrocellulose membranes. The membranes were hybridized with the labelled probe. Prehybridization was done at 62°C for 3 h, and hybridization was done at 62°C for 12 h. The buffer used for prehybridization and hybridization contained 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% (wt/vol) *N*-lauroylsarcosine, 0.02% (vol/vol) sodium dodecyl sulfate (SDS), and 1% (wt/vol) blocking reagent (provided in the kit). Bound probe was detected by using the reagents and procedures from the kit manufacturer.

Polymerase chain reaction. Oligonucleotide primers (19-, 23-, 24-, and 26-mer) were synthesized at the Regional DNA Synthesis Laboratory (University of Calgary). Amplification of DNA was performed as previously described (19) by using capillary tubes in a 1605 Air Thermo-Cycler from Idaho Technology (Idaho Falls, Idaho). Samples were subjected to 35 cycles of 1-s denaturation at 94°C, 5-s primer annealing at 60°C, and 25-s extension at 73°C. Polymerase chain reaction products were detected by gel electrophoresis and visualized by UV fluorescence after ethidium bromide staining.

Computer analysis. Sequences were analyzed by using MacVector version 3.3 (International Biotechnologies Ltd., New Haven, Conn.) and GeneWorks version 2.2 and PC-GENE version 6.01 (IntelliGenetics Inc., Mountain View, Calif.). Protein sequence similarity searches were performed in GenBank by the FASTA protocol of Pearson and Lipman (41) and at the National Centre for Biotechnology Information by using the BLAST network service (3).

T7 polymerase in vivo transcription. The procedure was based on the method of Rosenberg et al. (43), with modifications. Cells containing pGP1-2 and other plasmids were grown for 3 h at 30°C with shaking in L broth with antibiotics. Cells in 1.5 ml of this culture were harvested by centrifugation, washed in M9 medium, and resuspended in 3 ml of this medium. A 0.09-ml aliquot of methionine assay medium (Difco Laboratories, Detroit, Mich.) was added, and the cells were incubated at 30°C for 1 h before the temperature was shifted to 42°C for 45 min. After 30 min at 42°C, rifampin was added to a final concentration of 1 mg/ml, and 1 ml of the culture was incubated for an additional 45 min at 30°C. The cells were then pulse-labelled for 5 min with 10 μCi of [^{35}S]methionine. Bacteria were then pelleted by centrifugation and resuspended in 30 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer.

To localize in vivo-transcribed proteins, ^{35}S-labelled cells were fractionated by the method of Boeke and Model (8). The pellets from six 1-ml aliquots of labelled cells were resuspended in 500 μl of 20% sucrose in 10 mM EDTA–100 mM Tris-HCl (pH 8.0), 10 μl of a 5-mg/ml lysozyme solution was added, and the cells were kept on ice for 10 min. Spheroplasts were then separated from the periplasmic fraction by centrifugation for 2 min at 15,000 × *g*. The pelleted spheroplasts were then washed in 0.5 ml of sucrose buffer and resuspended in 50 ml of 20% sucrose in 10 mM EDTA–10 mM $MgCl_2$–100 mM Tris-HCl (pH 8.0) containing 50-mg of DNase per ml. After the addition of 200 ml of ice-cold distilled water, the spheroplasts were lysed by four

cycles of freeze-thawing, and the envelope fraction was sedimented (10 min, 15,000 × *g*). The cytoplasmic membrane was freed of outer membrane by differential solubilization with sodium lauryl sarcosinate and centrifugation at 45,000 × *g* as described by Filip et al. (13).

In vitro expression of *abcA* gene. A prokaryotic DNA-directed translation kit (Amersham Canada Ltd., Oakville, Ontario, Canada) was used for coupled in vitro transcription and translation. Plasmid DNA was digested, and fragments were separated by electrophoresis on an agarose gel. The template DNA fragment was cut out of the gel and purified by electroelution. After ethanol precipitation and drying, the DNA was resuspended in Tris-EDTA buffer. In each transcription-translation reaction, 5 to 10 μg of DNA was used. The reaction was carried out according to the protocol provided by the manufacturer. The reaction mix containing template DNA, 7.5 μl of supplement solution, 3 μl of amino acid solution minus methionine, 2 ml of [^{35}S]methionine, and 5 μl of S-30 extract was incubated at 37°C for 60 min. After adding 5 μl of methionine chase solution, the reaction mixture was incubated for an additional 5 min at 37°C. The reaction was then stopped by placing the mixture in an ice bath. A negative control was run in parallel without adding the template DNA. One-fifth of the reaction mixture was loaded onto an SDS–12% PAGE gel, and after electrophoresis the gel was dried and exposed to film. Prestained low-range-molecular-weight standards were used to facilitate apparent-molecular-weight estimations (Bio-Rad Laboratories, Richmond, Calif.).

Electrophoresis and Western immunoblotting. SDS-PAGE in 12.5% acrylamide was performed according to the method of Laemmli (32). Proteins in the gel were electroblotted to nitrocellulose paper (Schleicher & Schuell, Keene, N.H.) for immunological detection by the method of Towbin et al. (50). A-protein was detected by using a 1:3,000 dilution of affinity-purified anti-*A. salmonicida* 450 A-protein rabbit immunoglobulin, while *lacZ* fusion protein was detected by using a monoclonal antibody against *E. coli* β-galactosidase at 500 ng/ml (Boehringer Mannhein). A 1:5,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit or mouse immunoglobulin G (Caltag Laboratories, South San Francisco, Calif.) was used as the second antibody. Reactions were visualized by reacting the nitrocellulose paper with 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim) and nitroblue tetrazolium (Sigma Chemical Co., St. Louis, Mo.) (21). Densitometric analysis was done with a Personal Densitometer (Molecular Dynamics, Sunneyvale, Calif.).

