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Endogenous mutagenesis by an insertion sequence element identifies *Aeromonas salmonicida* AbcA as an ATP-binding cassette transport protein required for biogenesis of smooth lipopolysaccharide

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ABSTRACT **Analysis of an *Aeromonas salmonicida* A layer-deficient/O polysaccharide-deficient mutant carrying a Tn*5* insertion in the structural gene for A protein (*vapA*) showed that the *abcA* gene immediately downstream of *vapA* had been interrupted by the endogenous insertion sequence element IS*AS1*. Immunoelectron microscopy showed that O polysaccharides did not accumulate at the inner membrane–cytoplasm interface of this mutant. *abcA* encodes an unusual protein; it carries both an amino-terminal ATP-binding cassette (ABC) domain showing high sequence similarity to ABC proteins implicated in the transport of certain capsular and O polysaccharides and a carboxyl-terminal potential DNA-binding domain, which distinguishes AbcA from other polysaccharide transport proteins in structural and evolutionary terms. The smooth lipopolysaccharide phenotype was restored by complementation with *abcA* but not by *abcA* carrying site-directed mutations in the sequence encoding the ATP-binding site of the protein. The genetic organization of the *A. salmonicida* ABC polysaccharide system differs from other bacteria. *abcA* also differs in apparently being required for both O-polysaccharide synthesis and in energizing the transport of O polysaccharides to the cell surface.**

The Gram-negative bacterium *Aeromonas salmonicida* is an important pathogen of fish (1). A conserved property of *A. salmonicida* is the production of a paracrystalline surface protein array known as A layer. This array enhances the virulence of *A. salmonicida* (2) by protecting the organism against fish defense mechanisms and promoting host colonization (3–5). Indeed mutants unable to produce A layer display a $>10^3$-fold reduction in their virulence for salmonid fish (2). A layer is composed of a single M_r 50,000 species of protein (A protein/VapA) (6). A second conserved property of the *A. salmonicida* surface is the production of a lipopolysaccharide (LPS) with antigenically cross-reactive O polysaccharides of homogeneous chain length (7). These O chains contribute resistance to the bactericidal activity of complement (3) and also appear to tether A layer to the cell surface (8). Surface array-producing strains of *Aeromonas hydrophila* and *Aeromonas veronii* also have antigenically conserved homogeneous chain length O chains (9), and polysaccharide interaction is again required to retain S layer on the cell surface (10). In *A. hydrophila*, this polysaccharide interaction is with the carboxyl-terminal domain of the S protein (11).

The *A. salmonicida* gene encoding VapA has been cloned and sequenced (12), as has the gene *abcA* immediately downstream of *vapA* (13). The amino-terminal region of the AbcA protein contains a cassette belonging to a subfamily of ATP-binding cassette (ABC) proteins catalyzing export of drugs and carbohydrates (14), whereas the carboxyl-terminal region has a possible DNA-binding domain. AbcA appears to be required for maximal expression of the *vapA* gene in *Escherichia coli*, with the presence of *abcA* resulting in a 16-fold increase in VapA production (12). In this study we examined a Tn*5* insertion mutant that has lost the ability to produce both A protein and smooth LPS (A^-O^-; ref. 15). We found that in addition to a Tn*5* insertion in the *vapA* gene, this A^-O^- mutant carried an endogenous insertion sequence (IS) element in the *abcA* gene, and here we report that the gene and its ATP-binding site are required for biosynthesis of O polysaccharide-containing LPS in *A. salmonicida*. AbcA appears to represent an additional ABC carbohydrate transport protein.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Bacteriophage, and Media. *A. salmonicida* strain A449 and the Tn*5* mutant A449-TM4 were grown in L broth or on L agar at 20°C. *E. coli* strains DH5α, S17.1, BMH71-18 *mutS*, and KX100 were grown on the same media but at 37°C. When required, ampicillin and kanamycin were used at a concentration of 50 μg/ml. The plasmid vectors used were pTZ18R, pMMB67EH, pSUP202, pHC79, and pGP1-2. Recombinant plasmids constructed in the study are listed in Table 1. *A. salmonicida* bacteriophage 55 was from the collection of E. E. Ishiguro (Department of Biochemistry and Microbiology, University of Victoria, Victoria BC, Canada).

