

**Molecular Analysis of the Structure, Secretion and Anchoring of the
Paracrystalline Surface Array Protein of *Aeromonas hydrophila***

by

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ABSTRACT

Aeromonas hydrophila is a Gram negative pathogen of fish, amphibians, reptiles, birds, and mammals. High virulence strains of *A. hydrophila* produce a paracrystalline surface protein array (S-layer), and homogenous length O-polysaccharide side chains. Three dimensional reconstruction's of the native S-layer have shown that the subunit S-protein forms a tetragonally arranged array consisting of two structural domains with a lattice constant of 12 to 12.5 nm. The isolation of a Tn5 insertion mutant (TF7-ST1) producing a truncated S-protein of molecular weight 38,650 showed that self-assembly of the S-layer on, and anchoring to the *A. hydrophila* cell surface required the presence of the carboxy-terminus. The carboxy-terminus was also required for correct array morphology and formation of the minor tetragonal domain, while the amino-terminus was shown to form the major mass domain of the native S-layer.

The gene (*ahsA*) encoding the S-protein subunit of *A. hydrophila* TF7 was cloned into λ EMBL 3, and expressed in *Escherichia coli* from plasmid pUC18. The DNA sequence revealed a 1407 base pair open reading frame and a 450 residue 45,400 molecular weight mature protein with a predicted isoelectric point (pI) of 6.72 compared to the measured Mr of 52,000 and pI of 4.6. *In vivo* cell labeling, acid phosphatase digestion, ascending thin layer chromatography, and Western blot analysis with monoclonal anti-phosphotyrosine antibody showed that the S-protein contained phosphotyrosine.

Cell fractionation studies employing plasmid-encoded *ahsA* showed that in *A. hydrophila* the S-protein subunits were secreted by the native S-

protein secretion pathway, while in *E. coli* and *Aeromonas salmonicida* the cloned S-protein inserted into the outer membrane of the foreign host. Nucleotide sequence analysis of a 4.1 kb region terminating 700 bp upstream of *ahsA*, revealed the presence of a gene (*spsD*) encoding a 79.8 kDa polypeptide that shows high homology to the PulD family of secretion proteins. Insertional inactivation of the *spsD* gene resulted in localization of the S-protein to the periplasm of *A. hydrophila*. Use of the promoterless chloramphenicol acetyl transferase gene showed that *spsD* contains its own promoter. *A. hydrophila* has previously been shown to contain the *exe* operon, which is responsible for the secretion of a number of extracellular enzymes. A fragment of DNA was generated from the *exeD* gene of *A. hydrophila* Ah65 using the polymerase chain reaction, and used in hybridization studies to show the presence of an *exeD* homologue in *A. hydrophila* TF7. The *spsD* gene therefore encodes a second *pulD* homologue that displays high specificity for the secretion of the S-protein.

Immediately downstream of the *ahsA* gene, nucleotide sequencing revealed the presence of two open reading frames, *aosA* which encodes a 30.9 kDa protein, and *aosB*, which encodes a 48.1 kDa protein. Amino acid sequence analysis of AOsA revealed a hydrophobic membrane spanning polytopic protein, and analysis of AOsB indicated a polypeptide with a conserved ATP binding site. Insertional inactivation of *aosA* resulted in the expression of a lipopolysaccharide devoid of its O-polysaccharide side chains. This finding indicated a possible role for AOsA and AOsB in the export of the O-polysaccharide side chains across the cytoplasmic membrane in *A. hydrophila*.

Two *A. hydrophila* Tn5 insertion mutants were studied to determine the role of the S-layer and the homogeneous O-polysaccharide side chains in

serum sensitivity studies. Mutant TF7-ST1, which was unable to assemble or maintain an S-layer on its cell surface, showed susceptibility to the bactericidal effects of immune trout sera as did the parent *A. hydrophila* TF7, but not to fresh normal trout and fresh normal and immune rabbit sera. Mutant TF7-ST3, which does not express O-polysaccharide side chains, was sensitive to both fresh normal and immune trout sera, but less sensitive to fresh normal rabbit and immune rabbit serum. This result showed that the LPS O-polysaccharide side chains are important for conferring protection against the lytic action of serum components on the *A. hydrophila* cell, but the S-layer is not required for serum resistance.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Amp	Ampicillin
bp	Base pair
BSA	Bovine serum albumin
Cmp	Chloramphenicol
cm	Centimeter
DEPC	Diethylpyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Erm	Erythromycin
FPLC	Fast protein liquid chromatography
G	Gravitational force
Gem	Gentamycin
g	Grams
h	Hour
IPTG	Isopropyl β -D-thiogalactoside
Kan	Kanamycin
kb	Kilobase
kDa	Kilo Dalton
l	Liter
LB	Luria-Bertani broth
LBA	Luria-Bertani agar
lb/in ²	Pounds per square inch
LPS	Lipopolysaccharide
M	Molar
MES	4-Morpholinethansulfonsaure buffer

mg	Milligram
µg	Microgram
m	Minute
ml	Milliliter
µl	Microliter
mM	Millimolar
MOPS	(3-[N-Morpholino]propane-sulfonic acid)
Mr	Molecular weight
nm	Nanometer
ORF	Open reading frame
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
pfu	Plaque forming units
pI	Isoelectric point
PMSF	Phenyl methanesulfonyl fluoride
rbs	Ribosome binding site
RNA	Ribonucleic acid
s	Second
SD	Shine-Dalgarno ribosomal binding site
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLS	Sodium lauryl sarkosinate
TE buffer	10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)
TES buffer	50 mM Tris-HCl (pH 8.0), 5mM EDTA (pH 8.0), 50 mM NaCl
TBS	Tris buffered saline (10 mM Tris-HCl (pH 7.5), 0.9% NaCl)
TFA	Trifluoroacetic acid
Tris	Tris (hydroxymethyl) aminoethane

u.v. Ultraviolet

X-gal 5-Bromo-4-Chloro-3-indolyl- β -D-galactopyranoside

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DEDICATIONS

I dedicate this dissertation to the memory of my wonderful parents Ken and Rose Thomas, and my beautiful brother John, who was and always will be my hero.

INTRODUCTION

Bacterial surface layers

Surface layers or S-layers are an important class of secreted proteins. These regular two-dimensional paracrystalline surface protein arrays typically constitute up to 10% of total cellular protein and are widely distributed throughout the procaryotic kingdom, including both Archae and Bacteria (271). The majority of S-layers are composed of a one molecule thick layer of protein or glycoprotein subunits, which self-assemble into a supramolecular structure of precise ultrastructural morphology. The morphology of these structures typically include hexagonal, tetragonal, or linear oblique arrangements, which envelope the bacterial cell (figure 1) (263).

The protein subunits of bacterial S-layers interact with each other and with the underlying outer membrane or peptidoglycan cell wall by means of relatively weak noncovalent interactions. These interactions include hydrogen bonding, hydrophobic interactions, ionic bonds involving divalent cations, or by direct interaction of polar groups with the underlying cell membrane and LPS (20, 41, 72, 288). However, in the case of *Deinococcus radiodurans*, disruption of the outer membrane using SDS is required for the release of the S-layer, suggesting an intimate association between it and the outer membrane (290). Indeed, it has been shown that the *D. radiodurans* S-layer forms a stoichiometric complex with the membrane-integral exonuclease, and may be directly anchored to the lipid bilayer by means of a covalently attached fatty acid anchor (232). For Archae, a common feature in the anchoring of their S-layers is the formation of a distinct interspace between the plasma membrane and the array, which is maintained by a spacer element. This element, which is a domain of the S-protein monomer, is

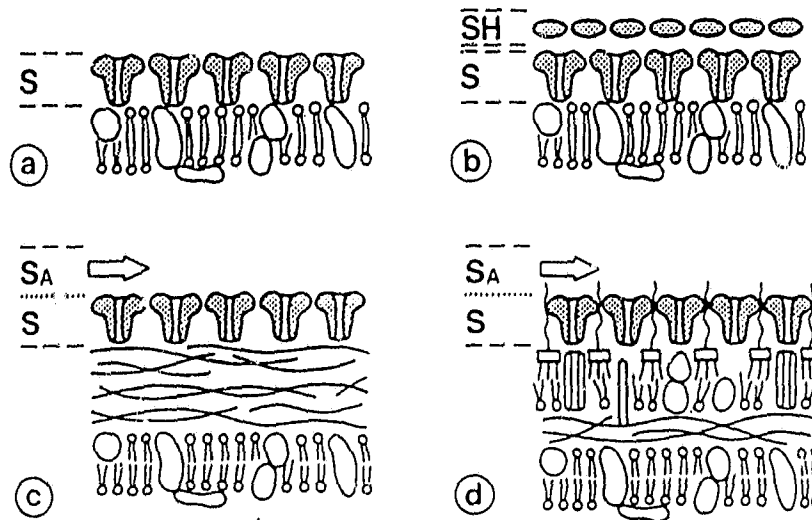


Figure 1. Schematic illustrations of the major classes of prokaryotic cell envelopes containing S-layers. (a) and (b) Cell-envelope structure of Gram-negative Archaea with S-layers as an exclusive cell-wall component (a), and with an additional regularly arranged sheath (b). (c) S-layers as observed in Gram-positive bacteria and Archaea cell envelopes (containing peptidoglycan or pseudomurein respectively). (d) The cell-envelope as observed in Gram-negative bacteria. SH indicates a possible additional sheath, SA is the location for an additional S-layer, and S shows the position of the paracrystalline S-layer. Taken from Messner and Sleytr 1992 (204).

probably inserted into the membrane, or is attached to the membrane via an interaction with a second membrane-integral protein (176, 239, 320).

S-layers serve as an interface between the bacterial cell and the environment, and in the case of bacterial pathogens the array proteins are ideally situated to potentially participate in sensing and signaling events, and to influence the outcome of a host-parasite relationship (figure 1). For comprehensive reviews on S-layers see (120, 140, 195, 203, 204, 271, 272).

Structure

The technique that has contributed most to our current knowledge about the morphological organization of S-layers is electron microscopy. Numerous studies have been performed utilizing a variety of methods including thin-sectioning, freeze-etching, freeze-fracture and negative-staining of samples. S-layers are generally composed of a single species of protein or glycoprotein that ranges in size from 40 to 200 kDa (19, 271). In bacteria, S-layer monomers are often described as having a heavy domain M, and a light domain C, allowing for a scheme of classification based on the disposition of these two domains relative to the crystallographic axes (263). Therefore layers are described as M_4C_4 if both the heavy and light domains join near the 4-fold axes of symmetry. Similarly, the S-layer is described as M_6C_3 if the heavy domains join near the 6-fold axes, and the light domains join at the 3-fold axes of symmetry (figure 2). The heavy domains then, are joined around the higher symmetry axes to form a massive core, while the light domains provide connectivity via the lower symmetry axes (263).

The following is a brief review providing examples of the morphology and chemical composition of select S-layers so far characterized from Archae, as well as Gram-negative and Gram-positive bacteria.

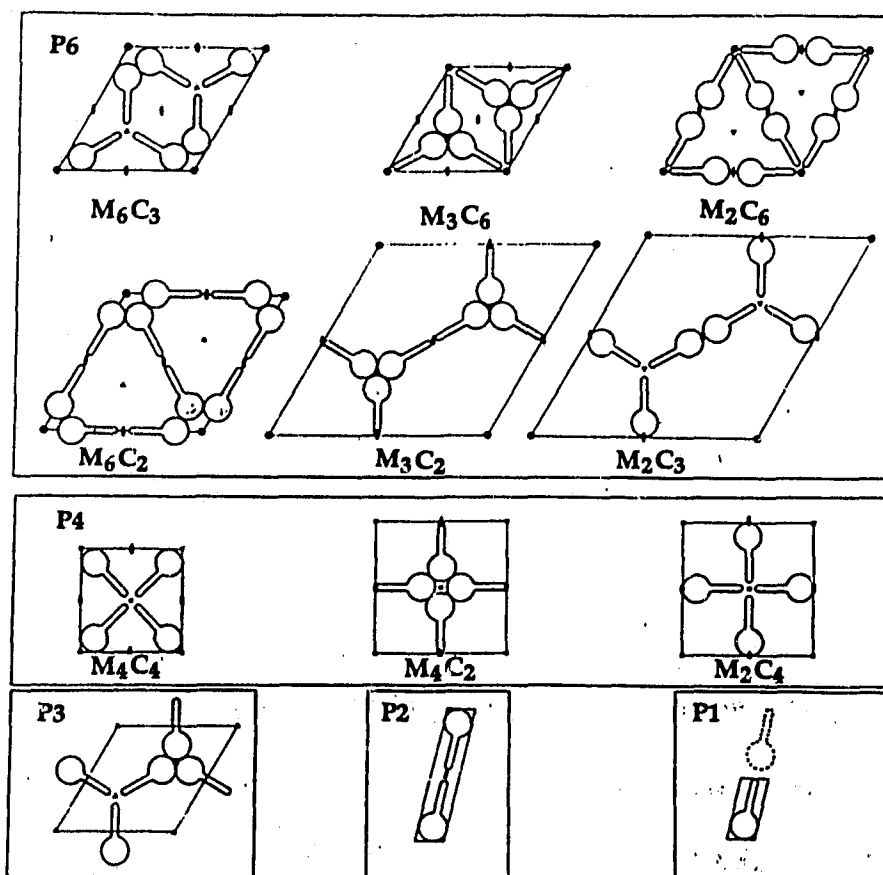


Figure 2. Principles of organization of bacterial S-layers with space groups P6, P4, P3, P2, and P1. Taken from Saxton 1986 (263).

Archae

The commonest type of Archae cell envelope consists solely of an S-layer, apposed to the cytoplasmic membrane, and is observed in members of the *Halobacteriales* (extreme halophiles), *Methanomicrobiales* (methanogens), *Sulfalobales* and *Thermoproteales* (sulfur-dependent extreme thermophiles), and *Thermococcales* (19, 158). Archae S-layers are often glycosylated, and based on gas-liquid chromatography and amino acid analysis, sugar contents of these can range from 1-20% (140).

The *Halobacteriales* are represented by neutrophilic or alkalophilic Gram-positive cocci or Gram-negative rods (101). The S-layer from *Halobacterium halobium* was the first prokaryotic glycoprotein to be isolated, and the structures of three different covalently linked glycopeptides have been determined (176, 196). The chemical characterization of the S-layer glycoprotein of *Haloferax volcanii* has shown that it contains seven N-glycosylation sites (195, 286). Four of the seven N-glycosylation sites were isolated as glycopeptides, and the structure of one of the corresponding saccharides was determined (195). Oligosaccharides consisting of β -1,4-linked glucose residues were shown to be attached to the S-protein via an asparaginyl-glucose unit, in contrast to the related glycoprotein from the extreme halophile *H. halobium*, which contains sulfated glucuronic acid residues rather than the glucose subunits. This difference causes a drastic increase in surface charge density, and may explain the relative stabilities of the S-proteins of the halophiles (195).

The order *Methanobacteriales* contains long rods, lancet-shaped rods, or cocci, with cells exhibiting an electron dense wall sacculi composed of pseudomurein (158). *Methanothermus fervidus* possesses an S-layer glycoprotein with an apparent Mr of 92,500 (218). Other members of the

methanogens which contain S-layers include the *Methanococcus* which live in marine habitats under mesophilic to thermophilic conditions, and display non-glycosylated hexagonal arrangements (218), and *Methanospirillum* and *Methanothrix* which form long filaments of individual cells surrounded by an outer envelope or sheath composed of protein and carbohydrate forming a 2D crystalline arrangement (139). *Sulfolobus* spp. B12 cells contain S-layers that are found a relatively large distance (approximately 8 nm) from the plasma membrane. This spacing is determined by what appears to be proteinaceous extensions of the S-layer (239). For *Sulfolobus sulfataricus*, a closely related organism to *Sulfolobus* spp. B12, a 3D reconstruction of the S-layer is available, but no spacer elements or membrane anchors are visible (19).

The *Thermoproteales*, is one of two groups of extremely thermophilic Archae, which along with the *Sulfolobales*, contain S-layers that are highly resistant to chemical agents (159). For example, the S-layer of *Thermoproteus tenax* has so far not been dissociated into its constituent subunits leading to speculation that the monomers are covalently crosslinked (329). The outer surface of this S-layer is smooth, with long spikes protruding from the inner surface at the 6-fold symmetry axis. *Pyrobaculum organotrophum* H10, a species of the recently discovered genus of rod shaped hyperthermophilic neutrophilic Archae which grow optimally at 100°C by sulfur reduction, contain a cell envelope composed of two distinct hexagonally arranged crystalline protein arrays (126). The outer layer forms a porous network of block-like dimers disposed around a six fold axes, and is loosely associated with the outer surface of the inner layer. As in the protein array of *P. islandicum* GEO3, the rigidity of the inner *P. organotrophum* H10 S-layer is thought to be important in the maintenance of cell shape (234).

Gram-positive bacteria

As is the case with Archaea, many of the S-layer proteins of Gram-positive bacteria are glycoproteins. This is interesting in light of the fact that until relatively recently, bacteria were considered not to possess the ability to glycosylate proteins. Glycosylated S-layers with varying carbohydrate contents have now been detected in a number of strains from the Bacillaceae family (171, 182, 202, 273, 324), as well as *Myxococcus xanthus*, *D. radiodurans*, and *Acetogenium kivui* (188, 230, 231, 233). However, some of these results have to be substantiated because of the possibility of contamination that was not detected in the earlier research. For example, the S-layer protein of *Campylobacter fetus* was originally reported to be glycosylated (323), but this could not be substantiated in later work (78). (For reviews of S-layer glycoproteins, see Konig (1988), and Messner and Sleytr (1991) (158, 203).) With the realization that many Gram-positive S-layers consisted of glycoprotein subunits, the determination of the significance of the carbohydrate moiety expressed in conjunction with the S-layer protein became a focus for many research groups.

The physical location of the covalently attached carbohydrate residues of the S-layer glycoprotein from *Thermoanaerobacter thermohydrosulfuricum* L111-69 (formerly known as *Clostridium thermohydrosulfuricus*) was determined using succinylation to convert the hydroxyl groups of the carbohydrate chains into carboxyl groups. The carboxyl groups were then labeled with polycationized ferritin. The amount of covalently bound ferritin was determined by freeze-etching and ultra violet measurement, and found to be located on the S-layer surface (259). The S-layer glycoprotein of *Cl. symbiosum* HB25, contains a carbohydrate chain covalently linked to the crystalline lattice protein subunits (198). Phosphate

groups are rare as constituents of these glycoproteins, however, the repeating unit of the *Cl. symbiosum* glycoprotein contains a tetrasaccharide repeating unit linked by monophosphate esters (198). Nuclear magnetic resonance data provided evidence for a charge interaction between the free amino group of the 2-N-acetyl-4-amino-2,4,6-trideoxy glucose (BacNAc) substituent of one glycan chain, with the phosphate group of an adjacent glycan chain. This direct electrostatic interaction between the two groups leads to an increase in the structural integrity of the S-layer lattice, and an overall net neutral surface charge (198).

Biosynthetic pathways have now been characterized for certain S-layer glycoproteins (5, 108, 109, 177, 285), and the nature of the covalent linkage of the carbohydrate residues of the glycan chain to the polypeptide has been determined. For the S-layer glycoprotein of *Ac. kivui*, the linkage occurs at four different Tyr residues, all of which are preceded by a Val residue (187, 230, 233). Due to the presence of the repeating Val-Tyr motif, it has been speculated that this may be a recognition sequence for glycosylation in bacteria. For the S-layer glycoprotein of *T. thermohydrosulfuricus* L111-69, Tyr is again the linkage amino acid, however, in this case the surrounding amino acid sequences vary (33, 199, 201). The Tyr-galactose linkage is quite novel, and has not been identified previously, although O-glycosidic bonds via glucosyl-Tyr have been described for S-layer glycoproteins in the related *T. thermohydrosulfuricus* S102-70 (199). In general for the S-layer glycoproteins of *T. thermohydrosulfuricus*, there is a large variation in glycan structures, even between closely related strains. Long chains can contain up to 50 disaccharide or trisaccharide repeats, while short chains can be composed of only a few sugar residues (201). Linear and branched carbohydrate structures can also occur in the *T. thermohydrosulfuricus* glycoproteins adding to the

variability, although in each case, the linkage amino acid was found to be tyrosine.

Gram-negative bacteria

A number of S-layers have now been characterized from both related and unrelated species of Gram-negative bacteria. Double S-layers have been described for *Aquaspirillum serpens* MW5 (281) and *Lampropedia hyalina* (15), where the two superimposed crystalline arrays are composed of different subunit species. Structural analysis was performed by Austin *et al.* (15) on the S-layer of *L. hyalina*. This S-layer is a composite structure consisting of an outer (punctate), and an inner (perforate) layer which specifically combine to form an arrangement of unusual complexity (15). The S-layer is shed easily by applying gentle mechanical forces probably because it is not directly attached to the cell surface as are most other S-layers, but rather attached loosely by a fibrous meshwork. Fixed, dehydrated and sectioned preparations of *L. hyalina* revealed the outer punctate layer to be composed of long, spine-shaped units closed at their tips which are connected about two-thirds of the way down the spine to adjacent spines by arm like structures. The punctate layer is unusual in that its outer surface is not flat as in most other S-layers, but tapers into fine tips. The spatial arrangement of the linker arms display M_6C_3 symmetry, the centers of mass on the 6-fold symmetry axes of the punctate layer extend both toward the cell surface (attached to the underlying perforate layer) and away from the cell to produce a spiny outer surface (15).

S-layers have been identified on a number of pathogenic Gram-negative bacteria, including *C. fetus* (78, 194, 228, 323), *Wolinella recta* (174), *Aeromonas hydrophila* (3, 72, 211), *Aeromonas salmonicida* (73, 144, 236, 308), and *Rickettsia rickettsii* (44).

The Gram-negative spiral bacterium *C. fetus* causes infectious abortion in sheep and cattle (264, 265). *C. fetus* has the ability to express S-proteins of varying molecular size, which is related to the presence of multiple S-protein gene (*sapA*) homologs (see section on genetics below) (93, 210). If an S-protein of Mr 97,000 is expressed, the resulting assembled S-layer is hexagonal in arrangement, whereas if the monomeric subunit expressed is either 127 or 149 kDa, the resulting paracrystalline layer is tetragonal in arrangement (30, 77, 93).

Another well characterized S-layer, is that of *Caulobacter crescentus* (276). Caulobacters exhibit a biphasic lifestyle alternating between a stalked cell and a non-stalked dispersal phase cell which is motile by means of a polar flagellum. The S-layer forms a hexagonal lattice composed of six subunits arranged in a circular structure arranged at 22 nm intervals (276). In side view, the structures form an upright structure sitting on the outer membrane, with linker arms connecting these assemblies approximately midway up its vertical height (275).

The 120 kDa S-protein of *R. rickettsia* is expressed from a gene encoding a predicted 168 kDa polypeptide (99). Cleavage at the C-terminal end of the protein produces a 120 kDa and a 32 kDa product, both of which remain associated with the outer membrane. Mutants that display a reduced ability to process the full length 168 kDa protein were shown to be avirulent, while studies attempting to remove the 168 kDa unprocessed precursor from the cell surface of this mutant suggested that the 32 kDa C-terminal fragment was required as a membrane anchor for the S-layer (106). In the case of the oral pathogen *Wolinella recta*, the presence of a highly structured paracrystalline layer external to and associated with the outer membrane was seen in low passage clinical isolates. However, *W. recta* is thought to alter the expression

of its S-layer and outer membrane associated proteins as a function of its growth environment (37). Following repeated *in vitro* subculturing, clinical isolates displayed a complete loss of the S-layer and the loss of high molecular weight proteins from the outer membrane (37).