β-Galactosidase activity. The β-galactosidase activity was measured as described by Sambrook et al. (44) with modifications. A 1-ml volume of an overnight culture in L broth was washed once with A medium [60 mM K_2HPO_4, 33 mM KH_2PO_4, 17.5 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate] and resuspended in an equal volume of A medium. This cell suspension (100 μl) was added to 900 μl of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol [pH 7.0]), 2 drops of chloroform and 1 drop of 0.1% SDS were added, and the tube was vigorously vortexed for 10 s. After equilibration at 28°C for 5 min, 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 μg/ml in A medium) was added, and after development of the yellow color, the reaction was stopped by the addition of 500 μl of 1 M Na_2CO_3. The A_{420} and A_{550} of the reaction mixture were measured and corrected for the A_{600} of the cell suspension in A medium, and β-galactosidase activity was calculated and expressed in Miller units (44).

Purification of β-galactosidase fusion protein. *E. coli* cells were collected from a 1-liter 18-h shaken L-broth culture and broken by passage through a French pressure cell, and unbroken cells and membrane debris were removed by centrifugation at 4,500 × *g* for 30 min. The soluble fraction was then subjected to $(NH_4)_2SO_4$ fractionation at 4°C by the addition of saturated $(NH_4)_2SO_4$. Precipitated proteins were collected by centrifugation at 31,000 × *g* for 30 min, and protein pellets were resuspended in 1 ml of TBSN buffer (50 mM Tris, 150 mM NaCl, 0.2% Nonidet P-40 [pH 7.5]). After dialysis against the same buffer, fractions were analyzed for the presence of β-galactosidase fusion protein by SDS-PAGE and Western blot analysis with anti-β-galactosidase monoclonal antibody.

The fraction containing the β-galactosidase fusion protein was applied twice to an immunoaffinity column containing 1 ml of *lacZ*-linked-agarose beads (ProtoSorb matrix) bound with anti-β-galactosidase monoclonal antibody (Promega, Madison, Wis.). After washing, bound proteins were eluted by a pH 10.8 buffer (0.1 M $NaHCO_3$-Na_2CO_3). Fractions were examined for the presence of β-galactosidase fusion protein as previously described, and positive fractions were pooled and concentrated by passage through a 30,000-M_r-cutoff centrifugal microconcentrator (Filtron Technology Corporation, Northborough, Mass.).

ATP affinity chromatography. ATP affinity column matrix (1 ml; Sigma) was swollen in a morpholinepropanesulfonic acid (MOPS) buffer (pH 7.2) containing 10 mM MOPS, 1 mM EDTA, 1 mM dithiothreitol, 0.1% (vol/vol) Triton X-100, and 1 mM phenylmethylsulfonyl fluoride overnight at 4°C and washed with 10 bed volumes of the same buffer. Purified β-galactosidase fusion protein in the MOPS buffer was applied to the column, and the column was washed at a flow rate of 0.1 ml/min with more than 10 bed volumes of the MOPS buffer. The protein was then eluted with 1-ml volumes of MOPS buffer containing increasing concentrations of ATP (0.125, 0.25, 0.5, 1.0, 2.0, 40, and 200 mM; Sigma). Fractions (1 ml) were collected and concentrated to 30 μl per tube by passage through a 10,000-M_r-cutoff centrifugal microconcentrator (Filtron). Fractions (15 μl) were examined for the presence of β-galactosidase fusion protein by Western blot, as previously described. Control assays employed β-galactosidase (Boehringer-Mannheim).

Nucleotide sequence accession number. The sequence reported here has been deposited in the GenBank data base under accession no. L11870.

RESULTS

Localization of *Aeromonas* DNA affecting *vapA* gene expression in *E. coli*. The *vapA* gene from *A. salmonicida* A450 was previously cloned on a 9.4-kb insert in the broad-host-range cosmid pLA2917 and subcloned on a 4.6-kb insert into pTZ18R to yield pTZ521 (Fig. 1) (10). This plasmid contained the entire 1,506-bp *vapA* gene, as well as 62 bp of upstream flanking DNA. Subclone pSC150 (Fig. 1), which was 1.3 kb shorter at the 3′ end, produced about the same amount of A-protein as did pTZ521 (10). However, when DNA fragments were removed from the 3′ end of pSC150 and A-protein production by the subclones was compared with that by pSC150 (Fig. 2, lane 6), it was found that removal of a 0.5-kb DNA segment (clone pSC151) (Fig. 1) resulted in decreased production of A-protein. A-protein could only be seen in Western blots of whole-cell lysates of clone pSC151 at heavy loadings (Fig. 2, lane 2), and comparison with dilutions of lysates from cells carrying pSC150 showed that the decrease was approximately 16-fold (Fig. 2,

