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Synthesis, Export, and Assembly of *Aeromonas salmonicida* A-Layer Analyzed by Transposon Mutagenesis

ROBERT J. BELLAND AND TREVOR J. TRUST*

Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 2Y2, Canada

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Suicide plasmid pJB4JI, containing transposon Tn*5* and phage Mu, was introduced into *Aeromonas salmonicida* 449 which produces a surface protein array known as the A-layer. Kanamycin-resistant exconjugants of 449 with altered ability to produce the A-layer were selected by virtue of their altered colonial morphology and color on medium containing the dye Congo red. Analysis of culture supernatants, periplasmic shock fluid, outer membranes, and whole-cell lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with a monoclonal antibody to A-protein revealed five classes of single-insertion mutations that affected the ability of cells to produce and export A-protein and to assemble the A-layer. These studies suggest that A-protein is produced from a single chromosomal gene. The subunits subsequently pass through the periplasm and across the outer membrane. At least one gene product is required for this export. Assembly of A-layer on the cell surface then requires the presence of O polysaccharide chains on the lipopolysaccharide. In one case, insertion of Tn*5* resulted in loss of ability to produce both A-protein and lipopolysaccharide with O polysaccharide chains, suggesting that synthesis of A-protein and synthesis of lipopolysaccharide may involve coordinate regulation.

Macromolecular arrays of protein subunits known as S-layers, RS-layers, or surface protein arrays are found on the outermost surfaces of a wide range of gram-positive and -negative eubacteria and archaebacteria (18, 19). Chemical analyses of these layers have shown that they are mainly composed of single protein or glycoprotein subunits with M_r ranging from 40,000 to 200,000 (19). In most of the cases examined, the protein subunits were arranged in a regular repeating hexagonal, tetragonal, or linear pattern and so represented a unique class of exported proteins which, in the case of gram-negative cells, need to cross the outer membrane (OM) before being assembled on the cell surface.

Assembly experiments with a number of isolated S-layer subunits have shown that they may reassemble when provided with cell wall templates and in certain cases self-assemble in vitro (19). This has implied that the information for the geometry of the lattices, and for the orientation of the arrays on intact cells, is determined by the molecular structure of the protein subunits (13, 21, 22) and not by the underlying cell wall or envelope structure onto which the subunits assemble. Before this assembly takes place however, the S-layer subunits need to be processed and exported. This aspect of S-layer production has received scant attention, even though the S-proteins are abundant cell proteins which characteristically are present on the cell surface throughout growth. These proteins therefore clearly represent ideal candidates for studying the protein export mechanisms of gram-negative organisms. The excreted proteins that have received attention have been enzymes or toxins, and evidence has been provided for two different protein excretion pathways (5, 11, 16, 24). One is represented by *Pseudomonas aeruginosa* exotoxin A, in which the periplasm appears to be bypassed and the protein has been proposed to flow from the inner membrane to the OM along the Bayer junctions before being released from the OM into the culture medium (11). The second pathway is seen in the case of several proteins of a strain of *Aeromonas hydrophila* (5), a hemolysin of certain strains of *Escherichia coli* (24) and a hemolysin and alkaline phosphatase of *P. aeruginosa* in which the exported proteins appear to pass through the periplasmic space (16).

One gram-negative organism with a well-characterized surface protein array is the fish pathogen *Aeromonas salmonicida*. This array (A-layer) is an important virulence property of the organism and appears to protect the cell from the defense mechanisms of the fish (6, 9, 14). The layer is composed of tetragonally arrayed subunits (A-protein) of M_r 50,000 (6). To explore the synthesis, export, and assembly of this important surface protein array we used transpositional mutagenesis as a genetic tool to obtain mutants blocked in A-protein synthesis, A-protein export, and A-layer assembly, and here we report our findings.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* 1830(pJB4JI) was the conjugal Tn*5* donor (1). *A. salmonicida* A449, a virulent A-layer-producing strain, was the conjugal recipient and was originally isolated by C. Michel (strain TG 36/75) (8).