Secretion and Assembly

In vitro self assembly of isolated S-layer subunits into lattices identical to those observed on intact cells can be induced in many cases simply by removing the disrupting agent used for the isolation of the subunits. S-layers can reattach to the cell walls from the bacteria from which they were isolated, or in some cases to those of other organisms. For example, the isolated S-layer protein of *Aq. serpens* VHA reassembles on templates prepared from phospholipids and LPS from either *Aquaspirillum* or *P. aeruginosa* (52). On the other hand, type A S-layer proteins from *C. fetus* can be reattached to S⁻ strains as long as they possess type A LPS, but not to S⁻ strains expressing type B LPS (326). For both *Aq. serpens* VHA and *Ca. crescentus*, Ca²⁺ seems to be a requirement for the attachment of the S-layer to the cell surface, as well as for subunit-subunit interactions (163, 317). Calcium independent mutants of *Ca. crescentus* lose the ability to express a surface molecule termed the S-layer associated oligosaccharide (SAO), and consequently fail to attach the S-layer to their cell surfaces (316, 317). SAO was isolated and shown to be a smooth LPS with a core sugar and fatty acid complement identical to the rough LPS and an O-polysaccharide of homogeneous length (316). Furthermore, the nucleotide sequence of the S-protein gene of *Ca. crescentus* encodes a mature polypeptide with four putative Ca²⁺ binding sites, which may be required for the interaction between the S-layer and the SAO (98).

Before assembly of individual proteins into S-layer lattices, the monomers must often be secreted across two membranes. The S-layer protomer of *A. salmonicida* does not seem to be transported via membrane fusion regions, but is secreted across the inner membrane, through the periplasm, and across the outer membrane before inserting into the S-layer on the cell surface (20). Also, the S-protein seems to have a completely different export pathway than do the secreted exoenzymes in this organism, as transposon mutants that accumulate S-layer in the periplasm secrete enzymes such as hemolysin normally (20, 216).

It would be expected that for the synthesis and export of a protein at a rate of 400 monomers per second in rapidly growing bacterial cultures (20 minute doubling time), some form of regulation would be required (204). This assumption is based on the fact that in the majority of organisms so far studied, very little of the S-protein can be found in the growth medium. However, this does not seem to be true for *Acinetobacter* which does not efficiently coordinate transport and assembly of S-layer proteins, approximately half the newly synthesized protein failing to incorporate into the S-layer lattice (291). For the 130 kDa S-protein of *Ca. crescentus*, synthesis of the S-protein transcript was found to be constant throughout the caulobacter life cycle. However, incorporation of the S-protein into the growing surface array was found to be temporally and spatially regulated by an unknown mechanism (88). Production or export of S-layer may be coupled to LPS synthesis in some bacteria. Thorne *et al.* found that if LPS synthesis in *Acinetobacter* was inhibited by bacitracin, S-layer protein was not produced (291).

The S-layers of thermophilic *Bacillaceae* display an inner surface that is negatively charged, whereas the outer surface is neutral, and such dipole

characteristics of charged surfaces may contribute to the proper orientation of the S-protein during local insertion in the course of lattice growth (241). By using restriction sites within the 3' end of the MWP (middle wall protein) gene of *Bacillus brevis*, Tsuboi *et al.* deleted segments of known size, cloned the truncated fragments, and isolated the translated protein products. Using the truncated polypeptides, *in vitro* re-assembly studies were performed to see whether the N-terminal was responsible for forming the hexagonal array on the *B. brevis* peptidoglycan. It was found that mutant MWP truncated in the C-terminal by only 20% failed to reassemble onto the peptidoglycan layer (305).

Function

Although the putative function of S-layers has been reviewed many times in recent years, an exact role for most S-layers still has to be established. Certainly in the case of Archae, the S-layer provides an important structural role in maintaining cell shape. For example, the highly ordered and stable S-layer of *Thermoproteus* probably has a shape determining role, in that isolated arrays maintain the same shape as the cell. The S-layer of *Pyrobaculum* spp. may well have a similar function, as does that of *Halobacterium*, as evidenced by the loss of the characteristic rod-like shape of the cell when treated with bacitracin, which inhibits proper assembly of the array (234).

A general feature of Archae S-layers is the formation of a distinct interspace of constant width between the plasma membrane and the S-layer, maintained by a regularly arranged spacer element (200). This space has been considered by some to be similar to the periplasmic space of bacteria, as defined by a compartment between the plasma membrane and the outer layer

of the cell envelope. For the S-layers of the Archae, *T. tenax*, *P. islandicum*, *P. organotrophum*, *Sulfolobus*, and *Halobacterium*, the limiting pore size of the crystalline array is in the range of 2.0 to 4.5 nm, giving a molecular weight cut off of less than 37 kDa for "average" globular proteins (19). By analogy with bacteria, certain proteins secreted by these cells could then be retained in this "periplasmic space" where they could carry out important functions (19).

One of the commonest functions attributed to S-layers is in inhibiting access of unwanted molecules or infective agents to the underlying components of the cell envelope. Examples of such a protective role include the blocking of bacteriophage receptors on LPS (127), and the resistance of S-layer containing species of *Aquaspirillum* and *Lamproedia* to the Gram-negative bacterial parasite, *Bdellovibrio* (40, 162 1941). Because the pore size varies depending on the particular S-layer under study, one must be careful in assigning a role to these barriers as molecular sieves for exclusion of specific substances. In some organisms ultrastructural studies indicate a pore diameter of 2 to 3 nm, which corresponds to a molecular weight exclusion of 3.5 to 11.0 kDa, this would inhibit the entry of enzymes such as proteases, phospholipases, and lysozyme, however, other layers have molecular weight limitations of 40.0 kDa (271) Studies by Sara *et al*, showed that for mesophilic *Bacillaceae*, original conclusions for the presence of lysozyme excluding pores in their S-layers were inaccurate, the protein could freely pass through the paracrystalline array but the underlying peptidoglycan was resistant to digestion with this enzyme (258).

Some of the best evidence for a function for S-layers has been provided for their role in virulence. This has been shown for *A. salmonicida* in that mutants having lost the ability to synthesize A-layer but retaining normal LPS are more susceptible to the lytic effects of complement compared to the

parent strains (127, 209). In only two cases so far reported has the role of S-layers in pathogenesis been determined, that of *A. salmonicida*, (see below), and *C. fetus*. The ability of *C. fetus* to cause disease seems to be associated with the presence of the S-layer (27, 29, 78, 228, 229, 318). The S-layer of *C. fetus* makes the cells resistant to phagocytic uptake and to the bactericidal activity of serum by the impaired binding of the complement component C3b to the paracrystalline array (30). Recently, Blaser *et al.* showed that when challenged in serum resistance studies, *C. fetus* cells expressing a truncated non-exported 50 kDa S-protein were capable of reversion at a high rate to S-layer positive cells (31). Furthermore, *C. fetus* can undergo antigenic variation at a relatively high frequency during the course of an infection due to expression of S-proteins displaying different antigenic specificities (see below) (77, 223).

A number of other possible functions exist for S-layers, e.g., in cell adhesion and recognition. Specific interactions are required to enable a bacterium to reach and accumulate at favorable sites in its host, and these interactions may require a mechanism of macromolecular recognition. In the case of the pathogenic bacteria *A. salmonicida* and *C. fetus*, hydrophobic surfaces mediating association with macrophages (299), and hydrophilic surfaces preventing attachment to phagocytes are found (30). S-layers can serve as receptors for bacteriophage, e.g., evidence for a transducing bacteriophage using the S-layer of *Ca. crescentus* has been published (80), and the A-layer of *A. salmonicida* has been shown to contain a receptor for bacteriophage (127). Some S-layers also have the potential to function much like the anionic exopolysaccharide glycocalyxes that act as ion exchange resins to attract and bind inorganic and organic nutrients or toxic metals close to the cell surface (241).

Baumeister and Hegerl hypothesized the movement of genetic material through what they termed "bacterial connexons" in an analogous manner to conjugation (18). They found that isolated detergent free S-layers from *D. radiodurans* associate spontaneously in vitro with their outer surfaces in perfect alignment forming pores with continuous channels allowing for the possible movement of DNA between cells. Page and Doran argue that transformation of *Az. vinlandii* can only take place when correctly assembled S-layer is present, disassembled layer essentially making cells non competent (220). Finally the S-layer of *Flexibacteriaceae* may be involved in a mechanism of gliding mobility, in that goblet shaped subunits serve as channels for the extrusion of slime causing locomotion or adhesion (245).

Genetics

Many S-layers have similar structures and characteristics, e.g., size, pI value, and ionic requirements, however, very little information is available at the gene level concerning common ancestors and the transferring of S-protein genes throughout the bacterial kingdom. Knowledge at the genetic level has been slow to accumulate, partly because many groups have experienced difficulty in the cloning of complete S-protein genes together with their controlling elements. This difficulty has often necessitated the cloning of S-protein genes on overlapping fragments because the expression of the complete S-protein even at low levels in *E. coli*, is unstable (34, 230, 304). On the other hand, there are examples where certain S-proteins have been expressed at high levels in *E. coli*. e.g., the RsaA protein of *Ca. crescentus* (98). Studies performed so far show that most S-proteins are expressed from monocistronic units transcribed from chromosomally located single copy genes. There are exceptions however, for example, the *B. brevis* S-protein

genes are part of an operon (304).

B. brevis 47 has a three-layered cell wall, the outer and middle walls (OWP and MWP) being protein layers that form hexagonal arrays on the cell surface (303). The genes encoding OWP and MWP form a gene cluster in the chromosome of *B. brevis*, and constitute a cotranscriptional unit when transcribed from their native promoter(s) (304). It is thought that these genes constitute an operon (*cwp*), that is under coordinate control from the promoter region which lies in the 5' position relative to the first gene that encodes the MWP protein. The cloning and characterization of the promoter region resulted in the finding that there are several tandemly arranged promoters in the 5' region of the cotranscriptional unit (1, 325). Several *Bacillus* spp. have genes that contain tandemly overlapped promoters resulting in quantitative or growth phase-specific regulation of gene expression (138, 319). Indeed two of the most active promoters in the 5' region of the *cwp* operon, P2 and P3, display different transcriptional activities at different stages of cell growth (325). MWP is synthesized from the *cwp* operon as pre MWP from two tandemly located translation initiation sites, resulting in either a 54 residue or a 23 residue amino acid signal sequence, both of which are processed at the same site resulting in a mature protein of the same size (2). In comparative studies with other S-layers, it was found that MWP is similar to the S-protein of *Ac. kivui* in many respects. For example, both contain similar lattice parameters and well conserved 3D structures, especially in the massive core domain. Also, their respective genes encode a 200 amino acid region in the N-terminal domain of both polypeptides which exhibit significant homology, and this region may contain the peptidoglycan interacting domain (core region) (230).

The *sapA* gene encoding the S-layer protein of *C. fetus* has been cloned

and sequenced (28). Southern blot studies have shown that there are several homologous copies of the *sapA* ORF which can lead to antigenic variation of S-proteins in this organism (31, 77, 78, 93, 306, 307, 318). Immediately upstream of *sapA* and its homologs, there is a 600 bp conserved region that extends into the coding region of each gene. This 5' conserved sequence is then followed by a region that diverges completely prior to another highly conserved sequence that is located immediately downstream of each S-gene. The antigenic variation is associated with the removal of the divergent region of *sapA* from the expression locus, followed by its replacement with a corresponding divergent region from a *sapA* homologue (306). Homologous recombination is a possible mechanism for the generation of antigenically different S-protein expressing variants, due to the presence of a putative RecBCD (Chi) site upstream of each *sapA* homologue (306).

A number of S-layer proteins are synthesized with a signal peptide to aid in secretion to the outer cell surface. In *A. salmonicida*, the S-protein gene has been cloned and sequenced, and contains a 21 amino acid residue leader peptide sequence that is not present in the mature processed polypeptide (56, 144). However, the *sapA* gene of *C. fetus* expresses an S-protein devoid of a leader sequence, indicating some other efficient mechanism for targeting the protomers for export (28). In the mature polypeptide there is a segment from residues 672 to 689, identified as a potential membrane spanning region. This region shows homology to leader sequences from a variety of bacterial fimbrial proteins, and may function to target the S-layer protein to the cell surface. Although searches of protein and gene banks with the *C. fetus* sequence showed some similarity with other S-layer protein sequences, there is little overall homology of either primary or secondary structure with these structural genes (28).

The *Ca. crescentus* S-protein is expressed as a 130 kDa polypeptide from a single copy gene which has been cloned and sequenced (98, 274). Transcriptional analysis of the *rsaA* gene of *Ca. crescentus* has revealed that the native promoter is not recognized by the *E. coli* RNA polymerase in vivo, and has to be expressed from an exogenous promoter in this foreign host (88, 274). This is an unusual result in light of the fact that the *Ca. crescentus* *rsaA* promoter region contains a conserved consensus -10 -35 sequence that is transcribed at a high level in its native environment. Fisher *et al.* suggest that the presence of the adjacent *sts1* promoter may have a blocking effect on transcription from the *rsaA* promoter in *E. coli*, possibly by steric hindrance of RNA polymerase binding (88). The *rsaA* S-protein gene encodes a product lacking a normal N-terminal signal sequence, the RsaA polypeptide not being processed to generate the mature protein (98). There is however, a 20 amino acid region at the N-terminal that is relatively hydrophobic, and which may act as a signal sequence during translocation (88).

Peters *et al.* determined the sequence of the *Ac. kivui* S-layer gene and found common structural features with other S-layer genes. Similar to the S-layer protein of *H. halobium* which contains threonine rich clusters, the *Ac. kivui* S-protein gene codes for an excess of acidic residues, and clusters of serine and threonine residues (231). The structural significance of this is unclear, but the serine-threonine clusters are flanked by moderately hydrophobic sequences. In some eukaryotic proteins, serine-threonine clusters are involved in adhesion phenomena, e.g., in the salivary glue protein of the fruit fly, or the contact site of the A protein of *Dictyostilium discoideum*. This adhesion property therefore may relate to some S-layers (231).

As it stands at present, more amino acid sequences of S-layers are

required to draw conclusions on phylogenetic relationships. In particular, the knowledge of S-layer protein sequences from organisms belonging to the same phylogenetic division as *Ac. kivui* and *B. brevis* should help us discover whether amino terminal sequence homologies reflect an interspecies gene transfer event, or whether strong evolutionary pressure has conserved a region or domain in two related proteins.

The genus *Aeromonas*

In 1936, Kluyver and van Niel proposed the genus *Aeromonas* to describe rod shaped bacteria possessing the general properties of the enteric group, but motile by means of polar flagella (154). This genus is included in the family *Vibrionaceae*, along with the genera, *Vibrio*, *Photobacterium*, and *Plesiomonas* (237). *Aeromonad* taxonomy, however, continues to evolve with regard to species identification, and is quite complex. Molecular genetic evidence suggests that the genus *Vibrio* should be further divided into three genera, placing the genus *Aeromonas* in a new family, *Aeromonadaceae* (59, 151, 253). *Aeromonads* have been divided into two distinct groups of species in the genus *Aeromonas*. The first group, which has *A. salmonicida* as the prototype species, are psychrophilic and nonmotile *aeromonads*. The second group consists of mesophilic and motile *aeromonads*, containing at least seven species, including *A. hydrophila*, *A. caviae*, and *A. veronii* biotypes *veronii* and *sobria* (45, 237). Table 1 describes the situation with strains phenotypically identified as *A. hydrophila*, *A. sobria*, or *A. caviae* by biochemical methods being separated on the basis of polynucleotide sequence relatedness, and comprising five to seven hybridization groups (HGs) based on DNA-DNA reassociation kinetics. The *Aeromonas* strains belonging to each HG can be verified with a 98% success rate by a method utilizing electromorphic variations at four enzymatic loci (129). More recently, members of the genus *Aeromonas* exhibited very high levels of overall sequence similarity (ca 98 to 100%) with each other using small sub-unit rDNA sequencing (192). In a separate study, species-specific probes for *A. hydrophila* and *A. veronii* were designed based on 16S rDNA sequences, and in PCR assays clearly discriminated these species from other *Aeromonas* isolates tested (76).

Table 1. Present Taxonomic status of the genus *Aeromonas*

DNA hybridization Group	Genospecies	Phenospecies
1	<i>A. hydrophila</i>	<i>A. hydrophila</i>
2	Unnamed	<i>A. hydrophila</i>
3	<i>A. salmonicida</i>	<i>A. hydrophila</i>
4	<i>A. caviae</i>	<i>A. caviae</i>
5	<i>A. media</i>	<i>A. caviae</i>
6	<i>A. eucrenophila</i>	<i>A. caviae</i>
7	<i>A. sobria</i>	<i>A. sobria</i>
8/10	<i>A. veronii</i>	<i>A. sobria</i>
9	<i>A. jandaei</i>	<i>A. sobria</i>
10/8	<i>A. veronii</i>	<i>A. veronii</i>
11	Unnamed	<i>A. veronii</i>
12	<i>A. schubertii</i>	<i>A. sobria</i>
13	<i>A. trota</i>	<i>A. sobria</i>

Adapted from Janda, 1991 (129)

Aeromonas spp. are important pathogens of fish, amphibians, reptiles, birds, and mammals, and cause severe economic loss to the fish industry (66, 92, 268, 308).

Psychrophilic nonmotile *Aeromonas* spp.

The psychrophilic and nonmotile species *A. salmonicida*, which can consist of both "typical" and "atypical" strains, is primarily a pathogen of fish, occurring in freshwater, estuarine and marine environments (21, 298). The prevalence and distribution of *A. salmonicida* is world wide, causing diseases in a number of different species of fish (193, 298). While "typical" strains of *A. salmonicida* cause furunculosis, a systemic fatal disease of salmonids, the "atypical" strains, have a more varied host range, including Atlantic salmon,

European carp, and herring, and cause chronic and inflammatory illnesses (35, 223, 268).

Virulence factors of *A. salmonicida* include a serine protease, which when injected intramuscularly produces a lesion similar to natural infections (94). Hemolytic activities of a number of hemolysins have been reported, including a 25.9 kDa H-lysin (294), a 56 kDa hemolysin (95), a T-lysin (293), and a 200 kDa salmolysin (215). Glycerophospholipid acyltransferase (GCAT) activity has also been identified (178). This protein forms a complex with LPS, and the complex is lethal for Atlantic salmon by injection.

A major virulence factor expressed by *A. salmonicida* is the paracrystalline surface layer, originally called the A-layer (143, 308). The A-layer forms an array displaying P4 symmetry composed of a major and minor tetragon, with 3D reconstruction showing a lattice constant of 12.5 nm (73). Monomers that assemble to form the A-layer are of approximate Mr 50,000, with a pI of 4.8 and a total measured hydrophobic residue content of 45% compared to the predicted value of 43.7% (56, 236). Assembly of the A-layer on the *A. salmonicida* cell surface requires specific interactions with the O-polysaccharide portion of the LPS, the absence of which leads to the accumulation of large amounts of A-protein as sheets in the culture supernatant (20). The organization of the A-layer is dependent on the availability of the divalent cation Ca²⁺ (96). Structurally, the A-layer is altered by limitation of Ca²⁺, with the alternative regular patterns of the array running at approximately a 45° angle to the original (96).

Secretion of the monomeric A-protein occurs through a specific pathway. A class of Tn5 insertion mutants of *A. salmonicida* have been isolated that are unable to secrete the A-protein across the outer membrane (20). In these mutants, the A-protein accumulates in large amounts in the

periplasm, but the secretion of the extracellular enzymes is unaffected. Recently, one of these mutants, TM1, was characterized more fully, and the Tn5 transposon was found to have inserted into a gene coding for a protein with high sequence homology to a member of the general secretory system from *Klebsiella pneumoniae* (63, 216).

The 1506 bp ORF encoding the surface array subunit protein (VapA) has been sequenced and shown to carry two 21 base pair direct repeats, which participate in a 816 bp deletion when the gene is expressed at high level in *E. coli* (56). As a result the *A. salmonicida* S-protein gene (*vapA*) has only been successfully cloned in the absence of its native high-level expression promoter.

An ORF (*abcA*) located immediately downstream of the A-protein gene *vapA*, has been characterized, and found to encode a polypeptide belonging to a sub-family of ATP-binding cassette (ABC) proteins (58). These proteins catalyze the export of a number of different substrates, including capsular and O-polysaccharides. However, the *AbcA* protein appears to be different because it has also been shown to influence the expression of A-protein in *E. coli*, as well as being involved in the transport and biogenesis of smooth LPS (57, 58).

Many biological functions have now been assigned to the A-layer. Initial work by Ishiguro *et al.* showed that loss of the A-layer during culture of *A. salmonicida* at high temperature led to a $>10^3$ -fold decrease in their virulence for salmonid fish (127). The A-layer protects cells against bacteriophages (127), and the killing activity of both immune and nonimmune serum (209). It is also responsible for binding several host proteins including porphyrins (145), immunoglobulins (235), and a number of extracellular matrix proteins (69, 146, 300). Presence of the A-layer on the cell surface confers increased hydrophobicity to the bacterium, which is

thought to be important for efficient phagocytosis by macrophages (299, 309). More recent work has shown that increased phagocytosis by murine macrophages may also involve specific adhesin effects of the A-layer (96).

Mesophilic motile *Aeromonas* spp.

The mesophilic motile *Aeromonas* spp., are widely distributed in stagnant and flowing waters, in sludge, and in sewage (43, 110, 246), as well as in marine environments (141, 321). VanderKooij *et al.* found that *Aeromonas* spp. can survive on low concentrations (microgram amounts per liter) of various compounds in drinking and tap water, including amino acids, carbohydrates, and long chain fatty acids (310). *Aeromonas* spp. have been isolated from retail fresh foods, e.g., seafood, raw milk, meats, poultry, and from fresh vegetables stored under a controlled atmosphere, possibly because of their ability to grow at 4°C under microaerophilic and anaerobic conditions (7, 150, 206, 221).

Pathogenesis

One of the major drawbacks in studying virulence determinants related to *Aeromonas* pathogenicity has been the inability to establish appropriate organ or animal models that faithfully reproduce specific diseases observed *in vivo* (129). Diseases caused by mesophilic motile *Aeromonas* spp. in humans can be subdivided into two major categories: (i) localized infections (e.g., gastroenteritis and cellulitis), and (ii) more invasive infections (e.g., bacteremia, meningitis, peritonitis, and myonecrosis) (91, 130, 133). In fish, mesophilic motile *Aeromonas* spp. are considered to be normal inhabitants of the intestinal tract (301), and are involved in the disease processes called variously, motile aeromonad septicemia, haemorrhagic

septicemia, and red sore disease (4). They cause a rapidly fatal hemorrhagic septicemia and are the primary bacterial aetiological agent of fish disease in the Southeastern United States (110). On one occasion in a North Carolina reservoir, more than 37,000 fish died over a 13-day period from red-sore disease (205).

Aeromonas gastroenteritis is the most prevalent human disease caused by these pathogens. In a study performed by Janda *et al.*, *A. hydrophila* gastroenteritis was detected in 12 pediatric patients during a 5-month period, with symptoms ranging from bloody diarrhea, fever, vomiting, to abdominal pain (131). Genetic studies conducted on fecal isolates indicate *A. hydrophila*, *A. caviae*, and *A. veronii* biotype *sobria* as being the most predominant species involved in aeromonad-associated diarrhea (8, 170). Altwegg *et al.* have isolated the same strain of *A. hydrophila* from the stools of a patient and from the ready-to-eat shrimp cocktail he had recently consumed (7). However, even with such evidence available, *Aeromonas* spp. are still not considered enteropathogens, primarily due to the fact that Koch's postulates have not been met because of the unavailability of an animal model, and the fact that no well-documented outbreak of aeromonad-associated gastroenteritis has been reported in the literature (129). Work is ongoing in the area of reproducing the diarrheal disease syndrome associated with *Aeromonas* infection, e.g., Pazzaglia *et al.* have used the intestinal tie adult rabbit model to produce histological changes consistent with colonization and infection of the ileum, however, this does not reproduce the diarrheal disease produced by the aeromonads (226).