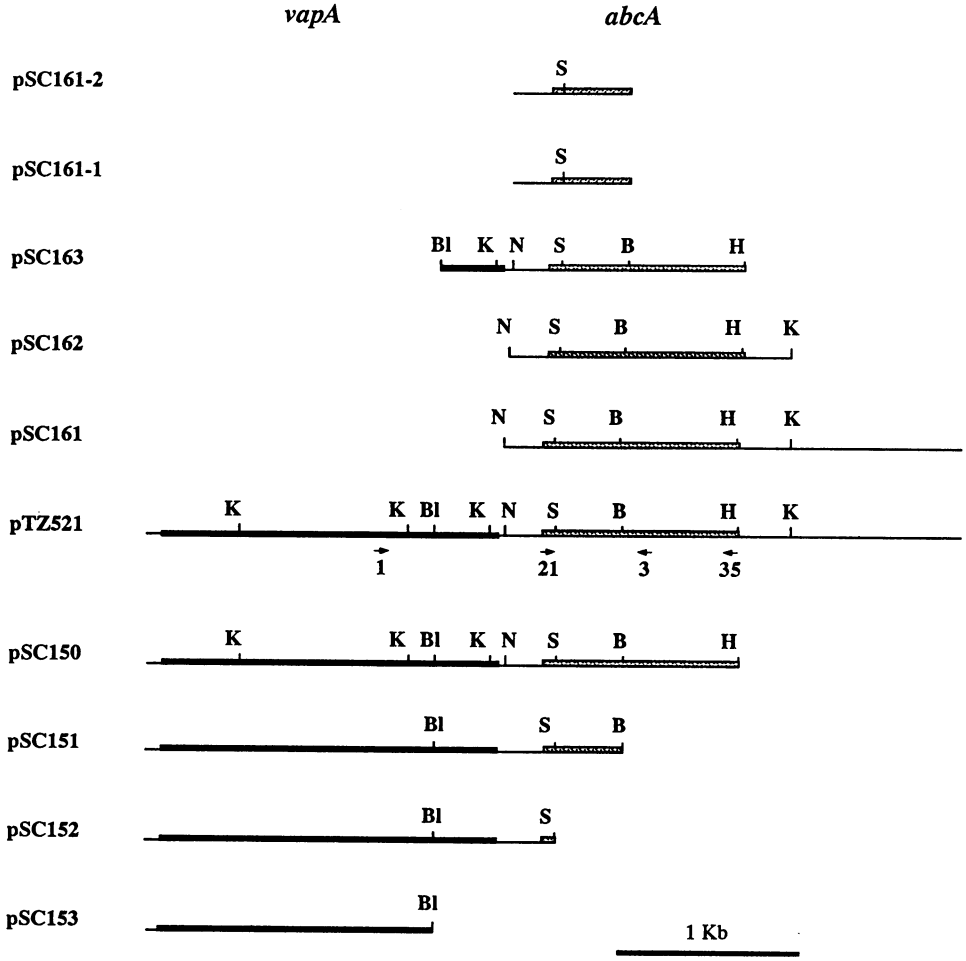

FIG. 1. Restriction maps of inserts in each subclone used in this study. Names of the subclones are given on the left. All subclones are vertically aligned to each other. Arrows indicate the positions of oligonucleotide primers used for polymerase chain reaction analysis of gene distribution. Numbers 1, 21, 3, and 35 beneath the arrows indicate primers AP-1, TJT21, RAA3, and TJT35, respectively. Primer RAA2 maps at bases −426 to −438 relative to the *vapA* gene (data not included in the map). Thick lines indicate *vapA* coding regions. Stippled boxes represent the *abcA* gene. B, *Bam*HI; Bl, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Nar*I; S, *Sph*I.

lane 7). Further deletion to the *Sph*I site (pSC152), which is less than 300 bp downstream from *vapA*, resulted in no further decrease in expression (Fig. 2, lane 4), while deletions in the *vapA* structural gene (pSC153) resulted in apparent total loss of production of A-protein or immunoreactive truncated A-protein products (data not shown).

To confirm that the downstream DNA influenced *vapA* expression in *E. coli*, complementation assays were performed. A 1.4-kb *Bgl*II-*Hin*dIII fragment from pSC150 was subcloned into a P15A replicon vector pSU2718 (pSC163) (Fig. 1) (35). This plasmid was then introduced into DH5α cells containing either pSC151 or pSC152. Western blot analysis showed that DH5α cells carrying either of the two pairs of plasmid, i.e., pSC151 and pSC163 or pSC152 and pSC163, produced A-protein in amounts indistinguishable from pSC150/DH5α (Fig. 2, lanes 3 and 5); i.e., production of A-protein was significantly increased by reintroduction of the 1.4-kb *Bgl*II-*Hin*dIII downstream DNA fragment.

DNA sequence analysis. Sequencing of the DNA downstream from *vapA* revealed an ORF (Fig. 3). This ORF is located on the same strand as *vapA* and starts 205 bp after the *vapA* termination codon. A number of additional stop

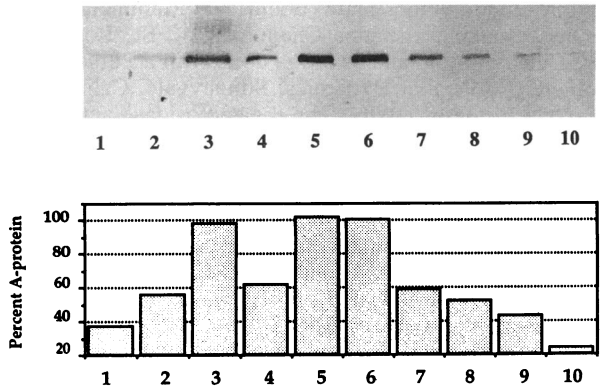

FIG. 2. Western blot detection of A-protein (upper panel) with 1:3,000-diluted affinity-purified anti-A-protein antiserum and densitometric analysis of the blot (lower panel). All samples were whole-cell lysates of *E. coli* carrying the following plasmids: pSC151/pSU2718 (lane 1), pSC151 (lane 2), pSC151/pSC163 (lane 3), pSC152 (lane 4), pSC152/pSC163 (lane 5), pSC150 (lane 6), and pSC150 for lysates diluted 1/16, 1/32, 1/64, or 1/128 (lanes 7 to 10, respectively).