DNA Manipulations. Preparation of DNA, restriction digestions, ligations, and transformation were carried out essentially as described by Sambrook *et al.* (16). DNA sequencing reactions were performed using the *Taq* DyeDeoxy terminator cycle sequencing kit [Applied Biosystems (ABI)] on an ABI model 373A automated sequencer. Custom primers were synthesised on an ABI PCR-MATE oligosynthesiser. PCR was performed using a Perkin–Elmer DNA thermal cycler (model 480).

Mutagenesis of the *abcA* Gene. Marker exchange mutagenesis was carried out using the kanamycin-resistance cassette from Tn*903* (17). Selection using bacteriophage 55 was performed by incorporating 100-μl volumes of phage lysate into the selection plates. Site-directed mutagenesis was performed on the *abcA* gene cloned in pTZ18R using synthetic primers with single-base-pair mutations and the repair-deficient *E. coli* strain BMH71-18 *mutS* (Clontech).

SDS/PAGE and Western Immunoblotting. SDS/PAGE was by the method of Laemmli (18). Western blotting was as described (19). Samples for LPS analysis were treated with proteinase K (Boehringer Mannheim) for 2 h at 50°C and analyzed by SDS/PAGE, silver staining (20), or Western immunoblot.

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Abbreviations: LPS, lipopolysaccharide; ABC, ATP-binding cassette; IS, insertion sequence; ORF3, open reading frame 3; Km^r, kanamycin resistance.

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Table 1. Plasmid constructs generated in this study

Plasmid	Description
pSC162	*Nde* I–*Kpn* I fragment containing *abcA* in pTZ18R
pSC-162-1	Insert from pSC162 in pMMB67EH
pSCGV58	Mutated *abcA* (Gly-58 → Val) in pTZ18R
pSCGV58-1	Insert from pSCGV58 in pMMB67EH
pSCGV61	Mutated *abcA* (Gly-61 → Val) in pTZ18R
pSCGV61-1	Insert from pSCGV561 in pMMB67EH
pSHKm	*abcA*::Km^r (*Sph* I site) in pSUP202
pBAKm	ORF3::Km^r (*Kpn* I site) in pSUP202

Km^r, kanamycin resistance.

***In Vitro* Transcription and Translation.** An *E. coli* S30-coupled transcription and translation system (Promega) was used to express mutated *abcA* genes. The procedure used for T7 polymerase *in vivo* transcription was based on Rosenberg's method (21) with modifications (13, 22, 23).

Immunogold Labeling and Electron Microscopy. Bacterial cell samples were prepared and examined as described (24). Samples were labeled with polyclonal rabbit anti-*A. salmonicida* LPS antiserum diluted 1:200.

RESULTS

Characterization of an *A. salmonicida* Mutant Defective in the Biosynthesis of A Layer and LPS-Containing O-Polysaccharide Chains. A449-TM4, the previously isolated Tn*5* mutant of *A. salmonicida* A449, has only a single Tn*5* insertion yet possesses two distinct phenotypic characteristics (15). A449-TM4 does not produce the structural protein (A protein) for the surface layer and is also lacking the O-polysaccharide side chains on its LPS. To determine the position of this Tn*5* insertion, a genomic library of A449-TM4 was constructed in the cosmid vector pHC79. One of these cosmid clones, pHC79-42, was found to contain the Tn*5* insertion. Restriction and DNA sequence analysis were then used to precisely determine the site of insertion of the Tn*5*. The Tn*5* in A449-TM4 was found to have inserted into the structural gene for the A protein, *vapA*, at a location 417 bp from the translation start codon (Fig. 1). This suggested that expression of A protein and O polysaccharide might be linked in some fashion (15).