Human wound infections are typically of environmental origin, and range from cellulitis in patients traumatized at seashores to individuals infected following leech therapy (4, 68). Ketover *et al.* suggests that *A.*

hydrophila along with *Erwinia* species and *Serratia marcescens* should be considered as significant causes of nosocomial septicemia's (149). In a number of patients with lesions thought to be ecthyma gangrenosum, the classical skin lesion associated with *P. aeruginosa* bacteremia, *A. hydrophila* was cultured from the site of infection, which evolved into a large hemorrhagic necrosis (149).

Aeromonas hydrophila

Globally *A. hydrophila* may be the bacterium most commonly associated with fish disease (298). It is also the most widely isolated of the mesophilic *Aeromonas* spp. from a variety of human infections. Almost all of the studies performed on the virulence factors of this group have been performed on this species, although these putative virulence determinants are not necessarily restricted to *A. hydrophila* alone. All high virulence strains of *A. hydrophila* belong to an important group of highly pathogenic *Aeromonas* strains. This sub-group possess a number of surface located phenotypic and structural properties which include the ability to autoagglutinate in broth, the presence of a somatic antigen (O:11), an S-layer (72, 207), and a homogeneous O-polysaccharide side chain often arranged as a doublet by SDS-PAGE analysis (74, 155).

Virulence factors

A. hydrophila secretes a number of extracellular enzymes, including a lipase (GCAT), an amylase, an alpha and beta hemolysin, a cytotoxic enterotoxin, and both thermostable and thermolabile proteases (12, 47, 100, 111, 180, 184, 249, 292). As well, there are a number of cell associated or

structural factors that may contribute to the virulence of this organism, including pili, LPS, flagella, S-layer, and an invasin (72, 74, 102, 116, 119, 262).

Pili

Ultrastructural studies of *A. hydrophila* have revealed two distinct morphologic types of pili from both clinical and environmental sources (46, 116, 119). These pilus types have been described as "straight," with a subunit Mr of approximately 17,000, and "flexible," with a subunit Mr of 4,000. Hokana and Iwanaga have described two pili isolated from *A. sobria* biotype *sobria* of subunit Mr 19,000 and 23,000, as being morphologically similar to the straight pili of *A. hydrophila*, but electrophoretically and immunologically distinguishable (117, 118). These pili from *A. sobria* were found to bind to human and rabbit intestines and agglutinate human and rabbit erythrocytes, with hemagglutination being inhibited by D-galactose and D-mannose, but not by L-fucose. The gene encoding the flexible pilin subunit has been cloned and sequenced, and is maximally expressed at 22°C in liquid media under reduced iron conditions (115). When purified, the flexible pili can agglutinate human, guinea pig, ovine, bovine, and avian erythrocytes (116).

Pearson *et al.* have identified a gene from the core region of the CTX (genetic locus containing the cholera toxin gene and flanking DNA) element from *Vibrio cholerae* that is highly similar in amino acid sequence to the flexible pilin of *A. hydrophila* (227). When this gene is deleted in *V. cholera*, the mutants show a defect in intestinal colonization, lending strength to the hypothesis that the *A. hydrophila* flexible pilus is a prerequisite to colonization of mammalian hosts due to its environmental regulation (116).

Flagella

Very little information is available on the flagellum of *A. hydrophila*, although environmental regulation and therefore a possible role in pathogenesis is in evidence. When *A. hydrophila* strain TF7 was grown in liquid media, a single polar flagellum was seen, but when cultivated on solid media, flagella expression became peritrichious (J. W. Austin and T. J. Trust unpublished data).

S-layer

The role of the S-layer of *A. hydrophila* as a virulence factor is poorly defined. One recent report has shown that the S-layer is not directly involved in mouse pathogenesis (156). Serological studies have shown that the S-layer of *A. hydrophila* is an immunodominant protein detected during the course of systemic infections and disseminated illnesses in humans (157). This dissertation deals with the S-layer of *A. hydrophila* in more detail in the experimental section.

Invasin

A problem that researchers have faced regarding the elucidation of an invasin in *A. hydrophila*, is the effect of the hemolysin, which interferes with invasion assays by producing irreversible, deleterious effects on cultured epithelial cell lines. There have been a few reports of evidence of an invasin in *Aeromonas* spp., typically *A. hydrophila*, or *A. sobria* biotype *sobria*. There are reports showing that certain *Aeromonas* strains are invasive in HEp-2 cells (102, 175).

LPS

The commonest pattern observed when *A. hydrophila* LPS is analyzed by SDS-PAGE is a heterogeneous length O-polysaccharide side chain profile that phenotypically consists of a series of ladder like bands that extend throughout the full length of the gel. A second pattern termed homogeneous consists of a small number of prominent O-polysaccharide side chain bands of equal length often arranged as a doublet (74, 155). This homogeneous phenotype has been associated with a high virulence subgroup of *Aeromonas* strains belonging to a specific serotype O:11, the core polysaccharide of which is most probably required for the anchoring of the S-layer to the cell surface (75). The LPS of *A. hydrophila* is discussed in more detail below.

Hemolysin

Hemolysins belong to a larger group of pore-forming bacterial cytolysins, which cause leakage of the cytoplasmic contents of target cells through disruption of the normal integrity of the cell membrane (25). Two major classes of hemolysins have been described for *A. hydrophila*, alpha and beta-hemolysins. The beta-hemolysins (originally termed aerolysins (24)), are heat labile proteins that fall within the 49 to 53 kDa molecular weight range, and are synthesized in a precursor form (preproaerolysin) prior to removal of their signal sequence and export across the outer membrane (12, 13, 24, 32, 113, 122, 164). The interaction of aerolysin with the target erythrocyte has been shown to occur in a multistep process ranging from the completely water-soluble form (proaerolysin) to insertion into lipid bilayers to form ion channels (222). The beta-hemolysin has been associated with two biological activities, demonstrating both cytotoxic effects, and enterotoxicity in rabbit ileal loops and suckling mice (165, 279). The alpha-hemolysins which

produce zones of partial hemolysis on blood plates are expressed during stationary phase at temperatures that do not exceed 30°C (183, 287).

Proteases

A. hydrophila produces two major proteases, a thermolabile serine protease and a thermostable metalloprotease (180, 247). Virulence studies have been performed on Tn5-induced metalloprotease deficient mutants of *A. hydrophila*, and the mutation was found to decrease virulence in trout (181), as well as to increase sensitivity to normal trout serum. Rivero *et al.* have cloned and sequenced the gene encoding the thermolabile serine protease, and the deduced nucleotide sequence encodes a protein of molecular weight 68,000 Daltons. Recently a novel zinc-protease from *A. hydrophila* was purified and characterized (185). This protease is present in the growth media of an *A. hydrophila* strain originally isolated from the intestinal tract of the leech *Hirudo medicinalis*. It is a 19 kDa protein which specifically hydrolyzes the Gly-Ala bond within the Gly-Gly-Ala sequence, which is located near the cross-link site in the gamma-chain dimer of fibrin, effectively inhibiting coagulation of the blood.

Enterotoxin

As early as 1979, enterotoxin production in *A. hydrophila* was correlated with the ability of isolates to cause diarrheal disease (62). Of 96 *A. hydrophila* isolates tested for cytotoxin and hemolysin production, 69% were found to be both cytotoxic and hemolytic. Later, this enterotoxin was found to cause fluid accumulation in rabbit ileal loops, and exposure of Y1 adrenal cells to this toxin led to rounding, concomitant steroid secretion, and increased levels of intracellular cAMP (183, 184). Three genes encoding enterotoxins

have been cloned from *A. hydrophila* (48, 54, 55), suggesting that *Aeromonas* species produce different structural types of cytotoxic enterotoxins that are functionally similar.

Glycero-Phospholipid-Cholesterol-Acyltransferase (GCAT)

Lipases may be important in bacterial nutrition, and may constitute virulence factors by interacting with human leukocytes thereby inhibiting the host immune response (42, 250), although their role in *Aeromonas* infections has not been characterized. Thornton *et al.* have cloned a GCAT gene from *A. hydrophila*, which shares many properties with mammalian lecithin cholesterol acyltransferase. Recently, Anguinita *et al.* have cloned a second gene from *A. hydrophila* encoding an extracellular lipase, that is capable of expressing a polypeptide of Mr 71,000 (10).

In addition to the above exoenzymes, *A. hydrophila* also produces extracellular amylase and chitinase, which may contribute to *Aeromonas* virulence, although it is not known whether these have any role in the pathogenesis of the organism (49, 50, 100).

***A. hydrophila* S-layer**

The surface characteristics of highly pathogenic strains of *A. hydrophila* are invariant with regards to the presence of an S-layer, as well as the homogeneous length LPS O-polysaccharide side chains (72, 207). The S-layer of *A. hydrophila* shares a number of structural features with the A-layer of the related organism *A. salmonicida*, for which the presence of the paracrystalline array is an essential virulence determinant. However, the A-layer is antigenically conserved, which is not the case for the *A. hydrophila* S-

layer, and none of the binding activities associated with the *A. salmonicida* A-layer are true for the *A. hydrophila* array. The differences between these two S-layers indicate that they share few if any biological roles, and suggest alternative functions in the pathogenesis of these organisms. Figure 3 shows three different views of the *A. hydrophila* S-layer, indicating the presence of the paracrystalline array on the cell surface.

Structure

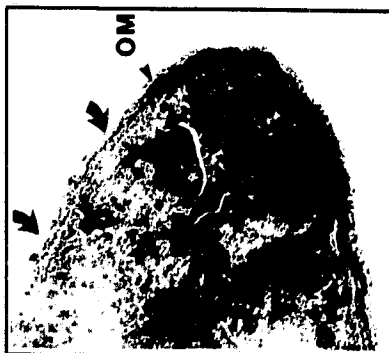
The S-layer of *A. hydrophila* is composed of a single species of protein of apparent Mr 52,000 (72). As is typical of S-proteins, amino acid composition analysis shows a high content of hydrophobic residues (41%), with an absence of cysteines and a single isoelectric form displaying an acidic pI of 4.6 (72). Two dimensional and three-dimensional reconstruction's of the S-layer have shown that the S-protein subunits constitute an array with a lattice constant of 12 to 12.5 nm containing a major tetragon at one 4-fold axis of symmetry, and a minor tetragon at the second 4-fold axis of symmetry (3, 211). The major tetragonal core of the array is composed of the heavy mass domains of four subunits, and is located towards the inside of the layer. The minor tetragon is raised and is composed of the lesser mass subunits which provide connectivity within the array and gives the surface of the layer three-dimensional architecture (211).

Protease digestion studies with the S-protein of *A. hydrophila* have provided biochemical evidence for two structural domains in the tetragonal array forming protein. Treatment of the S-protein with trypsin, chymotrypsin, or endoproteinase Glu-C under non-denaturing conditions resulted in a major peptide of approximate Mr 38,000 which could not be degraded further by these enzymes (160). The 38 kDa polypeptide mapped to

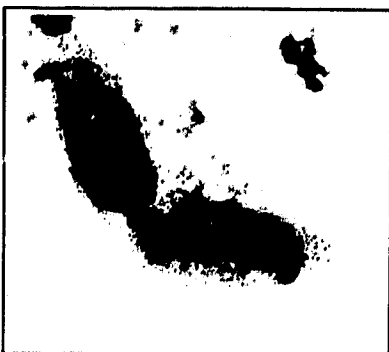
Figure 3. Localization of the *A. hydrophila* TF7 S-layer as shown using electron microscopy. (1) Immunogold labeling of the *A. hydrophila* TF7 cell surface using antisera JD3 as the primary antibody. (2) Thin section of the *A. hydrophila* cell, the small arrows point to the inner and the outer cell membranes, while the large arrow heads indicate patches of the S-layer located on the outer membrane. The inner membrane can be seen to have separated from the outer membrane at this pole of the cell. (3) Negative stain of a sheet of S-layer released into the culture medium by the Tn5 insertion mutant TF7-ST3. The negative stain used was 0.1% ammonium molybdate.



3



2



1

the N-terminal end of the S-protein. When treated with urea this 38 kDa polypeptide was capable of being further digested with these proteases (160). These data suggest that these structural segments probably reflected the morphological domains of the S-protein, the C-terminal peptide comprising the lesser morphological linker domain, and the N-terminal peptide corresponding to the inner major-mass core domain.

The S-layer proteins of mesophilic aeromonads are antigenically diverse. Using two different antisera, one raised against chromatographically purified S-protein, and one raised against denatured S-protein, Kostrzynska *et al.* showed that cross-reactive epitopes were non-surface exposed (160). Immunodominant epitopes were strain specific, surface exposed, and were localized by cyanogen bromide digestion and immunoblotting to a 26.0 kDa fragment within the protease resistant 38 kDa N-terminal domain. The N-terminal amino acid sequence was obtained for four antigenically different S-proteins, and even though these polypeptides were structurally similar, the primary sequence varied (160).

Secretion, assembly and anchoring

Recently, a main terminal branch of the general secretory pathway (GSP), the so-called *exe* operon, has been characterized in *A. hydrophila* (123, 128, 135, 136). This operon shows high sequence homology over its entire length to the pullulanase general secretory operon of *Klebsiella* spp. The products of the *exe* operon are required for both the secretion of the extracellular enzymes and normal outer membrane structure in *A. hydrophila*. Mutants in the *exe* operon have been shown to secrete the S-layer protein normally while the export of the extracellular enzymes was halted (121). The doubling time for *A. hydrophila* is very similar to *E. coli* in

rich media, at approximately 20 minutes. In order to maintain a complete S-layer, *A. hydrophila* must synthesize, secrete, and assemble the S-protein on the cell surface at an approximate rate of 400 monomers per second (204). The secretion system for the S-protein is therefore expected to be dedicated, and highly efficient. In the case of *A. salmonicida* secretion of the A-protein appears to be via the periplasm, and to involve a secretion system specific for this polypeptide (20, 216).

Studies performed on LPS mutants of *A. hydrophila* have shown that the LPS carbohydrate plays a major role in the anchoring of the S-layer to the cell envelope (75). Dooley and Trust found that the S-layer/LPS interaction had a minimum length LPS core carbohydrate requirement. They found that while a deep rough UV induced mutant, TF7/B, was unable to anchor the S-layer, a rough O-polysaccharide mutant TF7/U14 which contained two additional core sugar residues in its core stub, was capable of maintaining an intact array (75). This result is in contrast to *A. salmonicida*, where loss of the O-polysaccharide side chains lead to an inability to anchor S-layer to the cell surface and shedding of the S-layer into the growth medium (20).

The *A. hydrophila* S-layer apparently does not require Ca^{2+} or any other divalent cation for assembly or anchoring to the cell surface, in fact washing the S-layer of *A. hydrophila* with water, EDTA, or EGTA had no effect on its structure or presence on the outer membrane (211). In reassociation studies with the monomeric *A. hydrophila* S-protein, large sheets of paracrystalline array were found to re-form in the absence of any suitable template (71).

Genetics

At the beginning of this study, very little was known concerning the *A.*

hydrophila S-protein gene. Although the S-layer of *A. hydrophila* is structurally similar to the A-layer of *A. salmonicida*, it was found that they were not related at the genetic level (56).

Function

Purified *A. hydrophila* S-protein has been shown to bind hydrophobically to octyl-Sepharose gels, but the S-layer confers no overall hydrophobicity to whole cells, as determined by salt aggregation studies (72). However, it has been suggested that the S-layer of *A. hydrophila* has anti-phagocytic activity, which may aid in its systemic dissemination once invasion through the gastric mucosa has occurred (157). These results are based on the fact that upon intraperitoneal injection, S-layer negative strains were more virulent in mice when mixed with purified S-layer. Mittal *et al.* have demonstrated a relation between cell surface characteristics of S-layer producing aeromonads, and resistance to the killing activity of normal serum (207). More recently, Janda *et al.* showed that serum sensitivity generally correlated with the absence of the O-polysaccharide side chains, rather than a direct effect caused by the presence or absence of the S-layer (132).

***A. hydrophila* LPS**

All high virulence strains of *A. hydrophila* were shown to produce an S-layer, autoaggregated in liquid media, belonged to a single *Aeromonas* serogroup (O:11), and possessed homogeneous length O-polysaccharide side chains (74, 155, 156). The chemical structure of the O-polysaccharide of *A. hydrophila* LLI has been determined, and immunoblot studies with polyclonal antisera prepared against the LPS of both *A. hydrophila* strains LLI and TF7 showed that this O-polysaccharide structure contained a serogroup

specific epitope (74, 266). In a study of a number of S-layer containing O:11 serogroup strains consisting of both *A. hydrophila* and *A. veronii* biotype *sobria*, all were found to possess homogeneous length O-polysaccharide side chains (155). As stated above the LPS plays an essential role in anchoring the S-layer to the cell surface, and the homogeneous O-polysaccharide LPS structure is present in all S-layer containing strains of *Aeromonas*.

Purpose of this dissertation

Despite the number of bacteria known to express S-layers, very little is known about the role of these proteins in the interaction of prokaryotic cells with their environment, about the biological function of S-layers, or about the evolutionary relationships among S-layer proteins. This is surprising considering the surface location of these macromolecular assemblies, and in the case of pathogens, the potential importance of S-layers in the host parasite interaction. Furthermore, little knowledge has been gained concerning the genetics involved in the synthesis, regulation, secretion and assembly of these paracrystalline array proteins. These stages of S-layer maturation pose extremely interesting questions, especially considering that S-layers are one of the predominant proteins produced by the cell.

Bacterial infections of fish and homeothermic animals are often devastating economically, and are world wide problems. On a global basis *A. hydrophila* is possibly the bacterial pathogen most commonly associated with fish disease, and as such needs to be addressed with regard to a possible vaccine, or other form of treatment. Indeed, *A. hydrophila* causes economically significant disease in channel catfish in the Southeastern USA, trout in Spain, and European carp in other countries, and at this time there is no vaccine available to protect against *A. hydrophila* infections. All high

virulence strains of *A. hydrophila* produce an S-layer, therefore, this macromolecular protein assembly presumably gives this important pathogen a selective advantage either in its natural habitat, or during the infection process.

The current study was undertaken to provide the first molecular information on the S-layer of *A. hydrophila*. The results of this study are described in this dissertation, and have been organized into six major sections:

1. The cloning, nucleotide sequencing, and characterization of the S-protein gene (*ahsA*), and high level expression of the *A. hydrophila* S-protein in an *E. coli* background.
2. Biochemical characterization of the S-layer protomer.
3. The use of transposon insertional mutagenesis to characterize the two major domains of the S-protein and their roles in the formation, assembly, and anchoring of the paracrystalline array to the *A. hydrophila* cell surface.
4. The cloning, nucleotide sequencing and characterization of a gene which maps upstream of *ahsA* and encodes a protein involved in the specific secretion/translocation of the S-protein across the outer membrane of *A. hydrophila*.
5. The cloning, nucleotide sequencing, and characterization of a portion of a putative operon which maps downstream of *ahsA* which is apparently involved in the secretion of LPS O-polysaccharide side chains, and which concomitantly affects the anchoring of S-layer to the cell surface.
6. The role of the S-layer and LPS O-polysaccharide side chains in the serum resistance of *A. hydrophila*.

MATERIALS AND METHODS

Bacterial strains, vectors, and growth media

The bacterial strains from the culture collection of Dr. T. J. Trust are listed in Table 2, and vectors used in this study and their relevant properties are listed in Table 3. Stock cultures were maintained at -70°C in 15% (v/v) glycerol/LB. *A. hydrophila* and *A. veronii* biotype *sobria* strains were grown on LBA, or LB either overnight or for 18 h at 37°C , and for serum killing studies using rainbow trout serum, cells were grown at 15°C for 48 h. Other conditions used were as stated. *A. salmonicida* strains were grown at 22°C . All vector and plasmid containing strains were grown on LB media, and antibiotics were used in the following concentrations; Amp 50 $\mu\text{g}/\text{ml}$ (Boehringer Mannheim, Germany), Cmp 25 $\mu\text{g}/\text{ml}$ for vector containing strains and 10 $\mu\text{g}/\text{ml}$ for transconjugants, Ery 150 $\mu\text{g}/\text{ml}$, Gem 5 $\mu\text{g}/\text{ml}$, Kan 50 $\mu\text{g}/\text{ml}$, for vector containing strains, and 25 $\mu\text{g}/\text{ml}$ for transconjugants, and Tet 50 $\mu\text{g}/\text{ml}$, (Sigma Chemical Co., St Louis, MO). *E. coli* strain JM109 was maintained on minimal agar supplemented with 10^{-6} M thiamine.

Techniques used in the analysis of DNA

DNA Isolation

Chromosomal DNA

A. hydrophila chromosomal DNA was isolated by the method of Manning *et al.* (189). A 20 ml overnight culture of *Aeromonas* was grown with shaking at 37°C , and pelleted by centrifugation. The cells were resuspended in 2 ml of a 25 % (w/v) sucrose, 50 mM Tris-HCl (pH 8.0) solution to which 1 ml of a 10 mg/ml lysozyme made up in 0.25 M EDTA (pH 8.0) was added. The cell suspension was left on ice for 20 min prior to the addition of

Table 2. Bacterial strains used in this study

Species	Strain	Source or relevant properties
<i>A. hydrophila</i>	TF7	Trout lesion, Quebec
	Ah274	Sloth septicemia, Australia
	Ah423	Human diarrheal feces
	Ah598	Human diarrheal feces
	Ah77-115	Otary lung, Quebec
	Ah80-140	Aborted piglet liver, Quebec
	Ah80-160	Bovine brain, Quebec
	Ah300	Human diarrheal feces
	Ah65	Trout lesion
<i>A. veronii</i> biotype <i>sobria</i>	As701	Human septicemia, U. S. A.
	As702	Human septicemia, U. S. A.
<i>A. salmonicida</i>	A450	Brown trout, Tarn, France
	A449-TM4	Tn5 insertion mutant of strain A449
<i>E. coli</i>	DH5 α	<i>supE44DlacU169</i> (ϕ 80 <i>lacZDM15</i>)
	S17-1	Mobilizing donor strain; RP4 2-Tc::Mu
	JM109	<i>recA1supE44</i> Δ (<i>lac-proAB</i>)
	LE392	<i>supE44supF58hsdR514galK2</i> Δ (<i>lacIZY</i>)
	KW251	<i>supE44supF58galK2Tn10recD1014</i>
	1830	Containing plasmid pJB4JI

References for the *E. coli* strains include, (23, 36, 107, 212, 270, 327), reference for *A. salmonicida* A449 (20)

Table 3. Vectors used in this study

Vector name	Relevant details	Reference
EMBL 3	Lambda replacement cosmid	Promega
pBluescript(KS and SK)	High-copy-number plasmid	Stratagene
pUC18 and pUC19	High-copy-number plasmid	(327)
pSUP205	Mobilizable vector plasmid	(270)
pUC4SAC	Source of Kan ^R gene cassette	(16)
pUC4KISS	Source of Kan ^R gene cassette	(16)
pUC4KNOT	Source of Kan ^R gene cassette	(16)
pMMB206	Broad host range plasmid	(208)
pMMB67EH	Broad host range plasmid	(208)
pAT18/pAT19	Broad host range plasmids	(297)
M13mp18/19	Phage sequencing vectors	(197)
pRK2013	Conjugative helper plasmid	(86)
pJB4JI	Contains Tn5 transposon	(23)

0.75 ml TE buffer and 0.25 ml of lysis solution (5 % (v/v) sarkosyl, 50 mM Tris-HCl (pH 8.0), 62.5 mM EDTA), and 10 mg of solid pronase. The complete solution was mixed gently and left at 60°C for 1 h. The DNA was extracted 3 times with phenol (4 ml) equilibrated with Tris-HCl (pH 7.5), followed by extraction twice with ether (4 ml). The chromosomal DNA was dialyzed overnight in 2 l of TE buffer and stored at 4°C.