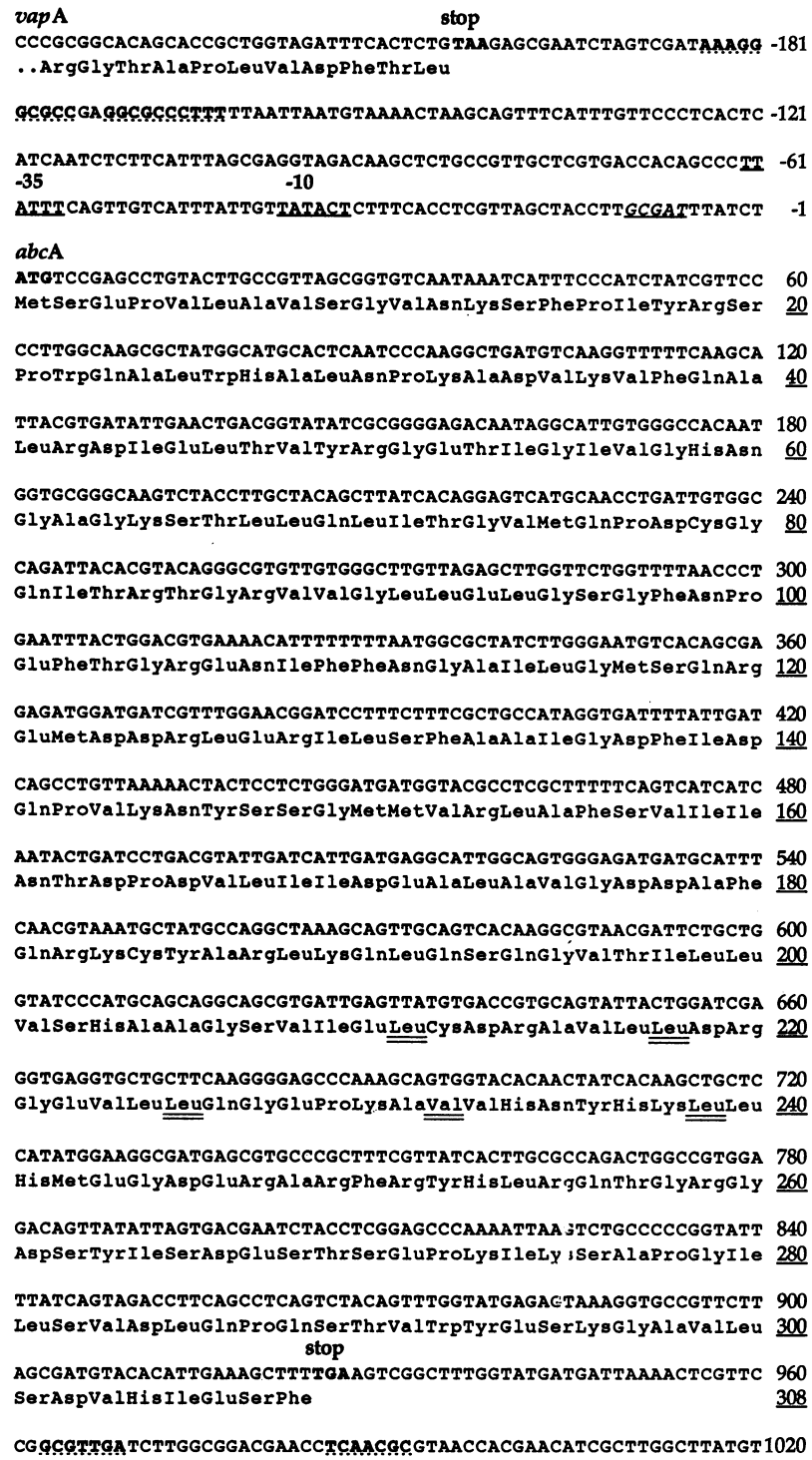

FIG. 3. Nucleotide sequence of the *abcA* gene and flanking DNA from *A. salmonicida* A450 and the translated amino acid sequence. The 3′ sequence of the *vapA* gene is indicated, as are C-terminal residues of the A-protein. The numbers on the right indicate nucleotide positions and amino acid residues (underlined) in *abcA*. The predicted translational start of the *abcA* gene is shown in bold, as are the termination codons of both the *abcA* and *vapA* genes. The possible ribosomal binding site and −10 and −35 RNA polymerase binding sites in front of the gene are underlined, residues composing the heptad Leu-Val repeat are double-underlined, and the inverted-repeat-forming stem of the possible transcriptional terminators are underlined with broken lines.