The recent finding that endogenous IS elements could insert in the *vapA* region of the chromosome of *A. salmonicida* (25), together with the sequence similarity of AbcA to ABC polysaccharide transporter proteins (13), suggested that the lack of O chains in this A$^-$O$^-$ mutant was not the result of the Tn*5* insertion in *vapA* but resulted from IS insertion into *abcA*. PCR analysis using primers TJT30 and CG4 (Table 2) to amplify *abcA* showed that the fragment amplified from the A449-TM4 chromosome was 1.2 kb larger than the fragment amplified from wild-type A449, verifying that an insertion had taken place in the *abcA* gene of A449-TM4. Southern hybridizations showed that the 1.2-kb difference was due to insertion of IS*AS1* (25), and sequence analysis indicated that IS*AS1* had transposed into the 3′ end of *abcA*, 885 bp from the translational start site (Fig. 1).

Restoration of O-Polysaccharide Chains on the LPS of A449-TM4 with Cloned *abcA*. To confirm that this mutation in *abcA* was responsible for the loss of O-polysaccharide chains on the LPS, the wild-type *abcA* gene was cloned into the low-copy-number vector pMMB67EH (pSC162-1) and mobilized into A449-TM4 by conjugation. As a negative control, the vector alone was also mobilized into A449-TM4. SDS/PAGE and silver staining analysis of exconjugant LPS (Fig. 2) and Western immunoblot analysis with rabbit polyclonal antisera raised against smooth *A. salmonicida* LPS (data not shown) showed that pSC162-1 complemented the O-polysaccharide chain phenotype in A449-TM4. Electron microscopy of immunogold-labeled thin sections further demonstrated that the O-polysaccharide chains being detected in A449-TM4 (pSC162-1) were located on the outer surface of the cell (Fig. 3). In contrast, little or no labeling was observed on the surface of A449-TM4 or A449-TM4/pSCGV61-1.

Marker Exchange Mutagenesis of the *abcA* Gene. To further confirm that the IS*AS1* insertion in A449-TM4, and not the Tn*5* insertion upstream in *vapA*, was responsible for the O-chain phenotype, the *abcA* gene was mutated in A449 by allele replacement. A mutated copy of the *abcA* gene was generated by inserting a cassette encoding a Km^r gene into the *Sph* I restriction site in the 5′ end of the cloned gene. Selective pressure was provided using a bacteriophage (phage 55) that has as its receptor the O polysaccharide of *A. salmonicida* (26), resulting in the isolation of the mutant, A449-M6. pSC162-1 was conjugated into A449-M6 and was shown to complement the LPS mutation (data not shown).

Site-Directed Mutagenesis of the ATP-Binding P Loop of AbcA. The *abcA* gene product has been previously shown to bind ATP, and the deduced amino acid sequence of the gene