Plasmid DNA

Two methods were used for the isolation of plasmid DNA:

1. According to Sambrook (257), a loopful of cells was harvested from an LBA plate and suspended in a microcentrifuge tube containing 200 µl of solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA). To this, 400 µl of solution II (0.2 M NaOH, 1% SDS) was added, the cells were mixed gently and left on ice for 5 m. To precipitate the chromosomal DNA and proteins, solution III was added (300 ml 3M sodium acetate pH 4.8), and again following mixing by inversion, samples were left on ice for 5 m. Samples were then centrifuged for 10 m at 16,000 X G and the supernatant collected. Plasmid DNA was extracted with a 1:1 ratio of phenol/chloroform, followed by extraction with chloroform alone. DNA was precipitated with 1.5 volumes of 100% ethanol on ice for 10 m, pelleted by centrifugation at 16,000 X G for 15 m, and then washed with 70% (v/v) ethanol prior to drying under vacuum. Dried samples were resuspended in TE buffer.

2. The Wizard mini-prep system supplied by Promega (Promega Corp., Madison, WI) was used for the isolation of plasmid DNA according to the manufacturers instructions.

EMBL 3 phage DNA

For the isolation of EMBL 3 phage DNA containing inserts, the high multiplicity of infection method was used as described by Sambrook *et al.* (257). Phage were collected from a confluent lysis plate, and titered. Ten ml of NZCYM (10 g NZ amine, 5 g NaCl, 5 g yeast extract, 1 g casamino acids, 2 g MgSO₄·7H₂O, H₂O to 1 l, pH 7.0), media was placed in a 50 ml flask and inoculated with a single *E. coli* KW251 colony. Following overnight growth, 500 ml of fresh prewarmed NZCYM media was inoculated with 1 ml of this culture and incubated at 37°C until an OD of 0.5 at A₆₀₀ nm was reached. The flask containing the 500 ml of culture was inoculated with 10¹⁰ pfu of phage, and incubation was continued with vigorous shaking. Signs of lysis occurred after approximately 5 h, at which point shaking of the flasks was increased and 50 ml of fresh NZCYM broth was added to ensure that the cultures did not reach stationary phase. Once complete lysis had occurred, 10 ml of chloroform was added and incubation was continued for a further 10 m.

The lysed cultures were then cooled to room temperature and pancreatic DNase I and RNase were added to a concentration of 1 µg/ml. Solid NaCl was added to a final concentration of 1 M and after standing for 1 h on ice, cultures were centrifuged at 11,000 X G for 10 m at 4°C to remove the bacterial debris. The supernatant was separated into a fresh flask and solid PEG 8000 was added to 10 % (w/v) and dissolved by stirring. After allowing the flask to stand on ice for 1 h, precipitated phage were pelleted by centrifugation as above, and the supernatant discarded. The phage particles were resuspended in 8 ml of SM (5.8 g NaCl, 2 g MgSO₄·7H₂O, 50 mM Tris-HCl (pH 7.5), 5 ml of 2% (v/v) gelatin, H₂O to 1 l), and extracted with an equal volume of chloroform. Phage particles were again collected by centrifugation at 140,000 X G and resuspended in 1 to 2 ml of SM, and left overnight at 4°C.

EDTA was added to 20 mM to dissociate the phage and release the DNA, and Proteinase K was added to 50 µg/ml prior to incubation at 56°C for 1 h. The DNA was extracted in phenol followed by phenol/chloroform as above, and precipitated in 0.3 M sodium acetate and 2 volumes of 100% (v/v) ethanol. Precipitated DNA was collected by winding around the stem of a pasteur pipette, and quantified by spectrophotometry at A_{260} nm.

M13 phage DNA

A single phage plaque showing the presence of an insertion by blue/white screening on IPTG/X-gal plates was inoculated into 2 ml of a exponential phase culture of *E. coli* JM109. Following growth for 2 h at 37°C with vigorous shaking, an aliquot of 1 ml was used to inoculate 45 ml of fresh LB broth. After incubation under the same conditions for a further 5 h, cells were removed by centrifugation and the phage particles precipitated by the addition of a 1/20 volume of 40% (v/v) PEG (PEG M_r 8000) and a 1/20 volume of 4M NaCl. The solution was left at room temperature for 15 m and the phage precipitate collected by centrifugation at 10,000 X G for 30 m at 4°C in corex tubes. Supernatants were removed by inversion of the tube and discarded, and the walls of the tube were dried by wiping with a Kimwipe tissue. Pellets were resuspended in 0.4 ml TE buffer and the phage DNA was extracted 2 times in equal volumes of phenol-chloroform (1:1), followed by extraction in chloroform. DNA was then precipitated as described above.

Oligonucleotides

Oligonucleotides were synthesized on columns on an Applied Biosystems 391 (Applied Biosystems, Inc., CA) DNA synthesizer as recommended by the suppliers. Beads containing the newly synthesized

oligonucleotides were removed from the columns, and placed in 1 ml of fresh concentrated ammonium hydroxide for 1 h at room temperature prior to being left at 55°C overnight. The beads were removed by centrifugation and the oligonucleotides desiccated under vacuum. The oligonucleotides were then dissolved in sterile distilled water, and concentrations determined by spectrophotometry at A_{260} nm.

Molecular Cloning

Preparation of competent cells

A single colony of the desired *E. coli* strain was removed from an agar plate stored at 4°C, and used to inoculate 5 ml of LB broth. Following overnight growth at 37°C, 1 ml of this was sub-cultured into 100 ml of fresh LB broth, and incubated under similar conditions until an OD of 0.5 at 600 nm was reached. Cultures were placed on ice for 30 m, followed by centrifugation at 7,000 X G at 4°C for 5 m to remove the cells. Cells were resuspended in 1/5th of the growth volume in solution CM1 (10 mM sodium acetate (pH 5.6), 50 mM $MnCl_2$, 5 mM NaCl) and left on ice for 20 m. Cells were again pelleted as above, and resuspended in 1/50th of the growth volume in solution CM2 (10 mM sodium acetate (pH 5.6), 5 % (v/v) glycerol, 70 mM $CaCl_2$, 5 mM $MnCl_2$). Aliquots of 50 μ l were removed into microcentrifuge tubes and stored at -70°C.

Transformation

For transformation, the ligation mixture was added to an aliquot of competent cells which had been removed from a -70°C freezer and thawed on ice. The DNA and competent cells were left on ice for 30 m, and heat shocked in a water bath at 37°C for 4 m. Cells were again placed on ice for 20 m, and

then sub-cultured into LB broth for subsequent growth with shaking at 37°C for 30 - 45 m. Transformed cells were plated onto LBA containing the required antibiotics.

DNA ligation

For the "Shot Gun" method of cloning DNA fragments into vectors, the procedures used were those described by Sambrook *et al.* (257). This involves the complete digestion of a plasmid containing the insert of interest with a specific endonuclease to give a number of fragments of varying length. A vector is digested with the same endonuclease, added to the plasmid digest, and the mixture is ligated. Selection of the vector containing the required fragment is performed by screening a number of recombinants on selective media. When specific inserts were required following endonuclease digestion, DNA fragments were separated on a 0.8% (w/v) agarose gel and isolated using the Quiex gel extraction system supplied by Promega (Promega Corp., Madison WI). This system is based on the specific binding of linear fragments of DNA by glass beads, followed by their elution after removal from the agarose. Fragments of DNA isolated in this manner were then ligated into the required vector as described by Sambrook *et al.* (257). Ligation reactions were routinely performed in One-Pho-All plus buffer (Pharmacia, Uppsala, Sweden) with the addition of 1 unit of DNA ligase (Pharmacia) for 2 h at 15°C, or at 4°C overnight.

M13 cloning

M13 mp18 or mp19 replicative form DNA was used for the initial cloning of double stranded DNA isolated from an agarose gel. Competent *E. coli* JM109 cells were transformed with the replicative form M13 DNA from

the ligation reactions, and stored on ice. Rather than plating onto LBA direct, transformed JM109 cells were added to 0.1 ml of fresh exponential phase JM109 cells, along with 10 μ l of 100 mM IPTG and 50 μ l of X-gal (2% w/v) in DMF). The complete mixture was added to 3 ml of soft LB top agar maintained at 50°C, and poured onto pre-warmed LBA plates. Plates were incubated upright at 37°C for approximately 8 h.

T4 DNA polymerase blunt ending of 3' overhangs

To remove 3' termini from *Kpn*I sites within the pUC18 vector, approximately 2 μ g of DNA was digested with the appropriate restriction enzyme in a total volume of 20 μ l. Following digestion, 1 μ l of a solution containing each of the dNTPs at a concentration of 2 mM was added along with 2 units of bacteriophage T4 DNA polymerase (Pharmacia), and the mixture was incubated at 12°C for 15 m. The T4 DNA polymerase was inactivated at 75°C for 10 m and the products were ligated as above. Prior to transformation, the ligated vector was redigested with *Kpn*I to select for those plasmids having lost this endonuclease site.

Bacteriophage plate lysates

10⁵ pfu of the EMBL 3S clone was added to 0.1 ml (approximately 1.6 X 10⁹ calls/ml) of *E. coli* strain KW251 grown in the presence of maltose and incubated for 20 m at 37°C. The infected bacteria were added to 3 ml of molten top agar/agarose (0.7% (w/v)) mix and poured onto a 90 mm LBA plate. Plates were incubated for 6 to 8 h until the bacteriophage plaques were in contact. At this time, either a small amount of the bacterial debris containing the recombinant S-protein was removed and solubilized in SDS-PAGE sample buffer, or the bacterial debris was suspended in 5 ml of SM (5.8 g NaCl, 2 g

MgSO₄·H₂O, 50 ml Tris-HCl (pH 7.5), 2 % (v/v) gelatin, H₂O to 1 l), and total protein was precipitated using TCA.

Conjugation

Bacterial conjugation was used for the generation of marker exchange mutants, and for the insertion of cloned genes back into *A. hydrophila* for complementation analysis. Conjugal donors consisted of plasmid pSUP205 containing the insert of interest as the suicide vector in the generation of marker exchange mutants, and plasmids pAT18/19, or pMMB67/206 as broad host range vectors for complementation studies.

Conjugation was achieved by tri-parental matings using logarithmic phase cultures of the donor, recipient, and *E. coli* strain DH5 α carrying a helper plasmid pRK2013 (86). These three strains were mixed on an LBA plate and incubated for 5 h at 37°C prior to plating on selective media unless otherwise noted. Conjugative recipients were diluted and plated in duplicate for the selection of transconjugants that displayed the correct antibiotic resistance phenotype. When bi-parental mating was used, plasmids were transformed into the mobilizing donor strain *E. coli* 517-1 (270).

To select for transconjugants affected in production or maintenance of the S-layer on the cell surface, a small loop of the conjugation culture was inoculated into LB broth containing the required antibiotics, and placed under stationary conditions at 15°C for 48 h. An aliquot (100 μ l) of the top phase was sub-cultured into fresh LB broth and grown for a further 48 h under similar conditions prior to selection of *A. hydrophila* transconjugants on LB antibiotic plates at room temperature or at 37°C.

Transposon insertional mutagenesis

Conjugal transfer was performed using a filter mating procedure essentially as described by Beringer *et al.* (23) and Belland and Trust (20). The suicide plasmid pJB4JI which carries the Tn5 (Kan^R) transposable element flanked by bacteriophage Mu sequences, was constructed from the broad host range plasmid RP4. Gentamycin resistance is also carried on the plasmid, therefore loss of this marker but maintenance of Kan^R, allows for confirmation of correct insertion of the transposon into the *A. hydrophila* chromosome.

E. coli 1830 (pJB4JI) was the conjugal donor, and *A. hydrophila* TF7 was the conjugal recipient. After conjugation, cells were suspended in 3 ml of PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, H₂O to 1 l) (pH 7.4), and appropriate dilutions were plated on LBA plates to select for Tn5 - containing Kan^R Gem^S transconjugants of strain TF7.

Southern Analysis

Approximately 5 µg of chromosomal or 1 µg of vector DNA was digested with the required restriction enzyme for 2 h at 37°C. When double digests were performed, the second enzyme would then be added to the reaction mixture and digestion would be allowed to continue for a further 2 h. The digested DNA was separated on a 0.8% (w/v) agarose gel at 90V to 100V in TAE. DNA was stained using ethidium bromide (0.5 µg/ml) for 15 m, denatured and transferred to a Nytran nylon membrane (Schleicher & Schuell, Inc., Keene, NH) as described by the suppliers. Ultra violet cross linking of the DNA to the nylon membrane was achieved using a u.v. Stratalinker (Stratagene, La Jolla, CA), at 1200 millijoules for 40 seconds.

Filters containing bound DNA were prehybridized for 3 h at 68°C in prehybridization solution (5 X SSC (pH 7.8), 5 X Denhardt's (67), 0.1 % (w/v) SDS, 2 mM EDTA, 100 µg/ml herring sperm DNA). The filters were then hybridized overnight at 68°C in the same solution following the addition of radiolabeled probe. Washing of the hybridized membrane included two brief washes at room temperature in 2 X SSC (pH 7.8), 0.1 % (w/v) SDS, followed by two 30 min washes in 0.1 X SSC, 0.1% SDS at 68°C. Membranes were exposed to Kodak X-OMAT film, and the autoradiogram was allowed to develop overnight.

Radiolabeling of DNA by nick translation

The probes used in the majority of the Southern blots presented in this study were products obtained from restriction enzyme digestion, or products generated by PCR amplification and purified from a 0.8% (w/v) agarose gel using the Qiaex system as described above. Radiolabeling was accomplished with 100 µCi of ³²P (α-³²P) dCTP (specific activity approximately 3000 Ci/mM) using the nick translation kit supplied by Amersham (Amersham International UK) as recommended by the manufacturers. The radiolabeled product was used to probe the immobilized restricted chromosomal DNA overnight at 68°C as described above.

Radiolabeling of DNA by random priming

Probes used in the analysis of immobilized DNA by random priming were isolated as stated above, and radiolabeling was accomplished with approximately 50 µCi of ³²P (α-³²P) dCTP (spec. act. approx. 3000 Ci/mM) using the random priming kit supplied by Amersham. The labeled product was used to probe the immobilized restricted chromosomal DNA overnight at

68°C as above.

Polymerase chain reaction

For PCR amplification of DNA, chromosomal templates were used at a concentration of 50 ng/ μ l, and vector templates were used at a concentration of 5 ng/ μ l. PCR primers were used at a concentration of 5 μ M. Each 10 μ l PCR reaction contained 1 μ l of template, 1 μ l of each primer, 1 μ l of 10x PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH8.3)), 1 μ l of 25 mM MgCl₂, 1 μ l of a dNTP solution (10 mM), 4 μ l of sterile distilled water, and 0.1 μ l of Taq DNA polymerase (Pharmacia). Reactions were performed in a Perkin Elmer Cetus Model 480 thermocycler (Perkin-Elmer Corp., Norwalk, Conn.) for 30 cycles. Denaturation was for 1 m at 94°C, annealing was performed at 55°C for 1 m, and extension was achieved at a temperature of 72°C for 1.5 m. PCR products generated in this manner were analyzed by 0.8% (w/v) agarose gel electrophoresis and stained using ethidium bromide as above.

Automated DNA sequencing

The dye terminator kit supplied by ABI (Applied Biosystems, Inc. Foster City, CA.) and a Perkin-Elmer Cetus model 480 thermocycler (Perkin-Elmer Corp., Norwalk, Conn.) was used for the sequencing reactions using parameters recommended by ABI. For each dye-primer sequencing reaction, 1 μ g of double stranded DNA template, 3.2 pM of primer, and 9.5 μ l of reaction pre-mix (from the kit) were mixed and the reaction was carried out in the thermocycler. The DNA in the sequencing reaction was extracted twice with a phenol/chloroform/water mix (68:14:18), ethanol precipitated, and dried. Before loading onto the acrylamide gel, the DNA was resuspended in 4.5 μ l of a buffer containing 5 volumes of deionized formamide, 1 volume of 50 mM

EDTA (pH 8.0), and denatured at 90°C for 2 m. Completed sequencing reactions were electrophoresed on a 6% (w/v) acrylamide-8.3M urea sequencing gel for 14 h at 2,200 V, using an Applied Biosystems 373A DNA sequencer. Sequence data were analyzed by using the ABI SeqEd program.

Computer analysis

Computer programs used in the analysis of nucleotide sequences included DNA Strider release 1.2 (Institut de Recherche Fondamentale, CEA, France), Geneworks release 2.4 (Intelligenetics Inc., Mountain View, CA), PC Gene release 6.70 (Intelligenetics Inc.), and MacDNASIS (Hitachi Software Engineering America, Ltd., San Bruno, CA). Protein sequence similarity searches of the SwissProt database were performed using the algorithm of Smith and Waterman (277), and GenBank using BLAST (6).

RNA preparation and detection

Isolation of total cellular RNA

For the preparation of total cellular RNA, the cells contained in 20 ml of an exponential phase bacterial culture were pelleted at 4,000 X G for 10 m, and resuspended in 10 ml of protoplasting buffer (15 mM Tris-HCl (pH 8.0), 0.45 M sucrose, 8 mM EDTA, 0.5 mg/ml lysozyme (Boehringer Mannheim, Germany)). This mixture was incubated on ice for 15 m, and centrifuged at 2,000 X G for 5 m. The supernatant was discarded, and the pellet was resuspended in 0.5 ml of lysing buffer (30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM sodium citrate, 1.5% (w/v) SDS, 3% (v/v) DEPC (Sigma)). To ensure minimal RNA degradation, the DEPC was added as the first constituent of the lysing buffer. Following incubation of the mixture at 37°C for 5 m, it was chilled on ice, and 250 µl of saturated NaCl was added to

precipitate the DNA, SDS and proteins. Precipitation was allowed to proceed for 10 m on ice, followed by centrifugation at 16,000 X G for 15 m at 4°C. The supernatant was transferred to a fresh tube, and the RNA was precipitated by the addition of 1 ml of 100% ethanol at -70°C for 30 m. The precipitated RNA was collected by centrifugation at 16,000 X G for 15 m. Following washing with 1 ml of 70% (v/v) ethanol, the RNA was air dried and solubilized in 0.2% (v/v) DEPC treated water. Samples were stored at -70°C until analysis by formaldehyde gel electrophoresis.

Northern hybridization

Total cellular RNA (approximately 10 µg) dissolved in DEPC treated water was added to 15 µl of electrophoresis sample buffer (0.75 ml formamide, 0.15 ml 10X MOPS (pH 7.0), 0.24 ml formaldehyde, 0.1 ml RNase free water, 0.1 ml glycerol, 0.08 ml 10% (w/v) bromophenol blue). Samples were heated to 65°C for 15 m, cooled on ice, and 1 µl of a 1 mg/ml solution of ethidium bromide added prior to separation on formaldehyde denaturing gels. Denaturing gels consisted of 1.2% (w/v) agarose, 1X electrophoresis buffer (0.02 M MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA), and 5.1% (v/v) formaldehyde. To determine the size of the reacting RNA species, a 0.24-9.5 kb RNA ladder was used (GIBCO BRL Life Technologies Inc., Ontario) as recommended by the suppliers. Gels were prepared in a fume hood 1 h prior to use, and electrophoresed at 100V until the dye had migrated approximately two thirds the length of the gel. RNA could be visualized with no further staining, and following a 45 m wash in 10X SSC the RNA was blotted from the gel on to Nytran nylon membrane by capillary action. The RNA on the membrane was fixed by baking at 80°C for 1 h. Prehybridization and hybridization was performed at 42°C in the same solution (25 mM KHPO₄, 5X

SSC, 5X Denhardt's, (67) 50 µg/ml herring sperm DNA, 50% (v/v) formamide), and stringent washing of membranes and exposure to film was as described for Southern analysis above.

Detection and purification of proteins

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie blue staining

SDS-PAGE separation of proteins was performed in a mini-slab gel (8 x 5 cm separating gel) apparatus by the method of Laemmli (173). Samples were boiled in solubilization buffer (10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 3% (v/v) SDS, 0.0625 M Tris-HCl (pH 6.8)), stacked in 4.5% acrylamide, and separated using 10.0% or 12.5% (w/v) acrylamide at 100V constant voltage. Staining of gels for visualization of proteins was achieved using Coomassie brilliant blue R250 (0.5 g (w/v) Coomassie R250, 90.8 ml methanol, 90.8 ml H₂O, 18.4 ml acetic acid). Gels were destained in 25% (v/v) ethanol and 7% (v/v) acetic acid in water.

Low pH extraction of S-layer

Low pH glycine extraction of S-protein from *A. hydrophila* has been described previously (72). Cells were harvested from LBA plates into 20mM Tris-HCl (pH 7.5) maintained at 4°C, and washed three times in this buffer. Following centrifugation at 12,000 X G for 20 m at 4°C, approximately 3 g wet weight of cells was resuspended in 100 ml of 0.2 M glycine-HCl (pH 3.0), and stirred on ice for 15 m. The cells were removed from the glycine solution by centrifugation as above. The supernatant containing the extracted S-protein was then used for further analysis.

For detection of S-protein in the TF7-D2 mutant, extraction using 0.2 M

glycine-HCl (pH 2.15) was performed for 1 m only to reduce the risk of leakage of the periplasmic content.

Purification of S-protein

Two methods were employed for purification of S-protein:

1. To purify the truncated S-protein from mutant TF7-ST1, cells were harvested into 100 ml of 0.2 M-glycine-HCl (pH 3.0) as described above, and the cells removed by centrifugation at 12,000 X G for 20 m. The supernatant containing the truncated S-protein was passed through a 0.45 μ m nitrocellulose filter to remove any remaining cells, neutralized with NaOH, and concentrated to approximately 30 ml by ultraconcentration using an Amicon YM30 membrane operating at 25 lb/in². The protein containing sample was subjected to macromolecular sieve chromatography using a Superose 12 FPLC column (Pharmacia Canada, Inc., Baie B'Urte, PQ, Canada). The FPLC system was run in 100 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl at a flow rate of 0.5 ml/m. Eluted proteins were monitored at 214 nm, and the major peak was found to contain the S-protein. Fractions constituting this peak were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) using either Spectra/Por (Spectrum Medical Industries, Inc., Los Angeles, CA) molecular porous membrane tubing of Mr cut off 12-14,000, or a Microsep centrifugal microconcentrator (Filtron Technology Corp., Northborough, Mass) of Mr 30,000 cut off. Microsep microconcentrators were also used to concentrate the protein samples to approximately 1 mg/ml, and PMSF was added to 0.1 mM prior to storage.

2. For a rapid purification protocol, S-protein was released from TF7-ST3 (decreased O-polysaccharide side chains) mutant cells by the low pH extraction

method described as above (72), with certain modifications. Cells were washed four times with vigorous pipetting to remove pili and flagella, the pH of the glycine-HCl used was 2.15, and the concentration of cells to buffer was 0.05 grams wet weight/ml. The extraction was allowed to proceed for 15 m, and following removal of the cells by centrifugation, the supernatant was further centrifuged at 100,000 X G for 1 h in the presence of the glycine-HCl and the pellet discarded.