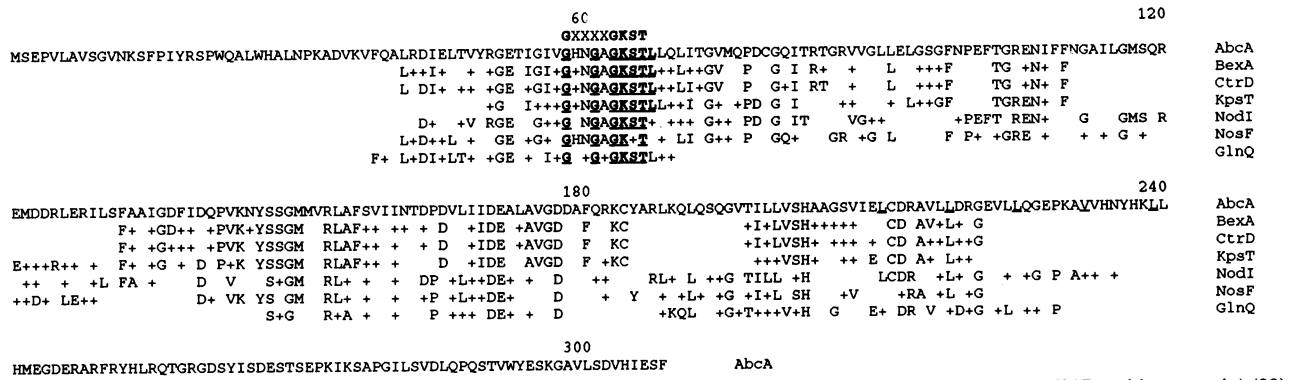

FIG. 4. Alignment of AbcA protein (308-residue protein) of *A. salmonicida* with ABC transporter proteins BexA (217-residue protein) (29), CtrD (216-residue protein) (15), KpsT (224-residue protein) (41), NodI (306-residue protein) (18), NosF (308-residue protein) (55), and GlnQ (224-residue protein) (54). Residues representing conservative substitutions are indicated by +. The AbcA residue number in the sequence is indicated, the consensus P-loop sequence is shown above the AbcA sequence on the top line, and residues of the putative leucine zipper are underlined.

codons are present in all three reading frames in the intervening sequence between the two genes. This downstream ORF is the only one in the area coding for a peptide of more than 300 amino acids. Other predicted ORFs coding for more than 150 amino acids are all from methionine codons within this larger ORF. The complete ORF, i.e., gene *abcA*, contains 924 bp and is terminated by a TGA stop codon. In pSC151 and pSC152, although the *vapA* gene is still intact, the ORF was truncated at the 3′ end by 541 bases and 846 bases, respectively.

The intact *abcA* gene has a GC content of 47%, which is similar to the 48% G+C content of *vapA* (10) but lower than the reported 55% G+C content for the *A. salmonicida* genome (7). Codon usage shows some bias for codons with T (35.7%) in the third position, and the codon adaptation index (48) according to *E. coli* was 0.242, much lower than the *vapA* codon adaptation index of 0.516. Codon usage in *abcA* was quite different from that in *vapA*. This was most obvious for the codons for Phe, Pro, Ala, Tyr, and Gly. For example, while UUU is used for 11 of the 12 Phe's in *abcA*, it receives only 36% usage in *vapA*. Also, in the case of Pro, CCA and CCG are not used in *abcA* but was used 70% in *vapA*. The only other codon not used in *abcA* is the Arg codon AGA. Possible −35 and −10 promoter regions are indicated in Fig. 3. The predicted −10 region promoter hexamer sequence TATACT is 33 bp upstream from the first codon ATG, and a poorly conserved putative −35 sequence TTATTT ends 17 nucleotides further upstream. There is also a possible ribosomal binding site 7 bp from the start of the gene. A predicted stem-loop structure was located 12 bases downstream from the stop codon, with a Gibbs free energy value (ΔG) of −6.2. It consists of a 7-bp stem and a 16-base loop, which could serve as a transcriptional terminator.

Because *vapA* had been shown to be conserved in *A. salmonicida*, we examined the possibility that *abcA* might also be conserved. Two approaches were used to determine the presence of this coding sequence in the chromosomal DNAs from a range of *A. salmonicida* strains, including the subspecies *salmonicida* (so-called typical strains), *nova* and *achromogenes* (so-called atypical strains) (7), and isolates from diverse species of fish, from diverse geographical regions, and from diseases with different pathogeneses. Southern blot analysis of *Kpn*I-digested chromosomal DNAs (data not shown) of nine representative strains (A362, A400, A419, A449, A450, A461, A477, A480, and A500) probed with plasmid pSC162 (Fig. 1), which contains a 1.4-kb *Nar*I-*Kpn*I insert including the entire 924-bp *abcA*, showed that a 1.6-kb *Kpn*I-*Kpn*I DNA fragment was conserved among the *A. salmonicida* strains tested (Table 1). No hybridization was seen with chromosomal DNAs of the tetragonal S-layer-producing strains of *A. hydrophila* (TF7 and A274) and *A. veronii* biotype sobria (A700 and A702) tested (Table 1). In addition, polymerase chain reaction analysis with primers allowing amplification of an internal sequence of the *abcA* gene (TJT21 and TJT35) or internal and 5′ sequences (AP1 and RAA3, RAA2 and RAA3 and AP1 and TJT35) (Fig. 1) resulted in the appropriately sized sequence (720, 1,170, 2,693, and 1,438 bp from the respective primer pairs) from so-called typical strains A202, A203, A251, A395, A440, A449, A450, A451, A488, A505, and A506, and so-called atypical strains A206, A401, A430, A442, and A475 (Table 1).

Predicted protein structure. The *abcA* gene of *A. salmonicida* A450 encodes a predicted M_r-34,015 protein containing 308 amino acid residues (Fig. 3). The protein contains 43.5% hydrophobic residues (including 10% Leu), and the Kyte-Doolittle (31) hydropathic index for an interval of nine amino acids gives an average hydrophobicity score of −0.8. By the method of Klein et al. (26), the protein has a possible membrane-associated segment between Val-196 and Ile-209 (peripheral:integral odds of 0.23). In addition, the AbcA protein contains 31.8% polar, 12.0% acidic, and 12.7% basic residues, a predicted pI of 5.7, and an overall negative charge (−5.58 at pH 7).