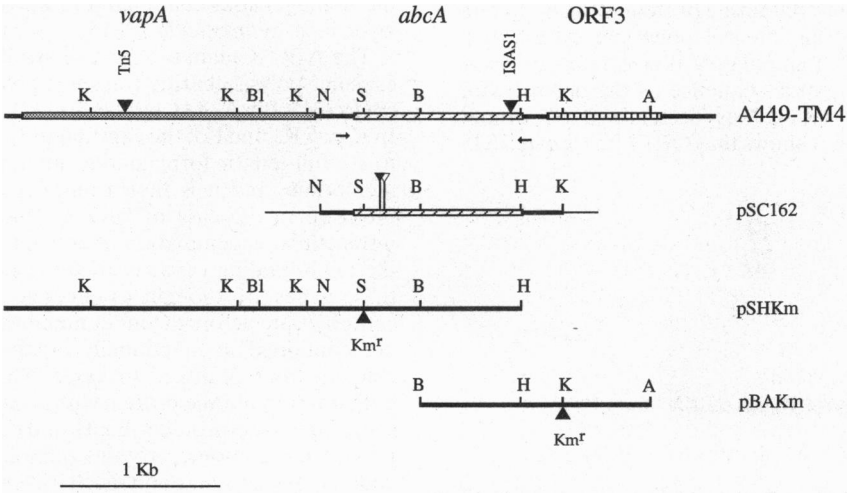

FIG. 1. Physical map of the chromosome of A449-TM4 in the region of *vapA*, *abcA*, and open reading frame 3 (ORF3). Also shown are plasmids pSC162, pSHKm, and pBAKm. The Tn*5* and IS*AS1* insertions in A449-TM4 chromosome DNA are indicated as solid arrowheads and are individually labeled. Small horizontal arrows represent DNA primers TJ30 and CG4 on the left and right, respectively. The two markers in the pSC162 map indicate the sites of base replacements in the ATP-binding site encoding sequence of *abcA* in pSCGV58 and pSCGV61, respectively. A, *Apa*LI; B, *Bam*HI; Bl, *Bgl* II; H, *Hin*dIII; K, *Kpn* I; N, *Nde* I; S, *Sph* I.

Table 2. Oligonucleotide primers used in this study

Primer	DNA Sequence	Position
TJT30	5′-GCCGTTGCTCGTG-ACCA-3′	−85 to −69 of *vapA*
CG4	5′-CAATGTGTACATC-GCTAAGAACA-3′	891 to 916 in *abcA*
SCI-V	5′-CTCACTATAGGGA-TATCGAGCTCGG-3′	*Eco*RI to *Eco*RV
SCG-V58	5′-GGCATTGTGGTCC-ACAATGGTG-3′	*abcA* (Gly-58 → Val)
SCG-V61	5′-GGCCACAATGTT-GCGGGCAAGTC-3′	*abcA* (Gly-61 → Val)

contains a highly conserved ATP-binding P-loop consensus sequence (GXXGXGKST, residues 58–66) (13). To determine if this P-loop consensus sequence was necessary for the complementation of A449-TM4, site-directed mutagenesis was performed to change two of the glycine residues (Gly-58 and Gly-61) to valine residues. Two oligonucleotide primers that span the ABC but that had a single base changed were synthesized (SCG-V58 and SCG-V61; Table 2) and used in conjunction with a selection primer SCI-V (Table 2) to synthesize the mutant genes in *E. coli* BMH71-18. The resultant mutant genes were sequenced to confirm the nature of the mutations.

Expression of Mutant *abcA* Genes in *E. coli*. To ensure that the mutant copies of *abcA* were expressed in *E. coli*, pSCGV58 and pSCGV61 were transformed into *E. coli* strain KX100, which carries the inducible T7 RNA polymerase gene on the plasmid pGP1-2. After induction and labeling with [^{35}S]methionine, the protein products were analyzed by SDS/PAGE. Both mutant copies of *abcA* were expressed although at a somewhat reduced level (Fig. 4).

The Native P Loop of AbcA Is Required for Complementation of A449-TM4. The two mutated forms of *abcA* were cloned into pMMB67EH to allow conjugation into *A. salmonicida*. Fig. 2 shows that neither pSCGV58 nor pSCGV61 was capable of complementing A449-TM4. Western blot analysis and immunogold labeling of thin sections (Fig. 3) confirmed that A449-TM4 containing either of these plasmids did not have O-polysaccharide chains on the LPS, and there was no accumulation of O polysaccharide at the inner membrane–cytoplasmic interface or in the cytoplasm.