Media and growth conditions. Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol-tryptic soy broth (GIBCO Laboratories, Madison, Wis.). Growth of *E. coli* 1830 (37°C) and *A. salmonicida* A449 (20°C) was in tryptic soy broth (GIBCO) or tryptic soy agar supplemented with the appropriate agents. Antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.) and were added to media at the following final concentrations: kanamycin, 50 μg/ml; gentamicin, 5 μg/ml; tetracycline, 12.5 μg/ml. Congo red was used in solid medium at a final concentration of 0.003% (wt/vol).

Bacterial matings. Conjugal transfer was done using a filter mating procedure essentially as described by Beringer et al. (1), except that mating was for 3 h at 20°C on tryptic soy agar. After incubation, cells were suspended in 3 ml of phosphate-buffered saline, and appropriate dilutions were plated on Congo red-tryptic soy agar containing kanamycin

* Corresponding author.

and tetracycline, to select Tn*5*-containing Km^r exconjugants of strain A449 (Tc^r).

Screening of Tn*5*-containing *A. salmonicida*. Putative Tn*5*-containing exconjugants were screened for the presence (Km^r Gm^r) or absence (Km^r Gm^s) of the conjugative plasmid pJB4JI by replica plating to Congo red-tryptic soy agar containing kanamycin and tetracycline, and kanamycin and gentamicin. Exconjugants unable to synthesize or properly assemble A-layer were detected by their altered colonial morphology or coloration since strains able to produce A-layer give red colonies while strains unable to produce A-layer give white colonies in the presence of Congo red (E. E. Ishiguro, T. Ainsworth, T. J. Trust, and W. W. Kay, submitted for publication).

Isolation of DNA. Rapid screening of bacterial strains for plasmid DNA was done using cells from solid media or from 2-ml broth cultures by the method of Kado and Liu (7). Chromosomal DNAs from selected strains were prepared as described by Stern et al. (21), but after centrifugation, chromosomal and plasmid bands were collected for subsequent restriction digests.

The plasmid pKC7 (15) containing Tn*5* was isolated by the alkaline lysis method of Maniatis et al. (12) and purified by ethidium bromide-cesium chloride equilibrium density centrifugation.

Electrophoretic analysis of DNA. DNA samples were electrophoresed for 5 h at 100 V in 0.8% agarose gels with Tris-acetate buffer (12). Gels were stained for 45 min with ethidium bromide (0.5 μg/ml) and were photographed with Polaroid film (type 667 or 665). Before electrophoresis, restriction digests of 2 μg of chromosomal or plasmid DNA were prepared with 12 U of *Eco*RI (Bethesda Research Laboratories) for 2 h at 37°C according to the manufacturer's directions.

Southern transfers and detection of Tn*5* DNA homology. Electrophoretically separated restriction endonuclease fragments were transferred to nitrocellulose by the procedure of Southern (20) as described by Maniatis et al. (12). Hybridizations and washing conditions were as described by Maniatis et al. (12), except that prehybridization was for 6 h at 68°C followed by hybridization for 16 h at 68°C in a fresh solution containing 10^7 cpm of pKC7 labeled with ^{32}P by nick translation. After the washes, autoradiography was performed at −70°C.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (10). Protein solubilized in sample buffer was stacked in 4.5% acrylamide (10 mA) and separated with 12.5% acrylamide (20 mA). Protein was stained with Coomassie blue.

Isolation of OMs. Cell envelopes were prepared by passing bacterial cells through a French pressure cell followed by centrifugation at 4,000 × *g* as previously described (2). OMs were prepared by differential solubilization of the inner membrane, using the detergent sodium lauryl sarcosinate as described by Filip et al. (3). The total membrane protein-detergent ratio used was 1:6 (mg/ml). After shaking for 30 min at room temperature, the preparation was centrifuged at 43,000 × *g* for 30 min at 4°C, and the pellet was washed three times in Tris buffer.

Silver staining of LPS. The lipopolysaccharide (LPS) morphology in whole cell lysates was determined by the proteinase K digestion procedure of Hitchcock and Brown (4) as previously described (2). After SDS-PAGE, gels were stained for LPS by the LPS silver staining procedure of Tsai and Frasch (23).