Protease digestion

Trypsin digests

Purified protein samples were made up to 1 mg/ml in 20 mM Tris-HCl (pH 8.0) and tosyl-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) was added to give a final enzyme to protein ratio of 1:10. Samples were incubated at 37°C for 60 m, soybean trypsin inhibitor (Sigma) was added at a 4-fold molar excess to terminate the reaction, and samples were analyzed by SDS-PAGE.

HPLC isolation of tryptic peptides

After digestion of S-protein with trypsin at a 1:10 enzyme to protein ratio, samples were diluted in TFA to give a final concentration of 1 % TFA. 120 µl samples were then loaded onto a C-18 Ultrasphere column (2.0 mm X 25 cm) (Beckman, San Ramon, CA), and the system was run in 100% buffer A for 10 m. The percentage of buffer B (0.8% (v/v) TFA in 100% acetonitrile) was then increased to 30% (v/v) over a period of 30 m until the peptides were beginning to elute from the column. The percentage of buffer B relative to buffer A was then increased from 30% to 50% over the following 40 m, prior to being increased to 100% over the next 30 m period (the ramp showing the

increase from 100% buffer A/0% buffer B to 0% buffer A/100% buffer B can be seen in figure 9). Peptides were detected at A₂₁₅ nm, (flow rate of 0.15 ml/m, chart speed = 0.2 cm/m, AUFS = 0.1) and isolated peaks were collected as separate fractions. To ensure that the base line remained constant as the concentration of buffer B increased relative to the concentration of buffer A, the absorbance of buffer A and buffer B were equilibrated at A₂₁₅ nm by titrating the TFA concentration in buffer B. This was found to be approximately 0.8% TFA.

Acid phosphatase digests

Purified S-protein was dialyzed against 10 mM glycine-HCl (pH 2.15) using a Microsep concentrator, and adjusted to a final concentration of 0.5 mg/ml. To this, one drop of concentrated HCl/0.5 ml of protein sample was added to maintain the S-protein at a low pH. This was then considered to be the stock solution. For the phosphatase reaction, 40 µl of stock was placed in a microcentrifuge tube, and pre-heated to 37°C. To this, 5 µl of a 1 M solution of MES buffer (pH 5.0) was added at the same time as 5 µl of a 10 mg/ml solution of potato acid phosphatase (Sigma) dissolved in 20 mM MES (pH 5.0). At timed intervals (5 m, 10 m, 15 m), 10 µl samples were removed and the reaction stopped by adding it directly to solubilization buffer, and heating to 100°C. In separate studies, 0.25 mg/ml protein samples were prepared in the same manner and acid phosphatase of the same concentration was added. The pH of the final reactions were checked in each case to ensure the reaction conditions were correct.

Partial acid hydrolysis

For partial acid hydrolysis, 200 µl of S-protein was dialyzed against 20

mM Tris-HCl (pH 7.5) and adjusted to a concentration of 0.5 mg/ml. This was made up to 6 normal using concentrated HCl, heated to 110°C for 45 m, and samples were then immediately analyzed by thin layer chromatography.

Thin layer chromatography

Partial acid hydrolysates were diluted 1:1 in the acetate/formic acid/water running buffer, and added directly to thin layer cellulose plates (Sigma C100), as were 5 µg samples of each of the phosphorylated standards O-Phospho-L-Serine (P-Ser), O-Phospho-L-Threonine (P-Thr), and O-Phospho-L-Tyrosine (P-Tyr) (Sigma). The samples were separated by ascending chromatography using an ethyl acetate/formic acid/water system (70:20:10 (v/v/v)) in either pH unadjusted, or pH 1.6, and visualized by spraying a thin mist of 0.3% (v/v) ninhydrin in 3% (v/v) acetic acid, 95% (v/v) ethanol. Plates were baked at 100°C for 10 m for development of the colour reaction.

Amino acid composition analysis and amino terminal sequencing

Protein samples for amino acid composition or amino terminal sequencing were separated by SDS-PAGE and electroblotted onto Problott (ABI) membrane. Staining of the protein on the membrane was achieved with Ponceau red (0.2% (w/v) Ponceau red, 1% acetic acid (v/v)) or Amido black (0.1% (w/v) Amido black in 40% methanol (v/v), 1% (v/v) acetic acid) and destained using distilled water. The required band was excised from the membrane and for amino acid composition analysis was hydrolyzed by 6M HCl and analyzed in an Applied Biosystems model 420 amino acid analyzer using procedures described by the manufacturers. Cysteine content was analyzed after oxidation of these residues was prevented by reaction with vapours of 5% (v/v) thioglycolic acid. Amino terminal sequencing was

achieved on an Applied Biosystems 470A gas phase sequencer running a standard operating program. Phenylthiohydantoin derivatives were separated by using an on-line analyzer (ABI model 120A). Data handling employed an Applied Biosystems model 900 Control Data Analysis module.

Solution molecular weight

Analytical centrifugation was used to determine solution molecular weights on a Beckman Model E ultracentrifuge equipped with an RITC temperature control unit and electronic speed control, using conventional low speed sedimentation equilibrium techniques (244). Double sector charcoal-filled Epon centerpieces (12 nm) were employed, and measurements were made using the u.v. absorption optics of the photoelectric scanner. The data was recorded on the chart paper of the scanner accessory. Equilibrium measurements were performed at 20°C with 0.1 ml of protein solution using rotor speeds chosen from graphical data (51). Runs were assumed to be at equilibrium when no further changes in pen tracing occurred. 100 mM NaCl, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes) was the pH 7.4 solvent; while 100 mM NaCl, 0.1% (v/v) TFA was used at pH 2.1.

Circular dichroism

Circular dichroism (c.d.) measurements were made on a Jasco spectropolarimeter (Jasco Inc. Easto, MD) interfaced to an Epson Equity 386/25 computer. The cell was maintained at 25°C with an RMS circulating water bath (Lauda, Westbury, NY). Near u.v. (320 to 250 nm) scans were performed in a microcell of path length 1 cm, volume = 90 μ l. In the wavelength range 255 to 190 nm, cells of path length 0.01 or 0.02 cm were used. The computer-averaged trace of 10 scans was used in all calculations. Signal due to solvent

was subtracted. The instrument was routinely calibrated with d-(+)-10 camphor sulfonic acid at 290 nm, and with pantoyl lactone at 219 nm, by following procedures outlined by the manufacturer. The data was normally plotted as mean residue weight ellipticity (expressed in degrees square centimeters per decimole) *versus* wavelength in nanometers. The mean residue weight was taken to be 100.5. The ellipticity *versus* wavelength data were analyzed by a computer program developed by Provencher and Glockner (238), which analyzes c.d. spectra as a sum of spectra of 16 proteins, the structures of which are known from X-ray crystallography. The input to the program was the mean residue weight ellipticities, in 1 nm intervals from the minimum value measured to 240 nm. The concentration of protein was determined by amino acid analysis after hydrolysis of suitable samples of the solutions used for spectroscopy.

Cell fractionation

Culture supernatant

Cells were removed from 18 h stationary broth cultures by centrifugation at 10,000 X G for 10 m. The supernatant was retained for use as the culture supernatant fraction. If required, samples were concentrated using the Microsep centrifugal concentrator of molecular weight cut off 30,000.

Periplasmic fraction

The osmotic shock method of Willis *et al.* was used to isolate the periplasmic fraction (322). Cells collected in the pellet (1 g wet weight) following centrifugation of overnight cultures were washed twice in ice cold 33 mM Tris-HCl (pH 7.5), and resuspended in 10 ml of a solution containing 0.15 M Tris-HCl (pH 7.5), 2mM EDTA, and 20% (w/v) sucrose. Suspensions

were shaken vigorously at room temperature for 10 m, and centrifuged at 10,000 X G for 20 m. As much as possible of the sucrose solution was removed by draining, and the cells were resuspended quickly in 2 ml of ice cold distilled water, followed by the addition of $MgCl_2$ to 1 mM. Samples were stirred on ice for 10 m, followed by the removal of bacteria by centrifugation. The supernatant containing the osmotic shockate was further centrifuged at 200,000 X G for 1 h to remove any contaminating membrane particles, and the resulting supernatant was retained as the periplasmic fraction.

Cytoplasmic fraction

The bacterial cell pellet isolated during preparation of the periplasmic contents was resuspended in 20 mM Tris-HCl (pH 7.5), and frozen at $-70^{\circ}C$ overnight to aid in cell breakage. After thawing a small amount of RNase and DNase were added to the suspension, and cells were disrupted by two passages through a precooled French pressure cell (16,000 lbs/in²). Unbroken cells were removed by two centrifugations at 4000 X G for 30 m. Cell envelopes in the supernatant were sedimented at 40,000 X G for 30 m. The pellet was removed as the cell envelope fraction, and the supernatant was collected as the cytoplasmic preparation.

Inner and outer membranes fractions

The envelope fraction collected during isolation of the cytoplasmic fraction was adjusted to 1 mg protein/ml in 20 mM Tris-HCl (pH 7.5), and SLS was added to a final concentration of 0.6% (v/v) to differentially solubilize the inner membrane (87). The preparation was shaken at room temperature for 30 m and the outer membranes sedimented at 40,000 X G for 30 m. The

supernatant containing the inner membrane was dialyzed to remove the SLS using a Microsep concentrator against 20 mM Tris-HCl (pH 7.5). The outer membranes contained in the pellet were washed 3 times in 20 mM Tris-HCl (pH 7.5), sedimented at 40,000 X G for 30 m, and resuspended in 20 mM Tris-HCl (pH 7.5).

In certain studies, to ensure that large sheets of S-layer was not co-sedimenting with the outer membranes, the cell envelopes were extracted twice in 0.6% SLS, and the outer membranes so isolated were further extracted with 6 M Guanidine Hydrochloride (GHCl) for 1 h with gentle shaking at room temperature. Following extraction, outer membranes were washed in 6 M GHCl once, and washed twice in 20 mM Tris-HCl (pH 7.5).

LPS fraction

Isolation of LPS was achieved using Proteinase K digestion of proteins following solubilization of whole cells (114) in SDS-PAGE sample buffer as above. Approximately 200 μ l of a logarithmic phase culture of cells at A₆₀₀ was boiled in the sample buffer for 5 m, and an equal volume of a 1 mg/ml solution of Proteinase K (freshly dissolved in H₂O) added. This preparation was placed at 60°C for 1 h, and then either stored on ice, or boiled for 5 m prior to loading directly onto an SDS-PAGE gel. For clear visualization using silver staining, 5 μ l to 10 μ l of preparations prepared in this manner and separated by 12.5% SDS-PAGE was generally ample.

Silver staining of LPS

LPS preparations separated by SDS-PAGE were silver stained by the method of Tsai and Frasch (302). In brief, following separation of LPS samples gels were fixed for 2 h (40% (v/v) ethanol, 5% (v/v) acetic acid), and oxidized

for 5 m in fixing solution containing 0.7% (w/v) periodic acid. The gels were then washed three times and stained with silver nitrate (2 ml NH_4OH , 28 ml 0.1 M NaOH, 5 ml 20% $\text{Ag}(\text{NO}_3)_2$, 115 ml H_2O) for 10 m with vigorous agitation. Following three washes in distilled water, the gel was placed in developer (50 mg citric acid, 0.5 ml 37% (v/v) formaldehyde H_2O to 1 l) until the desired colour was obtained. Reactions were stopped by placing the gels into a solution containing 10% acetic acid.

Detection of extracellular enzymes

Secreted enzymes were detected by culturing cells on LBA plates containing the substrate for each enzyme and comparing colony size and zones of clearing between wild type *A. hydrophila* TF7, and the mutant under study. The presence of aerolysin was detected on LBA plates supplemented with 0.6% (v/v) horse blood. For the secretion of protease, cells were sub-cultured onto Casein agar (2 g of sodium caseinate (ICM Biochemicals, Cleveland, OH) 1.0 g glucose, 0.2 g K_2HPO_4 , 0.2 g MgSO_4 , 0.001 g FeSO_4 , and 15 g agar per litre), and the presence of secreted amylase was detected on iodine flooded LBA containing 2 g of starch per liter.

***In vivo* ^{32}P orthophosphate radiolabeling of the *A. hydrophila* cell surface**

A. hydrophila TF7 cells were grown under conditions of limiting orthophosphate by adding 60 μl of a 1 mM solution of K_2HPO_4 , and 100 μl of ^{32}P -orthophosphate (100 μCi) (Amersham 200mCi/mM) to minimal media salts made up as in Sherman and Goldberg (267). Modifications to this media included the exclusion of casamino acids, and the addition of thiamine to 1% (w/v). Cells were grown for 20 h at 37°C, washed three times in 10 mM Tris-

HCl (pH 7.5), and the S-protein removed by low pH extraction. Radiolabeled samples were separated by SDS-PAGE as above, and exposed to Kodak X-OMAT film, allowing the autoradiogram to develop at room temperature overnight.

Immunochemical techniques

Western immunoblotting

For immunoblotting, proteins were transferred from the acrylamide gel onto nitrocellulose (NC) paper by the methanol-Tris-glycine system of Towbin *et al.* (296). Electroblothing was performed using the semi-dry system of LKB NOVABLOT (Pharmacia) at 12V for 0.6 h. Membranes were blocked using 2% (w/v) skim milk in TBS, and primary antibody was added (1:2000) in 2% (w/v) skim milk TBS. After incubation for 2 h, the membranes were washed three times in TBS and an alkaline phosphate conjugated goat anti rabbit IgG (Caltag Labs, San Francisco, CA) secondary antibody was added as per manufacturer's recommendations. Membranes were again washed three times in TBS, and substrate was added (Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP). The reaction was stopped when colour development was complete.

For detection of S-protein using anti-phosphotyrosine monoclonal antibody (Sigma), approximately 5 µg of S-protein isolated from various *A. hydrophila* or *A. veronii* biotype *sobria* strains by the low pH extraction method was separated by SDS-PAGE and blotted as above. The NC containing the S-protein was blocked overnight in 5% (w/v) BSA (fraction V, Sigma) in TBS at 4°C and the monoclonal antibody was added to a final concentration of 2 µg/ml and left with shaking for 5 h at room temperature. The blot was then washed three times for 10 m each in TBS, and goat anti mouse alkaline

phosphatase conjugated secondary antibody was added to a final concentration of 0.4 $\mu\text{g}/\text{ml}$. Washing and detection using NBT/BCIP was performed as above.

Bacteriophage plaque lift

50 μl of the EMBL 3S clone containing approximately 50,000 bacteriophage particles was incubated for 20 m at 37°C with 0.3 ml (approximately 1.6×10^9 cells/ml) of *E. coli* strain KW251 grown in the presence of maltose. 6.5 ml of molten top agar/agarose mix (0.7 % (w/v)) was added to each aliquot containing the bacteriophage infected bacteria, and the mixture was poured onto a 150 mm LBA plate. The plate containing the infected bacteria was incubated at 37°C until the plaques reached a diameter of approximately 1.5 mm, at which point the plates were chilled for 1 h to harden the top agar/agarose. A circular NC disc was placed in contact with the bacteriophage plaques for 1 m, removed, and placed plaque side up onto Whatman 3MM paper soaked in 0.5 M HCl. NC filters were rinsed in 20 mM Tris-HCl (pH 7.5), and then placed into 2% (w/v) skim milk TBS and processed as described for Western immunoblotting.

Colony blotting

NC discs were placed on agar plates containing bacterial colonies which had been grown overnight at 37°C. Plates were incubated for a further 1 h, after which the NC discs were removed and placed (colony side up) onto Whatman 3MM paper soaked in 0.5 M HCl. After 30 m at room temperature, bacterial debris was removed from the NC discs using 20 mM Tris-HCl (pH 7.5) applied from a wash bottle. Processing of the NC discs was as described in Western immunoblotting.

Inhibition ELISA

Samples (60 μ l) containing 0.9 ng of purified wild type S-protein were added to a microtiter plate previously blocked with 3% (w/v) BSA in TBS (pH7.5) containing 0.05% (v/v) Tween 20 (TBS-Tween), and mixed with 60 μ l of a 1:1000 dilution (v/v) anti S-protein polyclonal antiserum JD3 in TBS-Tween supplemented with 1% (w/v) BSA. After 2 h incubation at 20°C, the inhibition mixture was transferred to an ELISA plate previously coated with 0.1 μ g of wild-type S-protein/well. Coating of the ELISA plate was performed by adding purified S-protein to sodium carbonate buffer (pH 9.6) (82) to a final concentration of 1 μ g/ml and standing overnight at 4°C with 100 μ l of this solution/well. The ELISA plate was then blocked with Tris-Tween 3% BSA for 30 m at 20°C and washed 3 times with Tris-Tween before addition of the inhibition mixture. The protein and antiserum solutions were added in duplicate and allowed to react for 1 h at 20°C. Wells were washed 3 times with Tris-Tween and detection of bound anti-S-protein polyclonal rabbit antiserum JD3 was determined essentially as described by Engvall and Perlman (82). The developing antibody was goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Caltag). The absorbance was read at 405 nm with an EIA model 310 ELISA reader (Biotek Instruments Inc., Highland Parks, VT). BSA was used as the negative control.

Electron microscopy

Negative staining

Formvar coated grids were floated on droplets of sample suspended in TBS. After 5 m excess liquid was removed using Fisher brand filter paper. The grids were then negatively stained using 1.0% or 0.07% (w/v) ammonium molybdate for 2 m and allowed to air dry. Micrographs were

recorded on a Phillips EM 300 transmission electron microscope (Phillips Electronic Instruments, Inc., Mahwah, NJ) or a JEOL JEM-1200 EX transmission electron microscope (JEOL U. S. A., Inc., Peabody, MA) at an accelerating voltage of 60kV using standard imaging conditions. Images were recorded on 70 mm Fine Grain Release Film or Kodaline Rapid film 2586 (Eastman Kodak Co., Rochester, NY).

Immunogold labeling

Formvar coated grids were floated on samples of a suspension consisting of whole cells resuspended in TBS. After 10 m, the grids were removed and placed on a drop of 20 mM Tris-HCl (pH 7.5) containing 1% BSA for 3 . n. The grid was incubated on a drop of anti S-protein serum JD3 at a dilution of 1:100 for 1 h. After incubation, the grid was serially floated on three separate drops of 20 mM Tris-HCl (pH 7.5) for 10 m each to remove any unbound JD3 anti sera. The grid was then floated on a drop of TBS-BSA containing a 1:50 dilution of 15 nm colloidal gold particles coated with protein A (Janssen Pharmaceutica, Olen, Belgium). Non-specifically bound protein A gold particles were removed from the grid following a 1 h incubation period by three successive washes on drops of Tris buffer as above. The grids were then negatively stained and examined as above.

Isolation of fresh serum

Rainbow trout

Blood was collected from the caudal sinus of a 500 g to 1000 g animal and allowed to clot for 30 m at room temperature. Serum was separated from the clot by centrifugation at 2,000 X G for 10 m, stored at 4°C, and used within 2 h of collection.

Rabbit

Normal fresh serum was obtained by collecting blood from adult New Zealand white rabbits and allowing it to clot for 1 h at 25° C. The blood was centrifuged at 2,000 X G for 10 m and the serum separated from the clot, stored at 4°C, and used within 2 h of collection.

Preparation and isolation of immune serum**Rainbow trout**

Bacteria were grown overnight, and added to formalin and left for 24 h at 4°C. Cells were washed three times after formalin treatment, and resuspended in PBS. A 1,000 g animal was injected intramuscularly with 0.1 ml of a mixture of a formalinized suspension (1×10^8) of *A. hydrophila* TF7 in PBS in Freund's complete adjuvant (GIBCO laboratories, Life Technologies Inc., Grand Island, NY) at two sites in the dorsal muscle. After three weeks, a booster injection of formalinized cells in Freund's incomplete adjuvant (GIBCO) was given, and the animal bled within two weeks of this. Serum was collected from the caudal sinus.

Rabbit

Whole cell antisera was prepared by injecting a total of 2 ml (1 ml intramuscularly, 1 ml subcutaneously) of a mixture of formalin killed *A. hydrophila* TF7 cells in PBS 1:1 in Freund's complete adjuvant (GIBCO) (total of 1×10^8 cells) into New Zealand white rabbits. A booster injection of a similar preparation of formalinized whole cells was given both intramuscularly and subcutaneously in Freund's incomplete adjuvant (GIBCO) two weeks later, and a third similar inoculum was given after a further four weeks. Serum was collected within 6 days of the final

inoculation by exsanguination, and designated PF1.

Affinity chromatography purification of antisera

The purified S-protein from *A. hydrophila* strain TF7 was coupled to FMP (2-Fluoro-1 Methyl Pyridinium Toluene-4-Sulfonate) activated Avid-Gel F (Bio-Probe International, Inc., Tustin, CA) in 50 mM NaHCO₃/25 mM NaCl (pH 8.5) coupling buffer, according to the manufacturers recommendations. Unreacted sites were blocked by incubation in 0.15 M ethanolamine buffer (pH 10.5) and the Avid-Gel including the bound S-protein was packed into a Bio-Rad Econo-column (Bio-Rad laboratories Inc., Mississauga, Ontario). Polyclonal antiserum JD3 (72), which was prepared in rabbits to chromatographically purified S-protein from *A. hydrophila* TF7, was passed through the column and any unbound protein was removed with TBS until the absorbance of the eluate was zero at A₂₈₀ nm. The bound antibodies were eluted from the column using a 0.3 M glycine-HCl (pH 8.0). Fractions were collected directly into 1 M Tris-HCl (pH 8.0) and those containing the eluted antibody were pooled, and concentrated with a Microsep protein concentrator with a 100 kDa exclusion filter.

Serum killing

Approximately 5.0×10^6 washed cells in 0.1 ml PBS were added to 0.9 ml of serum solution (0.5 ml serum, 0.4 ml LB) and incubated at 15°C (rainbow trout serum), or 37°C (rabbit serum). At specified time intervals, 0.1 ml aliquots were removed, serially diluted, and plated onto LBA plates for counting viable cells. Control tubes contained either heat inactivated serum or LB broth alone. The inactivation of the serum was achieved by either heating to 56°C (rabbit), or to 45°C (trout) for 30 m.

Slide agglutination studies

A. hydrophila cells were cultured overnight in LB broth, harvested by centrifugation, and washed three times in 33 mM Tris-HCl (pH 7.5). Washed cells were resuspended in the Tris buffer to give a dense suspension when observed on microscope slides. The cell suspension was added to a microscope slide in volumes of 20 μ l, and an equal volume of the antisera dilution was added to this. Antisera to be tested was diluted serially (1:2) in the 33 mM Tris buffer. Agglutination was considered positive if a pronounced clumping of the bacterial cells could be seen compared to the negative control within 1 to 3 m. If no agglutination was seen following 3 m, samples were considered to be negative.

RESULTS

I. Cloning and Characterization of the *A. hydrophila* S-Protein Gene

In this study we undertook to clone and sequence the gene encoding the S-protein of *A. hydrophila*. This study was considered important, because, although the S-protein had been well characterized biochemically, little was known concerning either the primary structure or the synthesis of this important bacterial product. Due to their high level of expression and secretion, S-proteins may also be ideal for use as a vehicle for the expression of chimeric foreign epitopes in the field of vaccine development. The expression of foreign epitopes in a chimeric S-protein requires that the complete nucleotide sequence of the gene encoding the S-protein be known. Furthermore, for the study of the secretion of the chimeric proteins, stable expression must be obtained from a plasmid in an *A. hydrophila* background.