Characterization of the Gene Downstream of *abcA*. A characteristic of ABC-type transport systems is that the gene encoding the ATP cassette-containing protein is linked to one or more genes encoding an integral polytopic cytoplasmic membrane protein. DNA immediately downstream of *abcA* was cloned, and the nucleotide sequence of the downstream gene (ORF3 relative to *vapA*) was determined (GenBank accession no. L37077). Fig. 1 shows that ORF3 was located 325 bp after the end of *abcA*. The 741-bp ORF3 was translated in the same direction as *vapA* and *abcA* and had a G+C content of 39.5%, which is significantly lower than the reported G+C content of 55% for the *A. salmonicida* genome (27). The gene encodes a predicted 247-residue protein of M_r 28,611, and *in vivo* expression in *E. coli* by T7 polymerase allowed identification of a protein of apparent M_r of 30,000 by SDS/PAGE. Automated Edman degradation showed that the first 17 amino acid residues were identical to the residues predicted from the DNA sequence. Allele replacement mutants of ORF3 were not affected in their ability to produce O polysaccharides as determined by silver staining and Western immunoblot. Data base searches showed that the protein displayed highest homology to the *N*-heparan sulfate sulfotransferase of *Rattus norvegicus* (Swiss-Prot accession no. Q02353; ref. 28), and when analyzed by the algorithms of Higgins and Sharp (29) and Kyte and Doolittle (30), the protein showed no apparent structural, sequence, or phylogenetic relatedness to proteins present in the capsular polysaccharide transporter systems of *Neisseria meningitidis* (31), *Haemophilus influenzae* (32), and *E. coli* (33) or the *Yersinia enterocolitica* O:3 (34) or *Vibrio cholerae* (35) O-polysaccharide transporter systems.

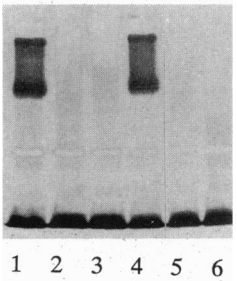

FIG. 2. Silver stain of LPS in proteinase K-digested whole cell lysates of *A. salmonicida*. The gels were overloaded in an attempt to demonstrate the presence of O polysaccharides on the LPS of the mutants. Lanes: 1, wild-type A449; 2, A449-TM4; 3, pMMB67/A449-TM4; 4, pSC162-1/A449-TM4; 5, pSCGV58/A449-TM4; 6, pSCGV61/A449-TM4.

DISCUSSION

This study has shown that the ATP-binding protein AbcA, whose gene maps immediately downstream of the *A. salmonicida* surface array protein gene *vapA*, and which has previously been shown to influence the level of expression of the *vapA* gene cloned in *E. coli*, is required for the biogenesis of smooth LPS in *A. salmonicida*. This smooth LPS is characterized by O polysaccharides having a repeat unit composed of a rhamnose-*N*-acetylmannosamine backbone substituted by glucose residues (36). These repeat units are polymerized to yield O polysaccharides of remarkably homogeneous chain length (7). There is increasing evidence that LPS with homogeneous length O-polysaccharide chains is important for the physical association of S layers with the bacterial cell surface. In addition to the S-layer-producing pathogenic aeromonads (7, 9) S-layer-producing Gram-negative bacteria including *Campylobacter fetus* (37, 38) and *Caulobacter crescentus* (39) have homogeneous chain length LPS. In the case of *A. salmonicida*, this study has shown that two genes essential for the biogenesis of smooth LPS and the A layer, and hence for the virulence of the organism, are tandemly positioned on the chromosome, further emphasizing the relationship between the homogeneous chain length LPS O polysaccharides and A layer in *A. salmonicida* (8, 15).

The ABC domain of AbcA shows 30–40% homology to the capsular polysaccharide transport proteins CtrD of *N. meningitidis* (31), BexA of *H. influenzae* (32), and KpsT of *E. coli* (33). In *E. coli* K1 and K5, the capsular polysaccharide is synthesized to its full-length form at the inner face of the cytoplasmic membrane, and it is then transported across the membrane (40–42). In the case of *kpsT* and *bexA*, mutation results in intracellular accumulation of polymer (33, 43), and because of the ATP-binding capacity of these proteins, the products of these genes are thought to energize the translocation of the carbohydrate across the inner membrane. *kpsT*, *bexA*, and *ctrD* are contained in functionally organized two- or three-gene clusters. *kpsT* is linked to *kpsM*, which encodes an integral polytopic membrane protein with six transmembrane segments (44). Similarly, proteins BexB and CtrC, which are integral polytopic membrane proteins homologous to KpsM, are encoded in the capsular polysaccharide synthesis gene clusters of *H. influenzae* type b and *N. meningitidis* group C, respectively.