The predicted secondary structure of this protein includes a 42.8% α-helix, 39.2% β-sheet, 9.4% β-turn, and 8.4% coiled structure (16). The longest α-helix-containing region is between Ser-202 and Arg-249, with all but four residues predicted to fall within the helix. In this region, a heptad repeat of hydrophobic residues is present, starting at residue 211, with Leu in four of the five heptad repeats and Val in the remaining heptad repeat. This leucine-containing sequence overlaps a highly positively charged sequence of 22 residues (Tyr-237 to Arg-259), which has a predicted pI of 11.02, and is within the longest hydrophilic region of the deduced protein.

Sequence similarity searches showed that the protein contained regions of the sequence with high similarities to a number of proteins belonging to a superfamily of ATP-driven membrane transporters (Fig. 4) (22). The NodI protein of

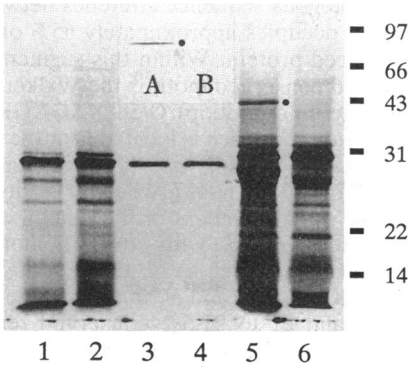

FIG. 5. In vivo plasmid expression and localization of AbcA protein (●) from pSC161 by using the T7 polymerase expression system. Lanes: 1 and 2, cytosol fractions; 3 and 4, periplasmic fractions; 5 and 6, cell envelope fractions of pSC161/DH5α and pTZ18R/DH5α, respectively. Molecular masses (in kilodaltons) are indicated on the right. (Inset) Presence of the apparent M_r-43,000 protein in the sarkosyl soluble (A) fraction and absence of the protein in the sarkosyl insoluble (B) fractions obtained from the cell envelope fraction.

Bradyrhizobium japonicum (18) (BLAST score of 160), the ctrD protein of *Neisseria meningitidis* (14) (BLAST score of 144), the polysialic acid transport protein KpsT of *E. coli* (40) (BLAST score of 141), and the BexA protein of *Haemophilus influenzae* (BLAST score of 130) (28) were examples of the proteins that gave the highest homology scores (Fig. 4). Homologies with the *Pseudomonas* ATP-binding protein NosF (56) and the *Bacillus* glutamate permease transporter GlnQ (55) are also shown in Fig. 4.

Identification and localization of the AbcA protein. Plasmid pSC161 (Fig. 1) and the T7 polymerase expression system were used to demonstrate the product of the *abcA* gene and to determine the cellular location of the gene product. This plasmid contains a 2.5-kb *Nar*I-*Pst*I insert, with the *abcA* gene located about 170 bases downstream from the vector's T7 promoter. To induce the T7 promoter, pSC161 was introduced into *E. coli* KX100, and a second plasmid, pGP1-2, was also introduced. pGP1-2 contains the T7 polymerase gene which can be induced by raising the temperature to 42°C (43). After induction of the T7 polymerase gene and [^{35}S]methionine labelling, the cells were fractionated, and the fractions were examined by SDS-PAGE. For the negative control, vector plasmid pTZ18R was used in place of pSC161. A ^{35}S-radiolabelled protein with an apparent M_r of 43,000 was detected in the envelope fraction in pSC161 assays (Fig. 5). This protein was not detected in the cytosol and periplasmic fractions or in the negative controls. After treatment of the envelope fraction with sodium lauryl sarcosinate and centrifugation to pellet the outer membrane fraction, the M_r-43,000 protein remained in the sarcosyl-soluble cytoplasmic membrane fraction (Fig. 5, inset). Two-dimensional electrophoretic analysis showed that the protein displayed a pI of 6.3 (data not shown). Another *Nar*I-*Pst*I insert-coded protein which was also detected in the envelope fraction was one with an apparent M_r of 30,000, which other studies (unpublished observations) have shown to be a membrane protein coded approximately 324 bp downstream of *abcA*.

To confirm that the M_r-43,000 protein was the gene product of *abcA*, a prokaryotic DNA-directed translation kit was used with a 1.7-kb *Nar*I-*Kpn*I fragment cut from pSC162 (Fig. 1) as template. This DNA contained the entire *abcA* gene but no other ORFs capable of coding for a peptide longer than 200 amino acid residues, and by using this 1.7-kb *Nar*I-*Kpn*I fragment the only protein observed in the test lane which was not present in the control lane had an apparent M_r of 43,000 (data not shown).

Analysis of expression. Expression of the *abcA* gene in *E. coli* was also examined by using a *lacZ* fusion. The vector used to construct the *abcA-lacZ* fusion was pMC1871, which contains a promoterless *lacZ* gene and a *Sma*I site at the beginning of *lacZ*, facilitating the creation of LacZ fusion proteins (47). *Aeromonas* DNA in pSC162 was digested by *Bam*HI, which cut at the vector polycloning site (168 bases in front of the *abcA* gene) and at the site 387 bases into the gene (Fig. 1). The *Bam*HI ends were made blunt by filling in with Klenow fragment. pMC1871 was then digested with *Sma*I and ligated to the blunt-ended *Bam*HI fragment. More than 500 colonies were obtained, and approximately 10% displayed blue color on plates supplemented with X-Gal. These colonies were selected, and whole-cell lysates were screened for the presence of an AbcA–β-galactosidase fusion protein by Western blot analysis with a monoclonal anti-β-galactosidase antibody. The fused gene selected, pSC161-1, produced a fusion protein with an apparent M_r of 130,000, which was confined to the cell cytosol (data not shown). The construction of the fusion was validated by restriction digestions, i.e., the insertion was confirmed to be 0.5 kb, and the *Sph*I site in the insertion was shown to be in the correct location. On the basis of the DNA sequence, this fusion protein should contain 129 residues coded by the *abcA* gene, a number consistent with the observed molecular weight of the fusion protein.