Periplasmic shock proteins. The periplasmic fraction was prepared by the osmotic shock procedure of Willis et al. (25). One gram of cells was washed twice in 0.33 M Tris buffer (pH 7.3) and suspended in 10 ml of this buffer. A solution containing 40% (wt/vol) sucrose and 4 mM EDTA in 0.033 M Tris buffer was added until a final concentration of 20% (wt/vol) sucrose was obtained. Cells were then shaken for 10 min at room temperature and collected by centrifugation at 12,000 × *g* for 15 min, and the pellet was rapidly suspended in 2 ml of ice cold, deionized water. This was adjusted to 1 mM with respect to $MgCl_2$, and the cells were stirred on ice for 10 min. Cells were removed by centrifugation at 12,000 × *g* for 15 min, and the supernatant was used as the periplasmic protein fraction.

Acetone precipitation of culture supernatants. Strains were grown in 50-ml volumes of tryptic soy broth supplemented with kanamycin (50 μg/ml) and tetracycline (12.5 μg/ml) at 20°C for 36 h. Cells were removed by centrifugation, and 1 volume of culture supernatant was mixed with 3 volumes of cold acetone. The precipitates which formed after standing at 0°C for 10 min were collected by centrifugation at 12,000 × *g* for 20 min at 4°C and allowed to dry. The dry pellet was suspended in 0.05 vol of 10 mM Tris-hydrochloride (pH 7.3) containing 5 mM $MgCl_2$.

Monoclonal antibody. Monoclonal antibody (MAb) AA1 to A-protein was used as mouse ascites fluid and was prepared as previously described (9).

Immunofluorescence. Surface-exposed A-protein on living cells of *A. salmonicida* was demonstrated by the indirect immunofluorescent antibody staining technique with MAb AA1 as previously described (2).

Immunoblotting. After SDS-PAGE, separated components were electrophoretically transferred from the slab gel to 0.45-μm (pore size) nitrocellulose paper using the methanol-Tris glycine system of Towbin et al. (22) as previously described (2).

Rapid screening of mutants or cellular fractions for the presence of A-protein was done using a dot-blot immunodetection technique. Small amounts of colony material or cellular fractions were applied directly to filter paper (Whatman no. 1) and dried at 37°C for 1 h. Cells were then lysed by placing the filter paper in 50 mM Tris buffer (pH 9.5) containing 1% (vol/vol) Nonidet P-40 for 30 min at room temperature. The filter paper was then soaked in 70% ethanol for 10 min and dried at 37°C. The dried filter paper was then blocked with Tris-gelatin-saline and reacted with antibody, and antibody-antigen complexes were detected with ^{125}I-labeled protein-A as described previously (2).

RESULTS

Transfer of pJB4JI to *A. salmonicida*. Suicide plasmid pJB4JI was constructed by Beringer et al. (1) from the broad-host-range plasmid RP4. pJB4JI carries the transposable element Tn*5* (Km^r) flanked by bacteriophage Mu sequences. The Mu sequences provide the suicide capacity presumably by interfering with plasmid maintenance in non-Mu hosts. Loss of the Gm^r marker (stably maintained on the plasmid) allows plasmid elimination to be monitored. pJB4JI was transferred to *A. salmonicida* A449 by conjugation with *E. coli* 1830. Km^r exconjugates were obtained from matings at a frequency of 1.2×10^{-5} per input donor cell. To determine coinheritance of the other plasmid marker, Gm^r, Km^r transconjugants were patched to gentamicin-containing medium. The proportion of Km^r Gm^s to Km^r Gm^r transconjugants was approximately 50%. Spontaneous Km^r in *A. salmonicida* occurred at a frequency of less than 1×10^{-9}

while spontaneous loss of A-layer/A-protein production occurred at less than 5×10^{-5}. Agarose-gel electrophoretic analysis of plasmid DNA isolated from Km^r Gm^r transconjugants revealed the presence of an additional plasmid which comigrated with plasmid pJB4JI. Km^r Gm^s strains had plasmid profiles indistinguishable from *A. salmonicida* A449. Of approximately 2.4×10^5 Km^r Gm^s transconjugants, 350 clones showed altered colony morphology or Congo red pigmentation properties. Preliminary structural analysis performed on 75 of these 350 transconjugants with the assay procedures detailed below revealed five distinct classes of structural mutants, each of which was homogeneous as far as tested. These classes were represented by *A. salmonicida* mutants TM1 (13 of 75), TM2 (26 of 75), TM3 (7 of 75), TM4 (17 of 75), and TM5 (12 of 75). While the parent produced granular adherent "hockey puck" colonies which were red on Congo red agar, all mutant classes produced smooth butyrous colonies on tryptic soy agar. Mutants TM1, TM2, TM3, and TM4 produced white colonies on Congo red agar while mutant TM5 produced red colonies. TM5 colonies were flatter than those of the parent.