Cloning of the S-protein gene into the EMBL 3 replacement vector

The use of either plasmids or cosmids for the cloning of some S-protein genes has proven difficult in the past. This is because expression of the recombinant product under the control of the native promoter often creates stability problems. However lambda vectors are potentially ideal for the initial cloning of toxic genes, because expression of the recombinant protein does not require the continued viability of the host cell. For this reason the lambda replacement vector EMBL 3 was selected to attempt to clone the *A. hydrophila* S-protein gene.

In this study we chose to use a timed partial *Sau3A* digest of *A. hydrophila* chromosomal DNA, followed by the isolation of fragments of approximately 10-20 kb in size as determined by agarose gel analysis. These

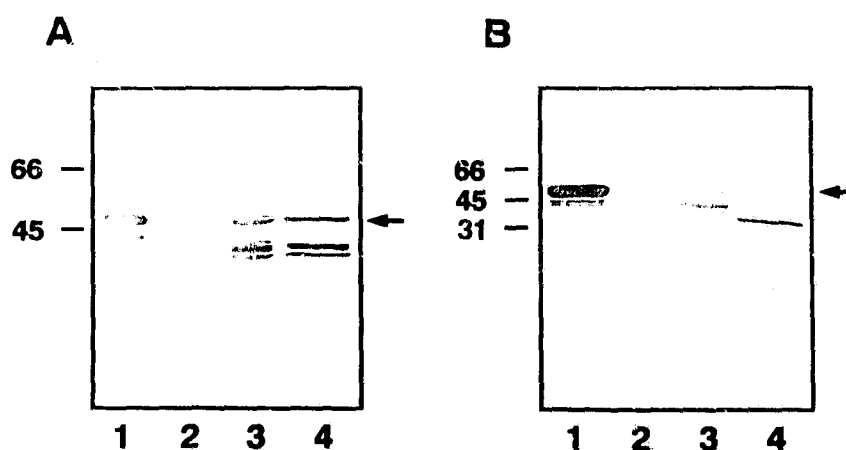


Figure 4. Western blot using affinity purified antisera JD3 of an SDS-PAGE of the *A. hydrophila* S-protein expressed in a confluent phage lysate from the homogeneous clone EMBL 3S (A), and from plasmid pUC18 in *E. coli* (B). Lane 1 in panels A and B shows the native *A. hydrophila* TP7 S-protein isolated by low pH extraction. (A), lane 3, TCA precipitated S-protein from the lysate plate; lane 4, cell debris from the phage lysate plate solubilized in SDS-PAGE running buffer; lane 2, whole cell lysate of *E. coli* strain KW251. (B) following the subcloning of the S-protein gene into *E. coli*, two subclones were isolated, pST100 expressed full size S-protein, and pST53 expressed an Mr 32,000 truncated S-protein. Lane 3, S-protein expressed from plasmid pST100; lane 4, truncated S-protein expressed from plasmid pST53; lane 2 contains a whole cell lysate of *E. coli* DH5 α . Numbers on the left of A and B indicate molecular weight markers, while the position of the native S-protein is shown on the right.

fragments were ligated into EMBL 3. Plaque lifts using *E. coli* strain LE392 were subsequently screened for S-protein expression, and a number of positive reacting plaques were identified using affinity-purified anti S-protein antisera JD3. Ten of these plaques were chosen at random for purification, but all showed instability when attempts at isolating a homogeneous S-protein expressing phage population were made. This continued to be a problem using strain LE392, but when *E. coli* strain KW251 was used, one plaque expressing the *A. hydrophila* S-protein was purified to homogeneity. Using the homogeneous phage plaque expressing the recombinant S-protein, a confluent phage lysate plate was prepared. Figure 4A shows an immunoblot of the recombinant S-protein from the confluent phage lysate plate containing the purified plaque. Lane 3 shows the presence of the Mr 52,000 immunoreactive polypeptide in TCA precipitated protein from the phage lysate plate, identical in size to the wild type S-protein (lane 1), while lane 4 also shows an immunoreactive Mr 52,000 protein in the bacterial debris from the lysate plate. The presence of a mixture of molecular size products representing degraded S-protein can be seen in lanes 1, 3 and 4.

Sub-cloning of the S-gene into pUC18 and expression of S-protein in *E. coli*

Restriction mapping of total phage DNA containing the S-protein gene showed the insert to be approximately 18 kb in size. Digestion of the 18 kb insert using *SalI* gave three fragments, one of which proved to contain between 10 and 12 kb of DNA that located to the 3' end of the *Sau3A* insert. Attempts at sub-cloning this large *SalI* fragment or a smaller *SalI*-*BglII* fragment into either high or low copy number plasmids proved unsuccessful. We therefore used a partial *Sau3A* digest of total EMBL 3 DNA containing the S-gene to try and remove the native promoter. *Sau3A* fragments were ligated

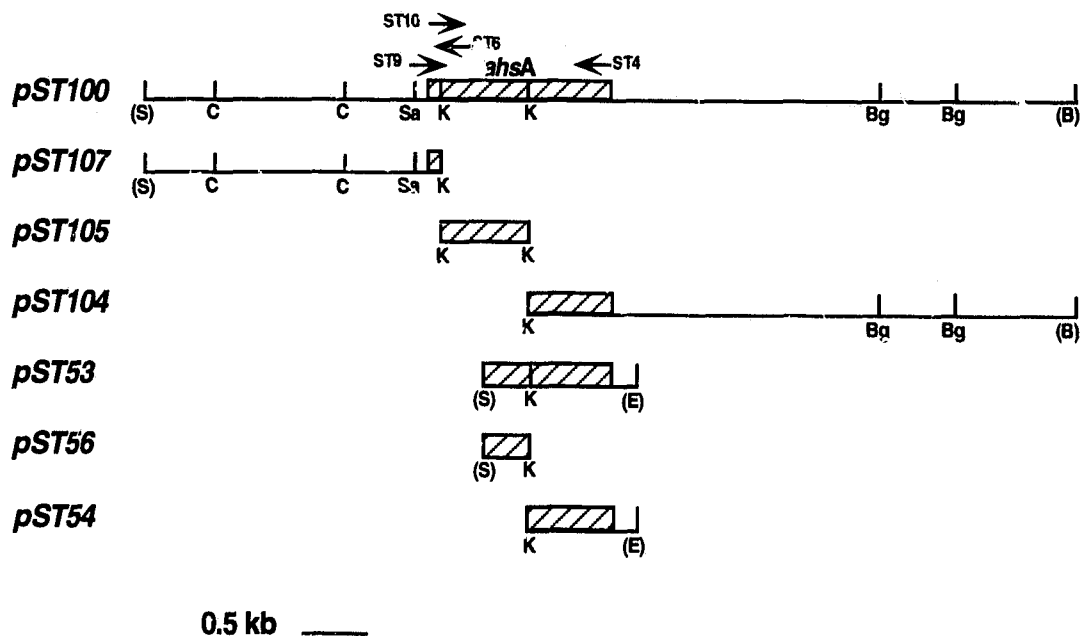


Figure 5. The plasmids pST100 expressing intact S-protein, and pST53 expressing a truncated S-protein were isolated from a *Sau3A* partial digest of the original EMBL 3S clone. Sub-clones used for the nucleotide sequencing of the *ahsA* gene are also shown. The primers ST9, ST10, and ST4 were used to verify the presence of the complete nucleotide sequence of the *ahsA* gene by PCR analysis, and for the generation of a probe used in Southern blot analysis. The primer ST6 was used for sequencing through the *KpnI* site in pST100. The insert from pST100 was sub-cloned into the broad host range vectors pAT18, and pMMB67(EH) generating constructs pST101 and pST102 (not shown) for conjugation of the *ahsA* gene into *A. hydrophila* TF7S, and *A. salmonicida* A449-TM4 respectively. Restriction sites contained within the multiple cloning sites of the vectors are indicated by brackets. B = *Bam*HI, Bg = *Bgl*II, C = *Cla*I, E = *Eco*RI, K = *Kpn*I, Sa = *Sac*I, and S = *Sal*I.

into *Bam*HI digested pUC 18 and transformed into *E. coli* strain DH5 α . A number of subclones expressing S-protein were identified by colony lift and immunoblotting, but of these, only two could be stably sub-cultured. These were found to contain plasmids pST53 expressing a truncated protein of approximate Mr 32,000, and pST100, which expressed S-protein of approximate Mr 52,000, identical in size to the native mature S-protein in *A. hydrophila*. Figure 4B is a Western immunoblot of glycine extracted S-protein reacted with anti-S-protein antiserum showing the Mr 52,000 immunoreactive protein expressed from plasmid pST100 (lane 3), and the immunoreactive truncated S-protein of approximate Mr 32,000 expressed from plasmid pST53 (lane 4).

Sequencing of the S-protein gene

Plasmids pST100 and pST53 were mapped using endonuclease restriction enzymes. Figure 5 shows the major restriction sites as well as the sub-clones used in the sequencing of the S-gene. Where endonuclease restriction sites were not available for sequencing in both directions, synthetic oligonucleotides were used. The *Kpn*I site at the 5' end of the *ahsA* (*A. hydrophila* S-gene) gene was sequenced across using oligonucleotide ST6 in pST100 to confirm that no small *Kpn*I-*Kpn*I fragment had been lost during the subcloning procedure. This showed that we had indeed sequenced the complete *ahsA* gene.

Following the completion of sequencing, and based on the Mr of the S-protein by SDS-PAGE, it was found that the *ahsA* gene was smaller than expected (see below). To ensure that the full length gene had been sub-cloned and sequenced, two methods were utilized. Firstly, PCR analysis was used to generate a fragment of DNA corresponding to a known size from the *vapA*

gene of *A. salmonicida* strain A449 as a standard, and compared to similarly generated fragments from the *ahsA* gene. Figure 6 shows the results obtained during this procedure, lanes 1 and 2 contain PCR generated products from *A. salmonicida* strain A449, which are 1525 and 1371 nucleotides in length respectively. Lane 4 shows a PCR generated fragment from pST100 using the primers ST9 and ST4 (figure 5), which generate a product of 1619 nucleotides in length. The *A. hydrophila* TF7 chromosome was used as a template for the generation of the PCR product shown in lane 5 (figure 6) (1350 nucleotides) using the primers ST10 and ST4 (figure 5), while lane 6 contains a PCR generated product from plasmid pST100, again using the ST10 and ST4 primers. Importantly, the product generated from the *A. hydrophila* TF7 chromosome is equal in length to the DNA fragment generated from plasmid pST100. These results proved that the *ahsA* gene was smaller than the *vapA* gene which was known to encode a protein of subunit Mr 50,000. Secondly, Northern blot analysis (figure 7) was used to determine the full size of the AhsA mRNA in *A. hydrophila* and *E. coli*. In both organisms the AhsA mRNA had an approximate size of 1480 bases when compared to commercial RNA markers. Figure 7 lane 1 contains total cellular RNA from *A. hydrophila* strain TF7, whereas lane 2 contains RNA from *E. coli* strain DH5 α as a negative control. In lane 3, total RNA from *E. coli* harboring plasmid pST100 can be seen, the increase in S-gene transcript compared to TF7 may be due to the high copy number of the plasmid leading to an increase in the amount of mRNA transcribed from the recombinant *ahsA* gene. The predicted size for the *ahsA* gene message at approximately 1480 bases, is close to that measured from the Northern blot, and indicates that the gene is not part of an operon.

For the sequencing of the 5' end of the S-gene, plasmid pST107 (figure 5

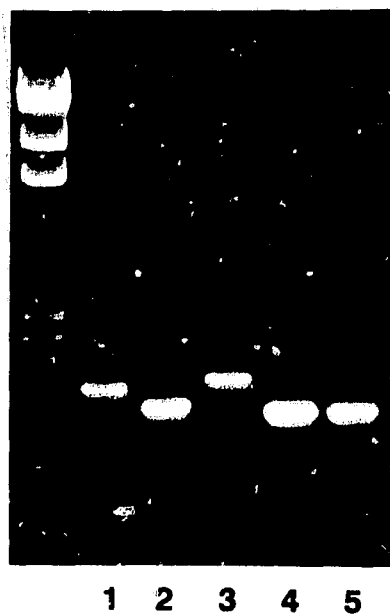


Figure 6. PCR analysis of the *ahsA* gene to ensure that no fragments were lost during the subcloning procedure. PCR was used to generate fragments from the *vapA* gene from the chromosome of *A. salmonicida* strain A449 as nucleotide markers of known length. Lanes 1 and 2 contain DNA fragments of length 1525 and 1371 nucleotides respectively from the *A. salmonicida* chromosome. Lane 3 shows a fragment of 1619 nucleotides generated from plasmid pST100 using the PCR primers ST9 and ST4 (see figure 5). Lanes 4 and 5 contain a fragment of 1350 nucleotides in length generated using the PCR primers ST10 and ST4 from the chromosome of *A. hydrophila* strain TF7, and plasmid pST100 respectively. The lambda size markers are shown on the left from the top in kb, 23.13, 9.42, 6.68, 4.36, 2.32, and 2.03.

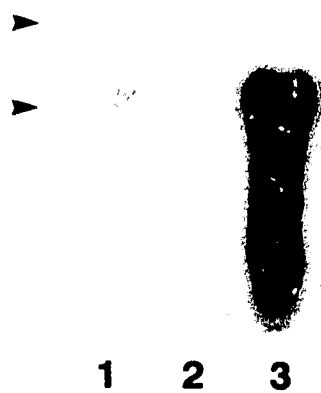


Figure 7. Northern blot analysis of the S-protein transcript from wild type *A. hydrophila* TF7 (lane 1), and from *E. coli* containing plasmid pST100 (lane 3) which contains the complete *ahsA* gene. *E. coli* strain DH5 α containing plasmid pUC18 with no insert is in lane 2, and does not transcribe any product that reacts to the *ahsA* gene probe. Approximately 20 μ g of total cellular RNA was added to each lane. Both transcripts appear to be the same size (approximately 1480 nucleotides), consistent with a monocistronic message transcribed from the *ahsA* gene. The high copy number of the pUC18 vector may be responsible for the quantitative difference of message seen in lane 3 compared to lane 1. Commercial RNA size markers are on the left from the top 2.4 kb and 1.4 kb.

was isolated, however, this proved difficult because we consistently had trouble in isolating large quantities of this plasmid DNA. This problem may have been due to a deleterious effect of the S-gene promoter on the copy number of the vector. Eventually the *SalI-KpnI* fragment from pST107 was sub-cloned into M13mp18 and mp19, and sequenced by the single stranded method.

The primary structure of the *ahsA* gene and S-protein sequence

Sequencing of the *ahsA* gene of *A. hydrophila* TF7, revealed an ORF of 1407 nucleotides encoding a protein consisting of 469 amino acid residues. Figure 8 shows the nucleotide sequence of the *ahsA* gene obtained following the sequencing of the complete region in both directions. Analysis of this region gives a G/C content of 49.61%. Labeled on figure 8, is the most likely ATG initiation codon, with a potential purine rich ribosome binding site the correct distance upstream. There are two regions internal to the *ahsA* gene capable of forming secondary structures due to their palindromic natures, at positions 520 to 541, and 1441 to 1458. Both of these sequences are capable of forming stem loop structures. Indeed, when the palindrome located at position 520 is read in the 3' to 5' direction, it is followed by a short poly-T sequence, which is typical of rho independent transcription terminators. The most probable rho-independent terminator for the *ahsA* gene is also indicated in figure 8, beginning at position 1513, with a stem of length of 8 base pairs, displaying a free energy of -19 kJ/mol. Codon usage for the *ahsA* gene is more typical of that used by the *A. salmonicida vapA* gene (56), rather than that used for other secreted proteins of *A. hydrophila*. Especially notable are the codons used for the amino acid residues Ser, Ala, Asn, Asp, and Gly. In each case the most abundant codon used was different to the codon used by the

Figure 8. Nucleotide sequence of the *ahsA* gene from *A. hydrophila* strain TF7. Partial amino acid sequences confirmed by Edman degradation are single underlined. The latter five underlined amino acid sequences were obtained from the isolation of tryptic fragments by reverse phase HPLC (see text and table 4). The putative Shine Dalgarno (SD) ribosomal binding site, and the terminator palindrome are double underlined. Also indicated are the translated amino acid signal sequence (dotted underlined) and signal peptidase cleavage site (▼). The two palindromes contained within the *ahsA* gene are dotted overlined. The AhsA amino acid sequence is also shown.

other *A. hydrophila* genes encoding secreted proteins, but the same as that used by the *vapA* gene. The complete codon usage for the *ahsA* gene is presented in Table 4, as well as the other genes sequenced in this study, and the *vapA* gene of *A. salmonicida*.

Edman degradation has been used previously to identify the N-terminal of the mature S-protein (72). We were therefore able to identify the cleavage site for the leader peptidase, which follows a typical sequence of three Ala residues. Based on the location of the N-terminal, the gene sequence predicts a signal peptide of 19 amino acid residues, with a 450 residue mature protein. The calculated molecular weight of the 450 amino acid mature protein is 45,400, with a pI of 6.72. As is usual for signal peptides, there is a basic region at the N-terminal position immediately followed by a hydrophobic internal sequence that consists of a high Ala/Leu content allowing for the formation of an α -helical configuration (240).

Several internal peptide sequences had been obtained previously, (figure 8) (72, 160), and were used for confirmation of the correct sequence of the *ahsA* gene. However, all of the previous internal peptide sequences were localized to the N-terminal portion of the S-protein and were contained within the trypsin resistant 38 kDa domain. In order to obtain more internal peptide sequences, we used trypsin cleavage of purified S-protein followed by isolation of the C-terminal fragments by reverse phase HPLC. Figure 9 shows the HPLC chromatogram following trypsin cleavage of the mature S-protein, and indicates the fractions isolated for sequencing. Table 5 contains the sequences obtained by this method, and these have also been indicated on the primary amino acid sequence (figure 8).

The Rost and Sander (252) algorithm was used to analyze the primary amino acid sequence of the S-protein for secondary structure content. S-

Table 4. Codon usage for the *A. hydrophila* *ahsA*, *spsD*, *aosA*, and *aosB* genes, and the *A. salmonicida* *vapA* gene.

Amino acid	Codon	<u>codon usage</u>					Amino acid	Codon	<u>codon usage</u>					
		<i>ahsA</i>	<i>spsD</i>	<i>aosA</i>	<i>aosB</i>	<i>vapA</i>			<i>ahsA</i>	<i>spsD</i>	<i>aosA</i>	<i>aosB</i>	<i>vapA</i>	
Phe	TTT	7	12	14	11	10	Ala	GCA	19	10	4	5	10	
	TTC	8	6	8	6	18		GCG	4	10	5	13	4	
Leu	TTA	0	6	2	1	1	Tyr	TAT	4	9	7	8	2	
	TTG	5	20	14	18	8		TAC	5	1	3	3	3	
	CTT	1	15	4	10	5	His	CAT	0	4	3	3	2	
	CTC	1	11	6	4	4		CAC	0	3	0	4	2	
	CTA	0	6	2	2	0	Gln	CAA	3	13	1	8	11	
	CTG	32	21	11	13	28		CAG	5	25	3	10	8	
Ile	ATT	6	10	9	19	11	Asn	AAT	12	28	4	9	14	
	ATC	14	23	7	9	15		AAC	18	13	4	3	25	
	ATA	0	5	2	3	0	Lys	AAA	20	12	3	10	8	
Met	ATG	6	21	12	10	3		AAG	17	21	3	10	19	
	Val	GTT	27	15	3	7	24	Asp	GAT	19	26	6	22	19
		GTC	3	9	6	11	3		GAC	9	13	1	4	11
		GTA	10	15	7	3	10	Glu	GAA	8	14	2	13	15
GTG	13	21	11	8	5	GAG	5		26	6	17	42		
Ser	TCT	10	5	3	5	11	Cys	TGT	0	2	2	5	0	
	TCC	10	6	2	4	8		TGC	0	0	0	4	0	
	TCA	1	7	4	4	0	Trp	TGG	0	5	9	4	4	
	TCG	0	10	1	2	0		Arg	CGT	5	19	7	10	10
	AGT	1	20	5	6	2	CGC		1	12	3	6	2	
	AGC	11	15	3	6	6	CGA		0	6	4	3	0	
Pro	CCT	3	11	4	4	4	CGG		0	0	2	2	1	
	CCC	1	12	1	5	0	AGA	0	0	0	3	0		
	CCA	4	5	4	4	1	AGG	0	1	0	3	1		
	CCG	3	16	5	4	8	Gly	GGT	35	14	3	17	35	
Thr	ACT	15	13	0	4	23		GGC	6	14	6	12	7	
	ACC	19	19	7	10	19		TGA	0	6	3	3	1	
	ACA	1	10	1	2	6		GGG	0	18	3	9	1	
	ACG	0	9	2	3	0								
Ala	GCT	48	14	3	5	42								
	GCC	14	22	12	12	8								

Figure 9. A chromatogram showing the isolation of tryptic fragments from a trypsin digest of purified *A. hydrophila* S-protein by reverse phase HPLC. Trypsin digests were diluted in buffer A (0.1% (v/v) TFA in HPLC quality water) at a ratio of 1:1, and TFA was added to 1.0% (v/v) making a total volume of 120 μ l. Samples were loaded onto a C-18 Ultrasphere column (2.0 mm X 25 cm) into 100% buffer A for 10 min, after which the percentage of buffer B (0.8% (v/v) TFA in 100% acetonitrile), was increased to 30% (v/v) over a period of 30 min. The ramp was decreased as the majority of peptides were eluted from the column, with the percentage of buffer B increasing from 30% to 50% over 40 min. The percentage of buffer B was increased to 100% over the following 30 min. Peptides were collected at A215 nm, (flow rate of 0.15 ml/min, chart speed = 0.2 cm/min, AUFS = 0.1). The absorbencies of buffer A and buffer B were equilibrated at A215 nm by titrating the TFA concentration in buffer B. The peaks isolated for Edman degradation are indicated (1 to 5, with peak 5 immediately following peak 4).

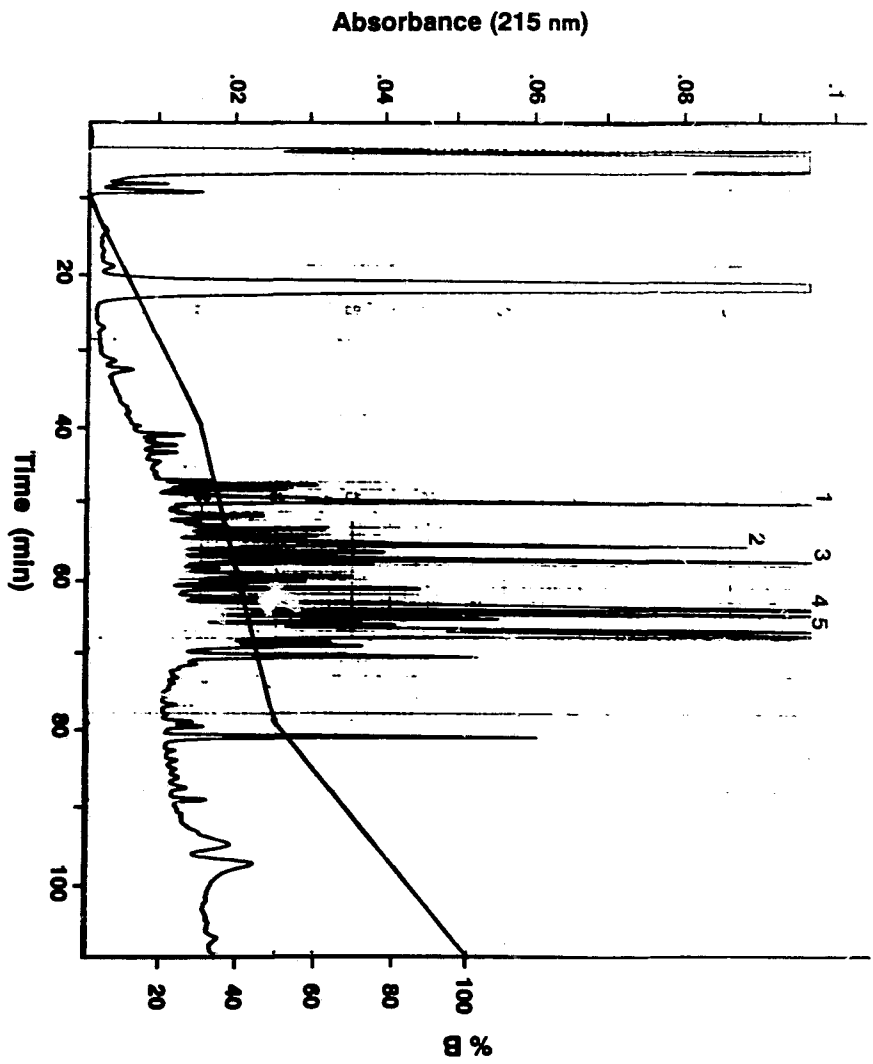


Table 5. Amino acid sequences obtained by Edman degradation of tryptic fragments of the *A. hydrophila* S-protein isolated by reverse phase HPLC

Peak number	Sequence
1	I S N T S
2	G L A L T G D
3	V A D I Y A A A
4	E A N L V L A P D
5	D G N A L N T M N

proteins typically contain a large amount of β -sheet, with the gene sequence predicting a content of 34% compared to 44% as determined by CD measurements in the far UV (72). Dooley *et al.* (72) found the measured α -helical content by CD at a pH of 7.4 to be 19%, while in the presence of 0.12% SDS, this dropped to 11%. Using Rost and Sander, the predicted α -structure content from the gene sequence is 23%.