To confirm that the *abcA* gene could also be expressed in *A. salmonicida* and to obtain a measure of promoter activity, the *abcA-lacZ* fusion gene was introduced into *A. salmonicida* A450 by using the broad-host-range plasmid pMMB67 (pSC161-2) (Fig. 1). In this clone, a 3-kb *Pst*I-*Pst*I fragment from pSC161-1 containing the whole fusion gene and 168 bp in front of the gene was inserted into the *Pst*I site in pMMB67. Since only a very short *Aeromonas* DNA fragment was included in front of the fusion gene in pSC161-2, it was possible that the gene could be transcribed from an outside promoter. Therefore, the fusion gene was also cloned into pMMB67 in the opposite orientation, and the β-galactosidase activity in both orientations was measured. The β-galactosidase activity in both directions was identical at 1,000 Miller units. This is essentially the same level as the 900 Miller units of β-galactosidase activity of pSC161-1 measured in *E. coli* DH5α. Control assays with pMC1871 in *E. coli* were negative, and pMMB67 in A450 gave less than 5 U of β-galactosidase activity.

Purification of the AbcA–β-galactosidase fusion protein and identification of ATP-binding activity. Because a sizeable portion of the ABC sequence was contained in the AbcA–β-galactosidase fusion protein, including a predicted ATP-binding site, the ATP-binding ability of the purified fusion protein was tested. This required that the fusion protein be purified from a pSC161-1/DH5α culture. Western blot analysis with monoclonal anti-β-galactosidase antibody showed that the fusion protein was precipitated in the 10 to 40% $(NH_4)_2SO_4$ fraction of the cell cytosol. Further purification was achieved by immunoaffinity column chromatography by using ProtoSorb *lacZ*-agarose beads bound with anti-β-galactosidase monoclonal antibody. Bound protein was eluted with pH 10.8 buffer. The majority of the fusion protein was found in the first 2 bed volumes of eluent. The first 6 bed

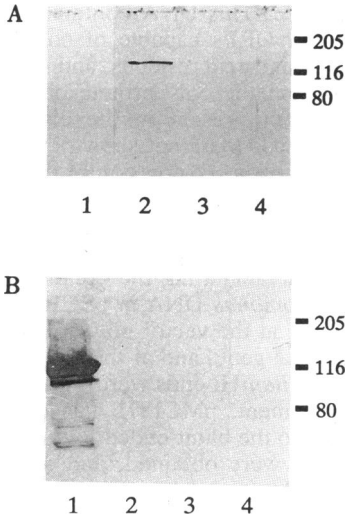

FIG. 6. Nucleotide binding ability of the purified AbcA–β-galactosidase fusion protein (A) and *E. coli* β-galactosidase (B) tested by using an ATP affinity column. The figures show Western blots of fractions eluted from the ATP affinity column. Lanes: 1, void volume; 2, 0.125 mM ATP; 3, 0.25 mM ATP; 4, 0.5 mM ATP. Molecular masses (in kilodaltons) are indicated on the right.

volumes of eluent were pooled and concentrated, and the purity of the preparation was confirmed by SDS-PAGE. The ATP-binding ability of the purified fusion protein was then tested with an ATP affinity column. Western blot analysis showed that the 130-kDa fusion protein bound to the ATP affinity column and required 0.125 mM ATP for elution (Fig. 6A), confirming the nucleotide-binding activity of the AbcA protein. In control assays, β-galactosidase did not bind to the ATP affinity column, eluting in the void volume (Fig. 6B).

DISCUSSION

This study has isolated and characterized an *A. salmonicida* gene, *abcA*, coding for a 308-residue protein which has ATP-binding activity and which appears to belong to the ABC transport family of proteins, or so-called traffic ATPases. Proteins belonging to this superfamily are associated with a variety of cellular functions, including cell division and DNA repair (23, 40), as well as a wide variety of transmembrane transport processes, including the transport of ions, heavy metals, sugars, amino acids, oligopeptides, and proteins (25, 30). Bacterial ABC-proteins include the well-characterized periplasmic permeases (39), capsule polysaccharide transporters (15, 29, 41), as well as protein secretion systems including the transporters for a *Bradyrhizobium* and *Rhizobium* nodulation protein (NodI) (11, 19), *E. coli* hemolysin A (HlyB) (12). *Pasteurella haemolytica* leukotoxin A (LktB) (49), *Bordetella pertussis* cytolysin (CyaB) (17), and *Pseudomonas aeruginosa* alkaline protease (PrtPA) (20). Eucaryotic members of the family include putative peptide transporters encoded at the locus for the class II major histocompatability complex (51) and the yeast Ste6 transporter which mediates export of a peptide hormone that lacks a classical hydrophobic signal sequence (30).