Localization of Tn*5* insertion. Tn*5* insertion was confirmed by Southern blot hybridization analysis with ^{32}P-radiolabeled pKC7 DNA-containing Tn*5* and *Eco*RI-treated total cellular DNA. The autoradiograph in Fig. 1 shows that Tn*5* did not hybridize with the *A. salmonicida* A449 parent DNA (lane 1). Hybridization of Tn*5* with DNA of the five mutants in each case resulted in a single hybridizing fragment, indicating that the mutations were due to single site insertions. In the case of mutants TM1, TM2, and TM5, Tn*5* hybridized to a DNA fragment of approximately 15 kilobase pairs (kbp) (lanes 2, 3, and 6). In TM3 hybridization was with a 9.2-kbp fragment (lane 4), and in TM4 hybridization was with a 7.0-kbp fragment (lane 5). Purified total plasmid DNA did not hybridize with the radiolabeled probe DNA (data not shown).

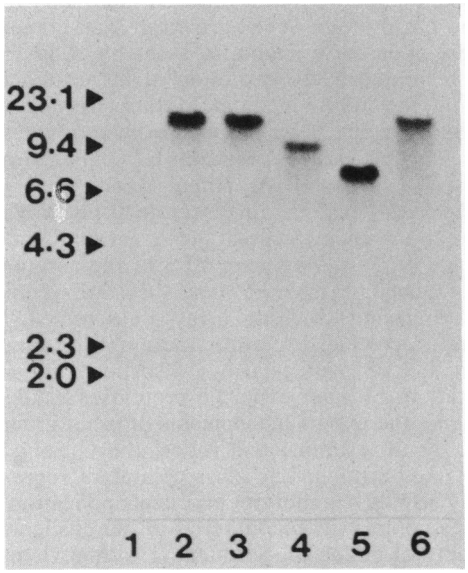

FIG. 1. Autoradiograph of a Southern blot of total cellular DNA digested with *Eco*RI from Km^r Gm^s exconjugants of *A. salmonicida* A449 hybridized with nick-translated pKC7 DNA containing Tn*5*. Lanes: 1, A449 parent; 2, TM1; 3, TM2; 4, TM3; 5, TM4; 6, TM5. The weights (in kbp) of fragments obtained by *Hin*dIII digestion of bacteriophage lambda DNA are shown at left.

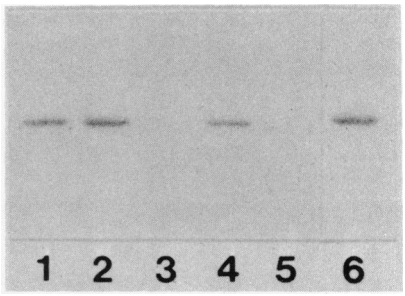

FIG. 2. Immunoblot detection of A-protein in whole cell lysates. Autoradiograph of Western blot of SDS-PAGE reacted with MAb AA1 to A-protein. Lanes: 1, A449 parent; 2, TM1; 3, TM3; 4, TM2; 5, TM4; 6, TM5.

Ability to produce A-protein. The ability of the different mutants to produce A-protein was first determined by dot-blot immunoassay of cells with MAb AA1, which is directed to an epitope on *A. salmonicida* A-protein, and with rabbit polyclonal anti-A-protein antiserum and was confirmed by reacting Western blots of SDS-PAGE-separated whole cell lysates of cells grown on solid medium (tryptic soy agar). Mutants TM1, TM2, and TM5 (Fig. 2, lanes 2, 4, 6, respectively) each produced an A-protein indistinguishable in apparent M_r from that of the parent (lane 1). Whole cell lysates of mutants TM3 and TM4 displayed no immunologically detectable A-protein (lanes 3 and 5).