Recently Zhang *et al.* (34) identified a different cytoplasmic membrane transport system for O polysaccharide in *Y. enterocolitica* O:3. These workers sequenced the *rfb* gene region of this species and identified a protein showing 34.4–37.5%

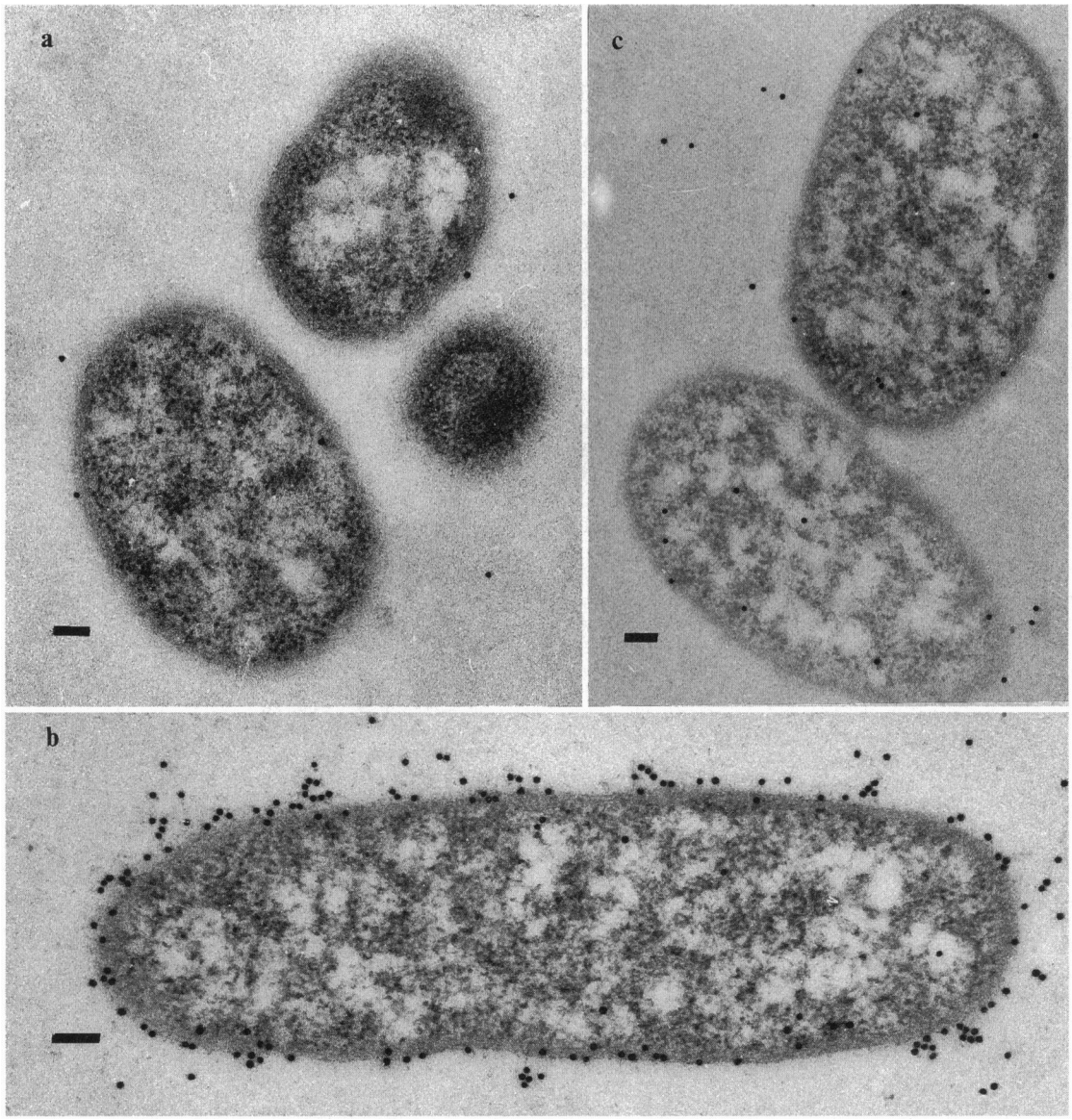

FIG. 3. Immunogold electron microscopy of thin sections of *A. salmonicida* cells incubated with a 1:200 dilution of polyclonal anti-*A. salmonicida* O polysaccharide. (*a*) A449-TM4. (*b*) A449-TM4/pSC162-1 (wild-type *abcA*). (*c*) A449-TM4/pSCGV61 (*abcA* with a mutated ATP-binding site). The O polysaccharides on A449-TM4/pSC162-1 are clearly labeled, whereas cells of A449-TM4, and A449-TM4/pSCGV61-1 show no labeling at either the surface or the inner membrane–cytoplasm interface. The small amount of labeling of A449-TM4 and A449-TM4/pSCGV61-1 cells represents background levels. (Bar = 100 nm.)

sequence identity with the CtrD, BexA, and KpsT capsular polysaccharide transport proteins. This *rfbE* gene was centrally located in the 9.5-kb *rfb* gene cluster of *Y. enterocolitica* and was preceded by *rfbD*, which encoded a protein with six putative membrane-spanning segments that showed ≈50% similarity to *E. coli* KpsM and *H. influenzae* BexB. Transposon mutagenesis of *rfbD* and *rfbE* resulted in cytoplasmic accumulation of O antigen, which led Zhang *et al.* (34) to suggest that RfbD and RfbE may act together to form an ATP-driven O-chain export complex. The *rfb* cluster of *V. cholerae* also contains centrally located ABC transporter protein encoding *rfbI* and *rfbH* polytopic membrane protein genes (35). Pairwise comparison of the predicted sequences of O polysaccharide-associated ABC proteins RfbE and RfbI shows that they share 31.8% and 32.4% sequence similarity, respectively, to the ABC domain of AbcA (45). The *A. salmonicida* ABC transport system described here appears to represent an additional ABC membrane polysaccharide transport system, one that differs in several regards from the previously described ABC capsular and