This superfamily of transporter proteins shares about 30% sequence identity over an ABC of about 200 amino acids (25). In the *A. salmonicida* protein, the conserved ABC transporter homologous sequence stretches between Phe-38 and Asn-236 and occupies approximately 65% of the length of the total deduced protein. Within this segment, the most readily recognized conserved motif is the Walker motif A or P-loop (phosphate-binding loop) G-58 XXGXGKST nucleotide-binding sequence, which is located near the N-terminal end of the protein (45, 54). *abcA* shares another loop structure, $(O)_4$-DEAXXXXD-177 (where O is a hydrophobic amino acid) with other members of the family, and several other segments of sequence with strong homology to the ABC transport proteins, such as *N. meningitidis* CtrD protein (15), *E. coli* KpsT (41), and *H. influenzae* BexA (29) (34 to 37% identity and 21 to 21.5% conserved replacements between residues 42 and 221), which are involved in capsular polysaccharide transport. These three proteins have recently been shown to belong to a new subfamily within the family of ATP-type transporters, the ABC-2 family (42).

One feature which appears to distinguish this *A. salmonicida* protein from other ABC proteins is the presence of a possible leucine zipper. This predicted α-helical region has Leu residues in four of five heptad repeats and a Val replacement in the fourth heptad repeat, which should allow the region to form a zipper (24, 46). The potential zipper sequence does contain a Pro; however, neither the Garnier (16) nor the Chou-Fasmann (9) algorithms predict disruption of the extended α-helix by this residue. The heptad repeat region is juxtaposed to a highly positively charged 23-residue sequence, which is a characteristic of leucine zipper proteins in eucaryotes (53). This region binds DNA in the eucaryotic proteins and is located N terminal to the leucine zipper, leading to the nomenclature bZIP protein. In the *A. salmonicida* protein, the basic region is carboxy terminal, providing a ZIPb-like structure (53). This differs from other reported procaryotic leucine zipper proteins where no typical basic regions have been observed near the zipper region and where the leucine zipper is usually at the N terminus of the protein (36, 46). The highly positively charged region in AbcA probably contributes to aberrant migration during SDS-PAGE, accounting for the difference in deduced (M_r of 34,015) and measured (M_r of 43,000) apparent molecular weight. Such effects have been noted before, for example for the HlyB protein of *V. cholerae* reported by Alm and Manning (2).

The *abcA* gene appears to be conserved in *A. salmonicida* and was not present in the other tetragonal S-layer-producing *Aeromonas* strains tested. The gene maps immediately downstream of the surface array protein structural gene, *vapA*, and appears to be followed by a gene coding for an outer membrane protein. As such, it is the first gene flanking an S-protein gene to be identified, and only the third gene to be shown to influence either the production or the export of an S-layer protein. The other two genes were identified in transposon insertion studies, and their products are required for the export of A-protein across the outer membrane (6). Preliminary studies indicate that these genes map several kilobases away from *vapA* and *abcA*. In the case of procaryotic ABC transport systems, the basic organization normally involves four distinct subunit components, either as domains of one or more proteins or as distinct individual proteins (25, 31). The various components interact to form a membrane-associated transport complex. The integral membrane proteins or protein domains which form part of this complex are believed to mediate the actual transport of the substrate across the lipid bilayer. Two of the subunits in the transport complex, usually ABC proteins, are normally encoded by a single gene and couple ATP hydrolysis to the transport. The

ABC-containing components are associated with the hydrophobic face of the membrane, and appropriately, the *abcA* gene product of *A. salmonicida* cofractionated with the cell envelope fraction when it was expressed in *E. coli* and indeed subfractionated into the sarkosyl-soluble putative inner membrane fraction.

In contrast to the *sec*-encoded protein secretion system which recognizes many different signal peptides (4), each ABC transport appears to be relatively specific for a single substrate or group of related substrates (25, 31). The genes encoding the ABC transport complex normally map contiguously (23). For example, the *E. coli hlyB* gene maps immediately downstream of the substrate of its transport system, *hlyA* (34), while the ABC transport complex proteins involved in capsular polysaccharide transport appear to belong to a single transcriptional unit which contains two to four other genes, including genes coding for integral membrane proteins (15). The organization of these capsular transport genes is very similar in different bacterial species, with the ABC-containing protein itself mapping after the membrane protein genes. Taken together, these various observations lend weight to the notion that VapA could be the substrate of an transport system involving AbcA. However, *abcA* and *vapA* do not appear to be arranged in a transcriptional unit. The two genes are separated by a sizeable stretch of DNA, they have significantly different codon usage, and Northern (RNA) blot analysis indicates that the *vapA* mRNA length is not sufficient for the two structural genes. *vapA* also has a strong terminator sequence, and in this study, *abcA* appeared to be expressed off its own promoter. The measured level of this expression off a plasmid was consistent with those of many other promoters (33), and the gene was expressed at similar levels in both *A. salmonicida* and *E. coli*.

The presence or absence of the *abcA* gene clearly influences production of the surface array protein of *A. salmonicida* in *E. coli*. Moreover, the gene is appropriately located, and its gene product has the appropriate characteristics to influence both the production and the transport of VapA in *A. salmonicida* in some fashion. The precise role of this gene in *A. salmonicida* now needs to be determined, and whether the effect on A-protein production in *E. coli* is indirect or is due to gene regulation needs to be elucidated. Unfortunately, attempts to knock out the gene in *A. salmonicida* have been unsuccessful, so we have not been able to determine the effect of AbcA on the expression of chromosomally encoded *vapA* in the natural host. However, studies are underway to determine whether the AbcA protein is capable of binding DNA and so has regulatory activity. Analogy with other ATP transporter systems suggests that the *abcA* gene should also affect transport, either of the surface array protein or some other surface or extracellular component such as a surface carbohydrate. Indeed, preliminary findings suggest that AbcA is a bifunctional protein, with both regulatory and transport activities.

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