Localization of A-protein. Cells were grown in liquid culture medium, and the periplasmic and OM fractions were analyzed for the presence of A-protein by SDS-PAGE. Acetone-precipitated culture supernatants were similarly examined for the presence of excreted A-protein. The presence or absence of A-protein in a given fraction was confirmed by dot-blot immunoassays with MAb AA1. A-protein was not detected in any fraction obtained from mutants TM3 and TM4. The results in Fig. 3 show that mutant TM1 contained a large amount of A-protein in the periplasmic fraction (Fig. 3B, lane 2), but no A-protein was detected in either the culture supernatant or OM fraction (Fig. 3B, lanes 1 and 3). Mutant TM2 also accumulated A-protein in the periplasm (Fig. 3C, lane 2). This mutant displayed a small amount of A-protein in the OM fraction (Fig. 3C, lane 1), but did not excrete A-protein into the culture medium (Fig. 3C, lane 3). In contrast to the other mutants and to the parent (Fig. 3A), mutant TM5 excreted A-protein and culture supernatants contained as much as 1 mg of A-protein per ml (Fig. 3D, lane 3). The OM fraction obtained from cells of this excretor mutant (Fig. 3D, lane 1) contained a much smaller amount of A-protein than that of the parent strain (Fig. 3A, lane 1). Since colonies of this mutant were red when grown on Congo red agar, plate-grown cells were examined for the presence of surface exposed A-protein by indirect immunofluorescent antibody staining analysis with MAb AA1. Cells taken directly from plates and not subjected to a washing step displayed surface-exposed A-protein which appeared as a continuous ring of fluorescence around the entire cell. However this fluorescence reaction was totally lost when cells were subjected to a single washing step. This was not the case with the parent strain, in which the A-layer remained intact and associated with the cell surface despite repeated washings. No other mutant displayed surface-exposed A-protein.

SDS-PAGE analysis of LPS. The ability of each mutant to produce LPS with O polysaccharides of homogeneous chain length typical of plate-grown wild type *A. salmonicida* (2)

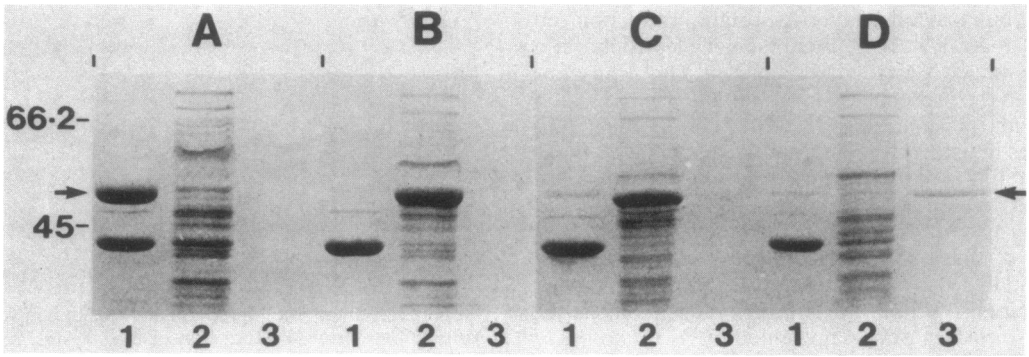

FIG. 3. SDS-PAGE of OM (lane 1), periplasmic shock fraction (lane 2), and culture supernatant (lane 3), stained with Coomassie blue. (A) parent A449; (B) TM1; (C) TM2; (D) TM5. A-protein is indicated by an arrow; molecular weight (×1,000) is indicated on the right.

was then examined by silver staining of proteinase K-digested whole cell lysates. The LPS of mutants TM2, TM4, and TM5 did not contain these O polysaccharide chains (Fig. 4, lanes 4, 5, and 6) and only showed the fast-migrating lipid A-core oligosaccharide fraction characteristic of the rough form of *A. salmonicida* LPS (4). The LPS of mutants TM1 and TM3 (lanes 2 and 3) did contain O polysaccharides of homogeneous chain length and provided electrophoretic profiles indistinguishable from that of the wild type (lane 1).

DISCUSSION

This study has shown that the suicide plasmid pJB4JI is an effective transposon delivery vehicle in *A. salmonicida* and can readily provide insertional mutations affecting A-layer production. With the aid of colony morphology and color on media containing Congo red we were able to identify five classes of insertion mutants with altered A-layer production. Southern blot analysis of the representative mutants indicated that each involved a single insertion in a chromosomal gene. In one case, TM3, the insertion appeared likely to be in the A-protein gene, since it resulted in complete loss of the ability to produce A-protein. This insertion was located on a 9.2-kbp *Eco*RI fragment.