O-polysaccharide transporters. *abcA* is not directly flanked by a gene encoding a protein displaying structural and evolutionary relatedness to the integral polytopic membrane components typical of these other ABC polysaccharide transporters. Importantly the genetic organization of *A. salmonicida abcA* also differs from the *Y. enterocolitica rfbD/rfbE* and

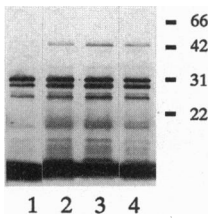

FIG. 4. Autoradiograph of SDS/PAGE showing *in vitro* gene expression of pMMB67 (lane 1), pSCGV61 (lane 2), pSC162-1 (lane 3), and pSCGV58 (lane 4). The M_r 43,000 band corresponding to ^{35}S-radiolabeled AbcA is clearly visible in lanes 2, 3, and 4. M_r markers ($\times 10^{-3}$) are on the right.

putative *V. cholerae rfbH/rfbI* transporter systems, which are both directly flanked by other *rfb* genes.

Site-directed mutagenesis studies demonstrated that the native ATP-binding P-loop consensus of the AbcA protein is required for complementation of the smooth O-polysaccharide phenotype, and based on the sequence similarity with the capsular polysaccharide ABC transporters, it is possible that one role for the protein is to energize O-polysaccharide export across the cytoplasmic membrane. However, AbcA also appears to be required for the maximal synthesis of O polysaccharides in *A. salmonicida*. While mutation of the *rfbD* and *rfbE* genes of *Y. enterocolitica* resulted in intracellular accumulation of O polysaccharides in quantities readily detectable by dot immunoblot assay (34), mutation of *abcA* resulted in significantly decreased synthesis of O polysaccharide. Phylogenetic analysis using the method of Nei (46) shows that AbcA appears to have diverged earlier in the evolution of this group of ABC polysaccharide transporter proteins. In summary, although sequence similarity places AbcA in a family of ABC carbohydrate transport proteins, the protein has evolved separately and appears to be essential for both the maximal synthesis and translocation of LPS O polysaccharides.

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