Conservation of the S-protein gene

Prior to performing Southern blots to determine the conservation of the *ahsA* gene, all the strains of *A. hydrophila* and *A. veronii* biotype *sobria* used were checked for the expression of S-protein. Figure 10 shows that all strains tested did indeed express S-protein, with their measured Mr ranging from approximately 50,000 (lane 7) to 55,000 (lane 2). This variation in the apparent Mr of the S-proteins from the motile aeromonads is interesting in light of the fact that the S-layer proteins from *A. salmonicida* are antigenically conserved amongst a variety of strains from diverse backgrounds. Because Kostrzynska *et al.* (160) had shown that certain S-proteins produced by *A. hydrophila* and *A. veronii* biotype *sobria* strains do not react with antisera produced against the TF7 S-protein, we decided to see if the lack of conservation was due to variation at either the genetic level or at the protein level due to modification of the polypeptide. Southern blot analysis of the S-protein genes from a number of *A. hydrophila* and *A. veronii* biotype *sobria* strains (figure 11) showed that in some cases partial conservation occurs, while for other strains no conservation is seen. When probed under stringent hybridization conditions using an isolated nick translated radiolabelled PCR gene product from the *ahsA* gene, strains Ah 274, and Ah 423 showed no sequence similarity to TF7 (figure 11, lanes 2 and 3). *A. sobria* strains As 701

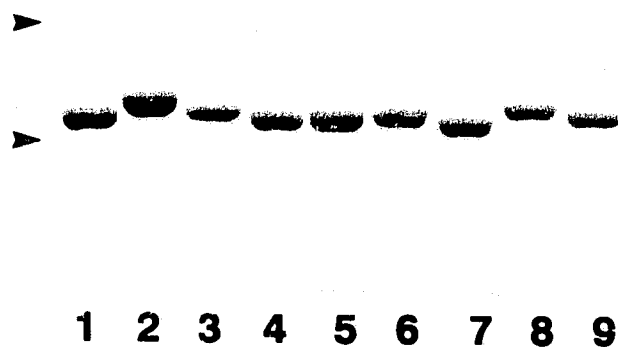


Figure 10. SDS-PAGE and Coomassie blue staining of S-proteins isolated by low pH glycine extraction from various *A. hydrophila* and *A. veronii* biotype *sobria* strains. Strongly staining bands representing the S-proteins can be seen for all strains tested.

Approximately 2 μ g of S-protein was loaded in each lane. Lane 1, TF7; lane 2, Ah 274; lane 3, Ah 423; lane 4, Ah 598; lane 5, Ah 77-115; lane 6, Ah 80-140; lane 7, Ah 80-160; lane 8, As 701; and lane 9, As 702. Molecular size markers are indicated by arrow heads, from the top, Mr 66,200, and 45,000.

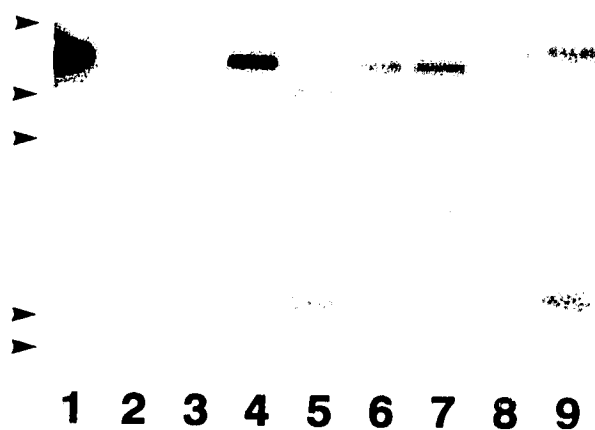


Figure 11. Southern blot analysis of *Sali*-*Bgl*II double digests of chromosomal DNA to determine the conservation of the *ahsA* gene in strains of *A. hydrophila* and *A. veronii* biotype *sobria*. The strains are in the same order as in figure 9: lane 1, TF7; lane 2, Ah 274; lane 3, Ah 423; lane 4, Ah 598; lane 5, Ah 77-115, lane 6, Ah 80-140; lane 7, Ah 80-160; lane 8, As 701; and lane 9, As 702. The probe used was a ^{32}P labeled nick translated PCR fragment generated using primers ST9 and ST4 (figure 5). On the left are the lambda standards in kilobases from the top, 23.13, 9.42, 6.68, 4.36, 2.32, and 2.03.

and As 702 showed some conservation, along with *A. hydrophila* strains Ah 598, Ah 77-115, Ah 80-140, and Ah 80-160 as indicated by the signal intensity. Second reacting bands can be seen in lanes 5, 8, and 9, possibly due to the presence of either *SalI* or *BglIII* sites within their respective S-genes.

Generation of the *A. hydrophila* S-protein negative strain TF7S

The generation of an S-protein negative strain of *A. hydrophila* was considered to be a pre-requisite to any studies regarding the synthesis and secretion of the polypeptide within its native environment. In order to isolate a marker exchange mutant unable to synthesize S-protein, we utilized the two *KpnI* sites within the *ahsA* gene (figure 5). This was accomplished by deleting the *KpnI* site within the polylinker of pUC18 using T4 DNA polymerase, followed by the sub-cloning of the *SalI-BamHI* fragment from pST100 into the *KpnI* deficient vector, generating pST100K. Plasmid pST100K was subsequently digested with *KpnI* removing a 676 base pair fragment of the *ahsA* gene, and isolated from an agarose gel giving pST106K (figure 12). The kanamycin resistance cassette from pUC4KISS (16) was also isolated from an agarose gel, and inserted into the *KpnI* site of pST106K. The resulting construct was excised and sub-cloned into the suicide vector pSUP205, leading to the isolation of pSTK106K.

Plasmid pSTK106K was then conjugated into *A. hydrophila* by the tri-parental mating method using the helper strain containing the plasmid pRK2013. Loss of the auto-aggregation phenotype by strains of *A. hydrophila* affected in their ability to maintain a complete S-layer on their cell surface is a characteristic that can be used for the isolation of S-protein deficient mutants. Therefore an enrichment procedure was used, where the conjugation mix was sub-cultured directly into static broth containing the required antibiotics,

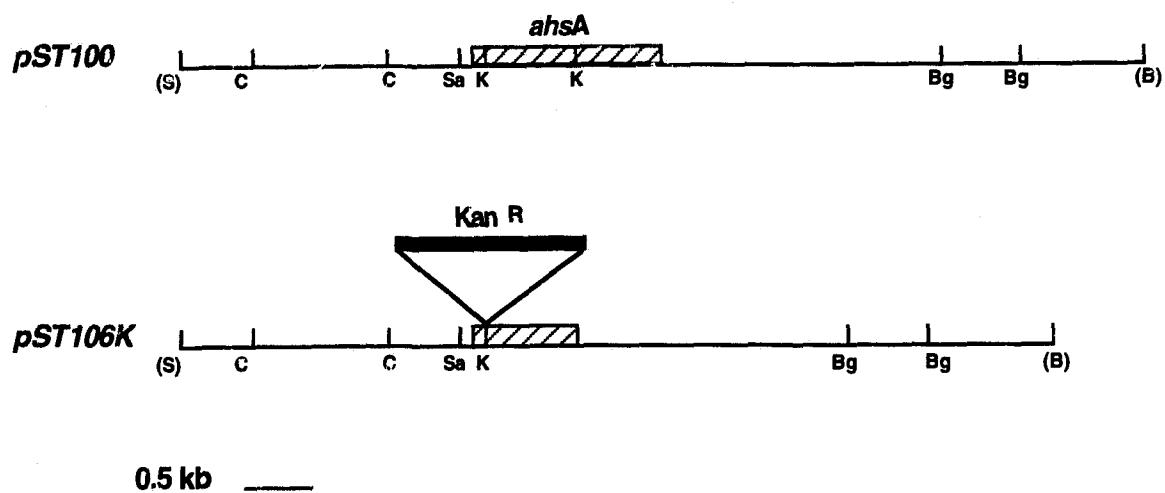


Figure 12. To generate the marker exchange mutant TF7S, unable to express S-protein, the *KpnI* fragment internal to the *ahsA* gene was excised from plasmid pST100 and the *Kan^R* marker was inserted in its place generating construct pST106K. The fragment containing the mutated *ahsA* gene was subcloned into the suicide vector pSUP205 on a *SalI*-*BamHI* fragment generating pSTK106K, and conjugated into *A. hydrophila* TF7.

and following growth for 24 h, the upper layer was sub-cultured further into static broth. The procedure was repeated twice. When the resulting culture was plated for isolation, all of the mutants tested were S-protein negative by Western immunoblotting (figure 13). The S-protein negative mutant TF7S showing a correct double cross over event resulting from the loss of the pSUP205 vector was isolated in this manner.

Conjugation of the *ahsA* gene into the S-layer negative mutants *A. hydrophila* TF7S and *A. salmonicida* A449-TM4

We wanted to study the expression and secretion of the S-layer protein in different genetic backgrounds. Especially interesting was whether the *A. salmonicida* Tn5 mutant A449-TM4 (A-protein negative) with the A-protein transport machinery still intact, was capable of secreting the *A. hydrophila* S-protein. The *ahsA* gene was conjugated into *A. hydrophila* TF7S generating TF7SS (figure 13), and into *A. salmonicida* A449-TM4 (20) producing strain A449-TM4S. Figure 14A shows the relative level of expression of recombinant S-protein in wild type *A. hydrophila* TF7, TF7SS, *E. coli* pST102 (see legend to figure 5), and *A. salmonicida* A449-TM4S. This was achieved by adjusting mid-exponential phase cultures to an OD₆₅₀ of 0.5, and loading 5 μ l onto an SDS-PAGE gel following solubilization in sample buffer. *E. coli* clearly expressed more S-protein than *A. hydrophila* TF7, whereas the levels expressed in *A. hydrophila* TF7SS and A449-TM4S appeared to be similar to native *A. hydrophila* (figure 14A).

Expression of the AhsA protein in S-protein negative *A. hydrophila* (TF7SS), *A. salmonicida* (A449-TM4S), and *E. coli* pST102

The expression and location of the *A. hydrophila* S-protein was studied

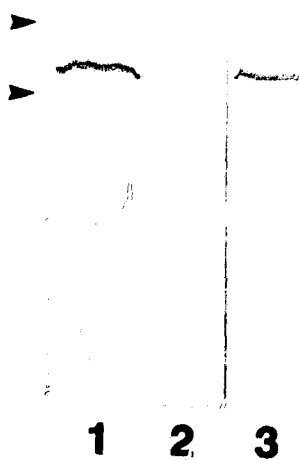


Figure 13. Western blot of a SDS-PAGE showing the expression of S-protein from the wild type *A. hydrophila* TF7 (lane 1); loss of expression of S-protein from the marker exchange mutant TF7S generated by mutant allele replacement (lane 2); and *A. hydrophila* strain TF7SS, which shows the S-protein expressed in the *ahsA* gene on a plasmid in the marker exchange mutant TF7S (lane 3). Arrow heads on the left indicate the molecular size markers Mr 66,200, and 45,000 from the top.

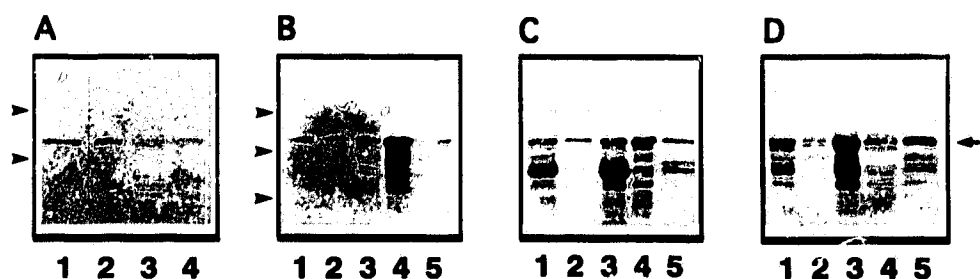


Figure 14. Western blot analysis of whole cell lysates (A) from strains TF7 (lane 1), TF7SS (lane 2), pST102 (lane 3), and A449-TM4S (lane 4) showing the amount of S-protein expressed in each background, and cell fractionation studies in *A. hydrophila* TF7SS (B); *E. coli* pST102 (C); and *A. salmonicida* A449-TM4S (D). Lane 1, cytoplasm; lane 2, cytoplasmic membrane; lane 3, periplasm; lane 4, outer membrane; and lane 5, culture supernatant. The whole cell lysates were separated on a 10% SDS-PAGE gel, and all other samples were separated on a 12.5 % SDS-PAGE gel. Indicated on the left are the positions of the molecular weight markers from the top, 66,200, 45,000, and 31,000. On the right the position of the S-protein is indicated by an arrow.

in these various host backgrounds using Western blot analysis. In the case of *A. hydrophila* TF7SS, S-protein was detected in the cytoplasmic, the inner membrane, periplasmic fraction, and the outer membrane (figure 14B). However, most of the S-protein co-fractionated with the outer membrane fraction, with only small amounts being found in the rest of the cell compartments. In the case of *E. coli*, and *A. salmonicida* the majority of the S-protein was found in the periplasmic shock fraction, although in these bacteria it was clearly suffering from significant proteolytic degradation (figure 14C, and 14D). Using GHI extraction of outer membranes, we determined that the S-protein present in this fraction in both *E. coli* and *A. salmonicida* was intrinsically associated with the membrane, and not simply co-sedimenting with the cell envelopes. Although S-protein could be found in the culture supernatants of *E. coli* pST100 and *A. salmonicida* A449-TM4S, this was likely due to leakage as opposed to specific secretion of the polypeptide. Immunolabeling with protein A gold conjugate to determine whether any S-protein was located on the surface of *E. coli* cells carrying pST100 or *A. salmonicida* A449-TM4S proved negative when examined by electron microscopy. However, when the outer membranes of TF7SS were studied by electron microscopy, the presence of complete sheets of S-layer were apparent (not shown), indicating that S-protein expressed from a plasmid in *A. hydrophila* was both secreted in large amounts and assembled into a macromolecular array.

II. Identification and Characterization of a Post Translational Modification of the *A. hydrophila* S-protein

The nucleotide sequence of the *ahsA* gene revealed a 1407 base pair open reading frame encoding for a protein consisting of a 19 amino acid signal peptide, and a 450 residue 45.4 kDa mature protein. This was smaller than the measured 52.0 kDa for the processed mature protein. The predicted pI of the AhsA protein was 6.72, which was also in contrast to the measured pI of 4.6. These differences between the measured and the predicted Mr and pI values suggested that the S-protein may be post-translationally modified. To analyze for the presence of a post translational modification, a large amount of pure S-protein was required. For the purification of the S-protein in quantity, we isolated a Tn5 insertion mutant that displayed a large reduction in the amount of LPS O-polysaccharide side chains on its surface, but at the same time maintained a full S-layer. This mutant allowed for ready isolation of S-layer protein because it released sheets of the paracrystalline array with very little contamination from the LPS fraction.

Isolation of Tn5 insertion mutant TF7-ST3, and rapid purification of the *A. hydrophila* S-protein

Due to the finding that a decrease in the amount of S-layer on the surface of *A. hydrophila* cells leads to a loss of the ability to auto-aggregate, we decided to use this characteristic to identify Tn5 insertion mutants showing alterations in this phenotype. This was achieved by inoculating the Tn5 insertion pool in static broth at 37°C, allowing growth to proceed until there was a pronounced pellet of bacterial cells at the bottom of the tube, and then removing a small aliquot from the top phase for further sub-culturing. This method enriched for non auto-aggregating mutants. Following three sub-

cultures, a small amount of the supernatant was removed, serially diluted, and plated for isolation of bacteria. Fifty colonies isolated in this manner were sub-cultured into separate tubes containing LB broth, and grown under static conditions. Any mutants showing loss of the auto-aggregating phenotype were studied further. The mutant TF7-ST3 which showed an intermediate auto-aggregating phenotype was isolated, and found to be affected in its ability to express normal amounts of O-polysaccharide side chains on its surface (mutant TF7-ST3 is further characterized in chapter 6).

Figure 15 shows the result of a rapid purification protocol for S-protein. Briefly this involved the shearing of flagella and fimbriae from the surface of *A. hydrophila* mutant TF7-ST3 by vigorous pipetting, followed by glycine extraction of S-layer from the cell surface. The low pH extract containing the S-layer protein was then subjected to centrifugation at 100,000 X G to remove contaminating outer membrane particles. Lane 1 contains a Coomassie blue stain of the glycine extracted S-protein contained in the supernatant following centrifugation, and shows the presence of a single band at approximately 52.0 kDa, typical of the wild type S-protein. Following Proteinase K digestion to remove the S-protein, both the supernatant and the pellet were analyzed by silver staining, and lane 2 shows the absence of any contaminating LPS in this fraction. In contrast, a large amount of LPS was seen when the pellet was analyzed (lane 3), indicating the removal of the LPS from the low pH extracted S-protein sample during the centrifugation.

In vivo* cell labeling of *A. hydrophila

The phosphorylation of proteins has been shown previously to be responsible for both an increase in molecular weight, and a decrease in isoelectric point (267). Following the growth of *A. hydrophila* cells in the

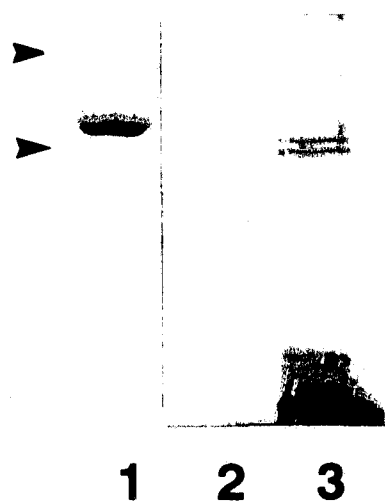


Figure 15. Coomassie blue (lane 1) and silver stain (lanes 2 and 3) of SDS-PAGE showing the results obtained during the purification of S-layer protein from Tn5 insertion mutant TF7-ST3. Lane 1 shows the S-protein in the glycine extract following the high speed centrifugation (100,000 X G for 1 h). Lanes 2 and 3 are silver stains of the supernatant and pellet respectively to detect LPS contamination following the removal of the S-protein by treatment with Proteinase K. Arrows at left indicate (top to bottom) Mr 66,200, and 45,000.

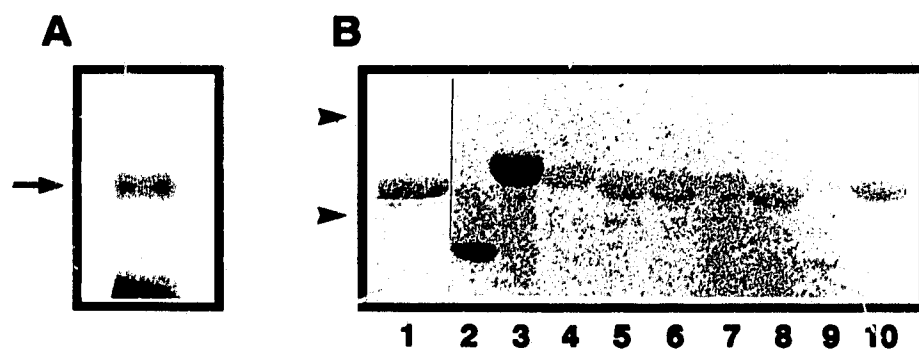


Figure 16. *In vivo* ^{32}P labeled S-protein (A), and an immunoblot of various S-protein producing strains of *A. hydrophila* and *A. veronii* biotype *sobria* using an anti phospho-tyrosine monoclonal antibody at a dilution of 1:500 (B). Lane 1, TF7; lane 2, trypsin treated TF7 S-protein; lane 3, Ah 274; lane 4, Ah 423; lane 5, Ah 598; lane 6, Ah 77-115, lane 7, Ah 80-140; lane 8, Ah 80-160; lane 9, As 701; lane 10, As 702. Molecular weight markers are indicated on the left of B, from the top Mr 66,200, and 45,000, and the position of the S-protein is shown to the left of A.

presence of limiting amounts of ^{32}P -orthophosphate, a discrete band was seen in autoradiograms of an SDS-PAGE gel containing low pH extracted S-protein (figure 16A). This radiolabeled band migrated at the identical position as the S-protein when compared to Coomassie blue stained SDS-PAGE gels, showing that $^{32}\text{PO}_4$ had been incorporated into the polypeptide by in vivo cell labeling. Phosphoproteins can also be distinguished from non-phosphorylated proteins using the carbocyanine dye "Stains-all," and we were able to successfully show a positive phosphoprotein reaction with the *A. hydrophila* S-protein (results not shown) (103).

Immunoblotting with monoclonal anti phosphotyrosine antibodies

The high specificity of monoclonal antibodies has been used successfully in the characterization of a number of phosphoproteins. A monoclonal antibody specific for phosphotyrosine residues is commercially available, and although it has on occasion failed to identify phosphorylated tyrosine residues, it rarely recognizes false positives (166). Approximately 5 μg of low pH extracted S-protein from a variety of *A. hydrophila* and *A. veronii* biotype *sobria* strains was separated by SDS-PAGE and analyzed by Western immunoblot using the anti-phosphotyrosine monoclonal antibody. Figure 16B shows the results obtained, with the protein from strains TF7 (both native and the trypsin digested product), Ah274, and As702 giving strong positive reactions, and strain As701 showing only a weak signal. No reaction was obtained when BSA was probed with this monoclonal antibody, confirming the specificity of the reaction.

The phosphorylation of bacterial proteins at tyrosine residues is considered to be a rare event. We were therefore interested in determining whether these residues might be exposed on the cell surface following

assembly of the S-protein. However, when *A. hydrophila* cells were immuno-gold labeled with anti-phosphotyrosine antibodies and examined by electron microscopy, no surface labeling was observed.