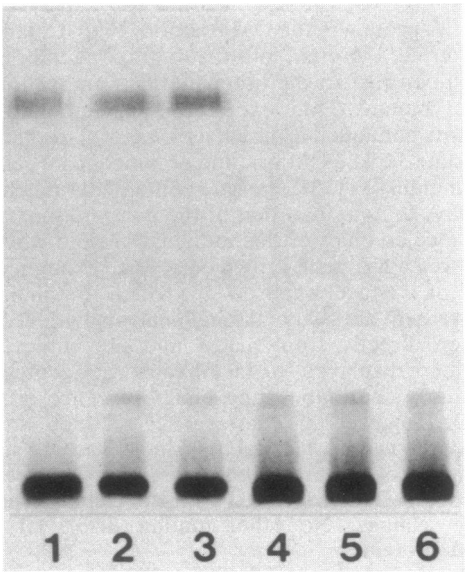

FIG. 4. SDS-PAGE of LPS in proteinase K-digested whole cell lysates stained with silver. Lanes: 1, A449 parent; 2, TM1; 3, TM3; 4, TM2; 5, TM4; 6, TM5.

A second step in the pathway of A-layer production was revealed by mutant TM1. This mutant accumulated A-protein in the periplasm and provided evidence that in this export pathway the A-protein passes through the periplasmic space. This mutant also provided evidence that at least one gene product is required to translocate the A-protein subunits across the OM. Mutant TM2 also accumulated A-protein in the periplasm. In this case, however, the insertion of Tn*5* also resulted in an inability to produce an LPS with O polysaccharide chains. While the precise nature of this third class of mutation affecting A-layer is still not understood, TM2 provided initial evidence that the ability of *A. salmonicida* to produce A-layer can be affected by the ability of the cell to produce a complete LPS.

The fourth class of mutation affecting A-layer production also involved LPS synthesis, and like TM1 and TM2, the Tn*5* insertion in mutant TM5 was in a 15.2-kbp *Eco*RI fragment. Mutant TM5 was unable to produce a smooth-type LPS with its characteristic O polysaccharides of homogeneous chain length, but still retained the ability to produce and export A-protein. When grown in liquid medium these cells were apparently unable to assemble A-layer on their surface resulting in the excretion of large amounts of A-protein into the culture medium. Mutants with this excretory phenotype have also been obtained from another strain of *A. salmonicida* by serial subculture (E. E. Ishiguro, W. W. Kay, and T. J. Trust, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, D24, p. 55). This excretory phenotype clearly points to an important structural role for the O polysaccharide chains of *A. salmonicida* LPS in the assembly of the A-layer. Indeed it may be that they are required as a framework around which the array is assembled. This identification of protein excretion mutants resulting from a mutation in LPS synthesis may also be of importance in considerations of other exported proteins. Certainly, workers studying the export mechanisms of other gram-negative bacteria should examine and report the O polysaccharide status of their strains. The class of mutant represented by TM5 may also have important practical application given the difficulties commonly encountered in the efficient recovery of a variety of biological products resulting when recombinant DNA technology is applied in gram-negative bacteria.

The fifth class of mutation with altered ability to produce A-layer was represented by TM4. In this case, insertion of Tn*5* in a 7-kbp *Eco*RI fragment resulted in the loss of both the ability to produce A-protein and the ability to produce an LPS with O polysaccharide chains. One possible explanation

for this class of mutation is that the insertion is in a gene which regulates the production of both A-protein and O polysaccharide synthesis.

This study has shown that the suicide plasmid pJB4JI can be used for Tn*5* insertional mutagenesis in the fish pathogen *A. salmonicida*. By this technique, the production of the A-layer of this organism was studied. The subunits of this surface protein array appeared to result from the transcription and translation of a single A-protein gene on the chromosome. The export pathway for A-protein involves passage through the periplasmic space and across the OM. A-protein is then assembled into a paracrystalline array on the cell surface, and this requires the presence of O polysaccharide chains on the LPS, implying that the information for the geometry of the lattice, and for the orientation of the array, involves both the array protein subunits and the underlying OM structure.

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