Acid phosphatase treatment of the S-protein

Alkaline and acid phosphatase, and lambda phosphatase studies were initiated to attempt to remove the phosphate groups and reduce the apparent molecular weight of the S-protein. Figure 17 (lanes 3 - 5) shows the result obtained using acid phosphatase during a timed digest ranging from 5 to 15 minutes. As can be seen, the mature S-protein could be reduced in apparent Mr to approximately 45,000 (lanes 1 and 2 contain the intact S-protein, and the 38.0 kD trypsin digested product controls respectively). Reaction parameters were varied to try and obtain more complete removal of the phosphate groups, including a rapid shift from a low pH (< 1.0) to pH 5.0 with varying concentrations of substrate and enzyme. However, we could not reduce the complete S-protein sample to its unphosphorylated form, possibly due to the fact that native S-protein is known to aggregate strongly in solution (72). Either re-aggregation as the reaction conditions are met occurs at an extremely rapid rate, and mostly precludes binding of the substrate by the phosphatase, or alternatively, the S-protein may already be in various stages of assembly prior to the addition of the enzyme. When the reactions were allowed to continue for extended periods of time at 37°C, some degradation became evident, as can be seen in figure 17 lanes 4 and 5. Samples containing S-protein but no phosphatase also showed a limited amount of degradation at 37°C (data not shown).

Presumably, for the same reason that complete removal of the phosphate group using the acid phosphatase was not possible, both lambda

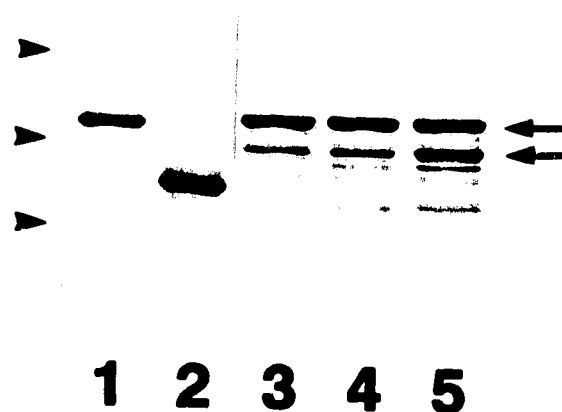


Figure 17. Western blot of SDS-PAGE of acid phosphatase treated purified S-protein using antisera JD3. Lane 1 contains the native untreated S-protein at Mr 52,000, and lane 2 contains the N-terminal Mr 38,000 polypeptide following trypsin treatment. Acid phosphatase treated samples were digested for 5 min (lane 3), 10 min (lane 4), or 15 min (lane 5) and the reaction stopped by adding SDS-PAGE sample buffer and heating to 92°C. No increase in the intensity of the 45 kD band (lower arrow on the right) was noticed after 15 min. The reaction contained approximately 20 μ g of S-protein, and the acid phosphatase was added to a final concentration of 1 mg/ml at a pH of 5.0. Molecular weight markers are shown on the left, at 66,200, 45,000, and 31,000, from the top, and on the right the position of the S-protein is indicated.

phosphatase and alkaline phosphatase were incapable of reducing the S-protein in size. Again, this may be due to exclusion of the substrate from the active site of the enzyme because of assembly or aggregation of the S-protein. Sherman and Goldberg (267) also experienced difficulty in removing all of the phosphate from GroEL, possibly because this protein is known to form a multimer in solution.

Ascending thin layer chromatography

The tyrosine phosphomonomer bond is more labile to acid hydrolysis compared to the bond in phosphoserine and phosphothreonine (190). Therefore, partial acid hydrolysis rather than complete acid hydrolysis is the method of choice for analyzing phosphorylated tyrosine residues. To examine for the presence of phosphotyrosine, purified S-protein was subjected to partial acid hydrolysis in 6 N HCl and immediately separated by ascending chromatography on thin layer cellulose plates. Following separation of the hydrolysates, a specific spot that migrated at the same position as the P-Tyr standard was identified (figure 18). The study was repeated using the same ethyl acetate/formic acid/water buffer but at different pH's (unadjusted, and adjusted to a pH of 1.6) to ensure that we were not detecting an acid resistant polypeptide. However, the same result was obtained in each case.

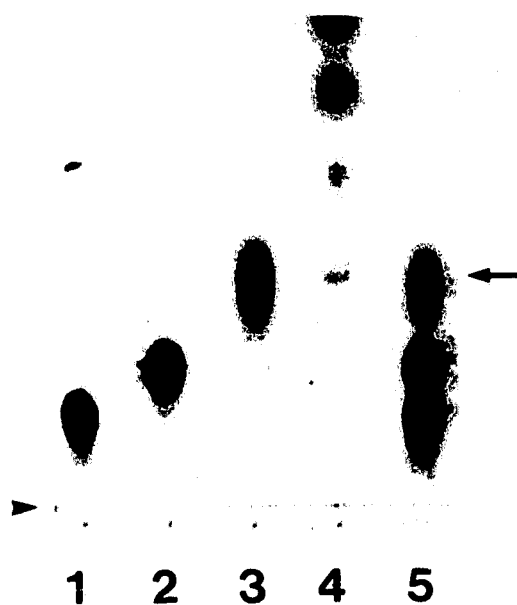


Figure 18. Ascending thin layer chromatogram of partial acid hydrolyzed purified *A. hydrophila* TF7 S-protein. Samples were separated on cellulose plates in a ethyl acetate/ formic acid/water (70:20:10 (v/v)) (pH 1.6) system and visualized using a spray of 0.3% (v/v) ninhydrin in 3% (v/v) acetic acid, 95% (v/v) ethanol. The phosphorylated amino acids phosphoserine, phosphothreonine, and phosphotyrosine were used as standards. Lane 1, P-Ser; lane 2, P-Thr; lane 3, P-Tyr; lane 4, acid hydrolyzed S-protein; lane 5, mixture of the three phosphorylated standards. The point of origin is marked on the left (►), and the position of P-Tyr is shown on the right. Further acid hydrolysis products can be seen in lane 4 that migrated faster than P-Tyr, and may represent other amino acid residues

III. Roles of Structural Domains in the Morphology and Surface Anchoring of the S-Layer, and Biochemical Characterization of the Major Structural Domain

Three-dimensional reconstructions of the *A. hydrophila* and *A. salmonicida* S-layers have shown the presence of a major tetragon at one four fold axis of symmetry, and a minor tetragon at the other four fold axis of symmetry (3, 73, 211, 280). Protease digestion studies have further provided evidence for two structural domains, e.g., treatment with trypsin gave a major peptide of Mr 38,000 for the *A. hydrophila* S-protein, and a major peptide of Mr 39,439 for the *A. salmonicida* S-protein (56, 160). In this study, a Tn5 insertion mutant (TF7-ST1) was isolated which expressed a truncated S-protein monomer of approximate Mr 38,000, allowing for the biochemical and structural characterization of a major fragment of the *A. hydrophila* S-protein.

Isolation of a Tn5 insertion mutant expressing a truncated S-protein monomer

The Tn5 insertion mutant TF7-ST1 was isolated by placing a 1 ml aliquot of an overnight culture of the Tn5 insertion pool into a microcentrifuge tube, and adding polyclonal anti S-protein antisera to a final dilution of 1:100. This had the effect of immunoprecipitating S-layer positive cells which were removed by a brief centrifugation in a bench top centrifuge, and at the same time enriching for cells deficient in surface located S-layer. Following two rounds of immunoprecipitation, isolated colonies were obtained from plates of the supernatant fraction, and screened for loss of the S-layer by colony lift and Western blotting techniques. Mutant TF7-ST1 was obtained using this method, and figure 19 shows a Western blot of the wild

type S-protein from *A. hydrophila* together with the truncated protein expressed by mutant TF7-ST1 (lane 2). Lane 3 shows the trypsin resistant core of Mr 38,000 obtained by digestion of the native S-protein. This resistant core is similar in size to the truncated polypeptide expressed by mutant TF7-ST1.

Cell fractionation studies showing the location of the truncated protein relative to the native protein in *A. hydrophila*

The expression of a truncated S-protein by mutant TF7-ST1 provided an opportunity to study the effect that such an abbreviation might have on the normal secretion of the S-protein. Fractions of cells grown in liquid media, as well as the culture supernatant, were analyzed by Western immunoblotting. In contrast to wild-type Mr 52,000 S-protein, which was only detected in the outer membrane fraction (figure 20A), the Mr 38,000 immunoreactive truncated polypeptide was detected in the periplasmic and cytosol fractions, and a quantity was also secreted into the culture medium (figure 20B). The periplasmic and cytosolic fractions also contained small amounts of another immunoreactive protein of slightly higher apparent Mr which likely represented a precursor form of the protein produced during the truncation and processing of the S-protein. When cells were grown on solid media, gentle washing in TBS removed large amounts of the truncated polypeptide, indicating that this protein was not anchored to the cell surface. To confirm that the failure of the truncated S-protein to anchor and assemble on the cell surface was not due to an altered LPS structure, SDS-PAGE analysis of this fraction was performed, and silver staining showed that the LPS of TF7-ST1 was indistinguishable from wild-type smooth LPS (see chapter 6).

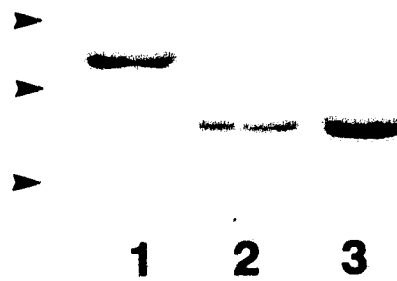


Figure 19. SDS-PAGE comparison of Coomassie blue stained truncated S-protein expressed by *A. hydrophila* Tn5 insertion mutant TF7-ST1 (lane 3) with the N-terminal peptide obtained by trypsin cleavage of purified wild type S-protein (lane 2). The native Mr 52,000 S-protein is in lane 1. Arrows at left indicate (top to bottom) Mr 66,200, 45,000, and 31,000.

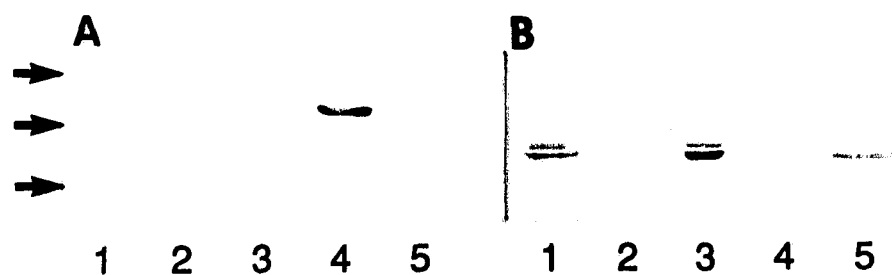


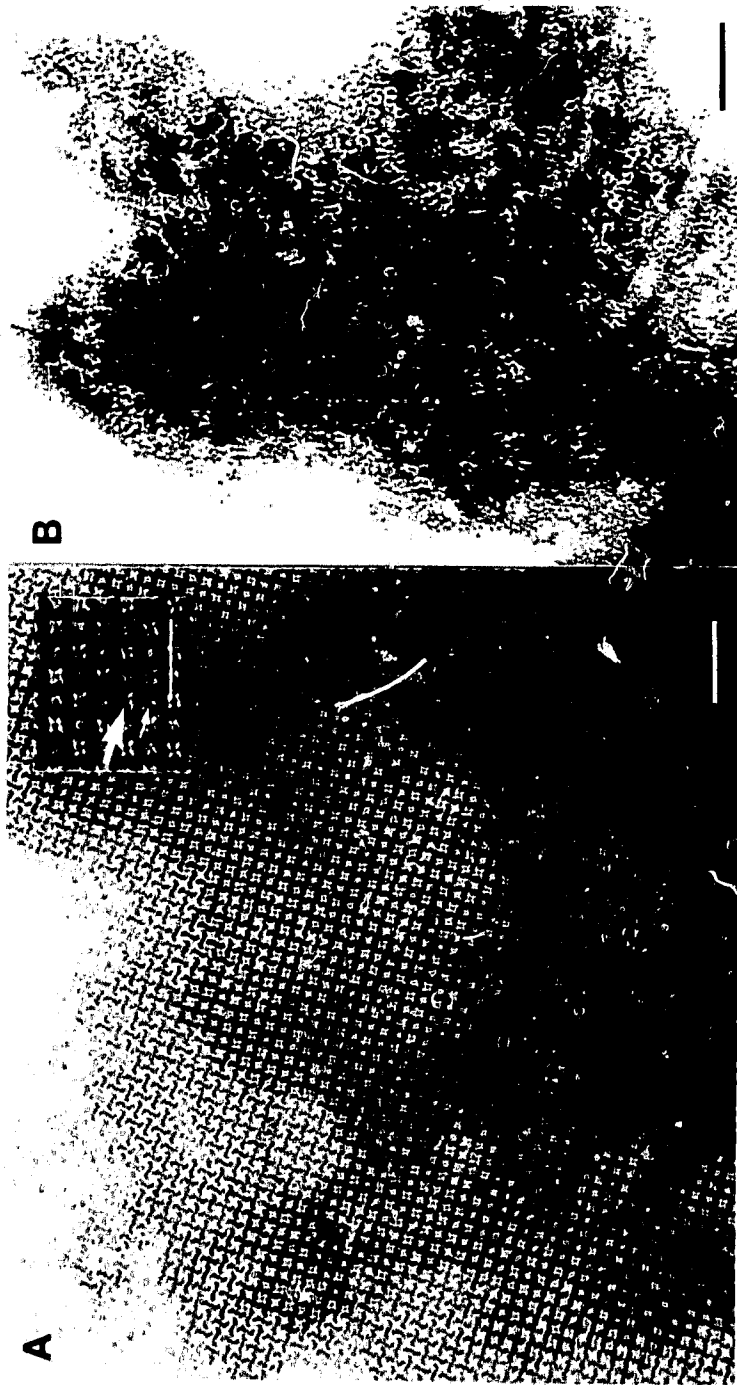
Figure 20. Western immunoblot using antisera JD3 to show the sub-cellular location of the wild type S-protein from *A. hydrophila* TF7 (A), compared to the truncated polypeptide expressed by mutant TF7-ST1 (B). Lane 1, cytosol; lane 2, cytoplasmic membrane; lane 3, periplasmic fraction; lane 4, outer membrane fraction; lane 5, culture supernatant. Arrows at left indicate (top to bottom) Mr 66,200, 45,000, and 31,000.

Morphology of aggregates formed by the truncated S-protein compared to that of the wild-type protein

Figure 21A shows an electron micrograph of negatively stained S-layer sheets released from the surface of *A. hydrophila* Tn5 mutant TF7-ST3, displaying the characteristic tetragonal morphology. Electron microscopy of culture supernatants of mutant TF7-ST1 showed that the Mr 38,000 polypeptide produced assemblies which had a totally different morphology from the S-layer sheets obtained from TF7-ST3. The secreted truncated protein did not assemble into a tetragonal array either on the cell surface or in the culture supernatant. Rather the Mr 38,000 subunits formed ring-like macromolecular assemblies in vivo after natural secretion from the bacterial cells, and in vitro after purification by low pH denaturation and gel filtration. Figure 21B shows a large number of typical ring-like assemblies that could be found in the TBS washes of cells grown on solid media.

Other than the ring-like structure, another predominant morphology that could be formed by the truncated polypeptide, was an assembly with two parallel lines separated by a stain filled center (figure 22). By the measurement of the external diameter of the ring structure and the stain filled center (6.5 nm and 3 nm respectively), it was determined that the ring represents a view down the 4 fold axis of the major S-layer tetramer. The structure formed by the two 12 nm parallel lines joined at one end and separated by a stain filled central line was 6.5 nm across and probably represents the major S-layer tetramer lying on its side. The majority of assemblies found were of four major tetramers lying on their sides, connected by their bases. Other aggregates formed by the truncated S-protein, included the association of two or three major tetramers, connected by their bases, presumably via hydrophobic interactions (figure 22).

Figure 21. Electron micrographs of negatively stained (0.07% ammonium molybdate) native tetragonally arranged wild type S-layer of *A. hydrophila* TF7 (Bar = 50 nm) (A), compared to the structures formed by the secreted truncated S-layer from mutant TF7-ST1 (Bar = 60 nm) (B). Inset in (A) shows a higher magnification of the native S-layer with the large arrow indicating a major tetramer, and the small arrow pointing to a minor tetramer (Bar = 30 nm). A flagellar filament can be seen in panel B.



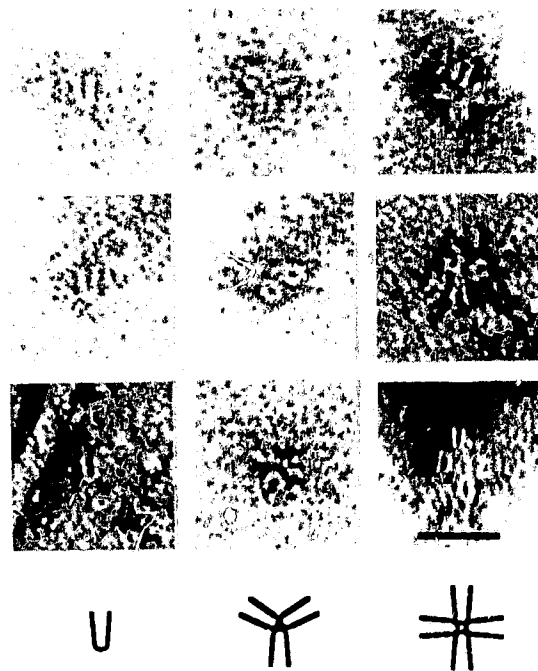


Figure 22. Electron micrographs of negatively stained structures formed by the truncated S-layer protein in the culture supernatant. The panels on the left and center indicate side views of single major tetramers and triple major tetramers respectively, which were most common. The panel on the right indicates side views of four major tetramers which were rare. Very few views of double major tetramers were seen (not shown). Some of the major tetramers are lying flat on their sides, showing an elongated cup joined at their base. Other major tetramers are arranged vertically with respect to the electron beam resulting in a ring shaped structure. Bar = 30 nm.

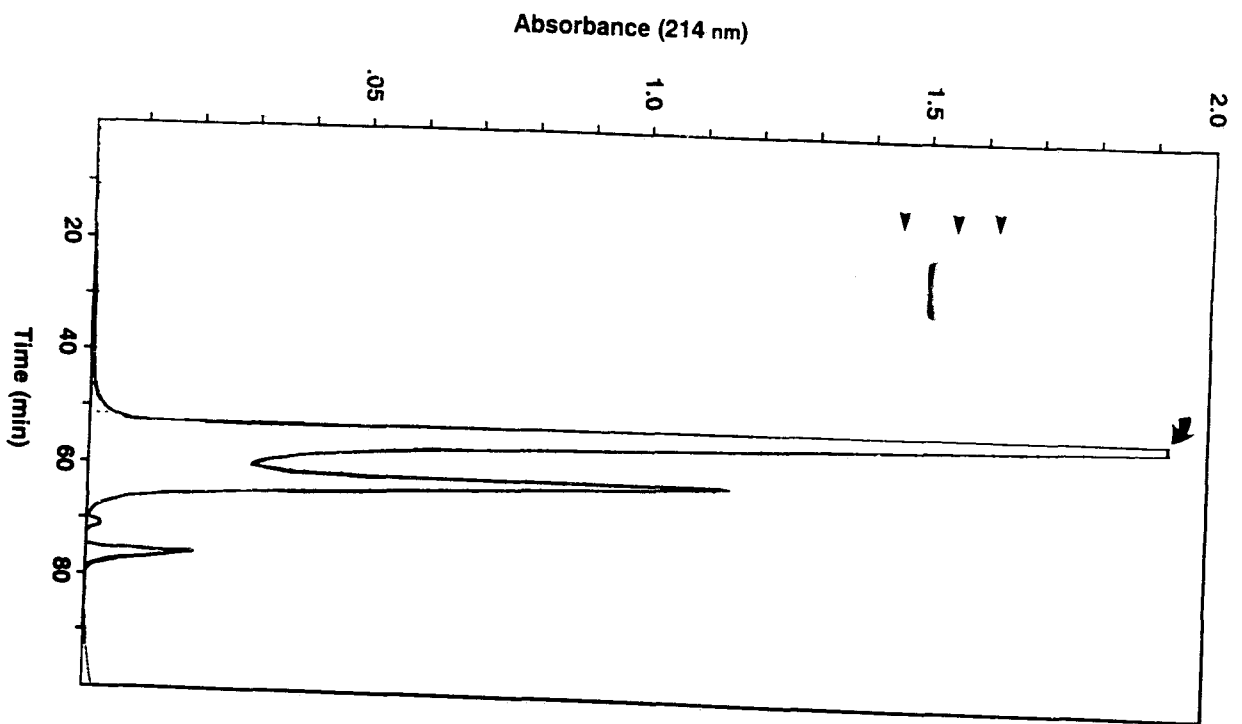
Purification of the truncated S-protein by FPLC

Truncated S-protein was enriched by using the low pH extraction method on LBA grown mutant cells. After concentration by ultrafiltration, the extract was applied to a Superose 12 FPLC column in 100 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl. The Mr 38,000 polypeptide was in the major peak eluted from the column (figure 23). Following concentration of the fractions containing the S-protein using a Microsep centrifugal microconcentrator, SDS-PAGE analysis showed the truncated polypeptide to be the only observable protein (figure 23). LPS silver staining determined that protein isolated in this manner was not contaminated by fragments of the outer membrane.

Characterization of the truncated protein

With a good source of purified truncated S-protein in quantity, it was possible to perform a number of studies that were important for a thorough analysis of this polypeptide. Amino terminal sequencing by Edman degradation (179) of the protein after SDS-PAGE separation and electroblotting onto Problot membrane localized the Mr 38,000 fragment to the N-terminus. The first 20 amino acids were found to be identical to those previously found for the mature S-protein (160). SDS-PAGE comparison of the 38 kDa truncated polypeptide with the N-terminal 38 kDa peptide produced by TPCK trypsin treatment showed no apparent difference in electrophoretic mobility between the two polypeptides (figure 19). The morphological analysis described earlier suggested that the region of polypeptide present in the wild-type S-protein but absent from the truncated polypeptide was responsible for the tetragonal array formation and morphology, and also participated in the anchoring of the array to the cell

Figure 23. FPLC chromatogram of purification of the truncated protein from mutant TF7-ST1 using a Superose 12 column. Samples were eluted in 100 mM Tris-HCl (pH 7.4) containing 100 mM NaCl at a flow rate of 0.5 ml/min. The eluted protein was monitored at 214 nm, collected, pooled, and dialyzed against 20 mM Tris-HCl (pH 7.5). The SDS-PAGE Coomassie blue stain of the major peak (indicated by an arrow) is shown on the left, and represents the Mr 38,000 truncated S-protein. Arrows at left indicate (top to bottom) Mr 66,200, 45,000, and 31,000.



surface. We could therefore assign these functions to the C-terminal portion of the S-protein.

Kostrzynska *et al.* have shown that virtually all the reactivity of the *A. hydrophila* S-protein from strain TF7 to polyclonal antisera resides in the Mr 38,000 fragment (160). Using an inhibition ELISA with anti TF7 S-protein antiserum, we were able to show that the majority of the epitopic reactivity of the wild-type S-protein is carried on the truncated polypeptide of TF7-ST1 (figure 24).

When comparing the measured amino acid composition of the truncated S-protein to the TF7 wild-type protein, it was found that the content of hydrophobic residues was higher for the abbreviated protein, and the content of basic residues was decreased (Table 6). The truncated S-protein contained 387 amino acid residues/molecule compared to the intact TF7 protein, which contained 526 amino acid residues/molecule. With the availability of the *ahsA* gene sequence, we were able to compare the amino acid composition between the measured and the predicted values for both the full size S-protein, and the truncated Mr 38,600 polypeptide. Table 6 shows the predicted amino acid composition for the S-protein from the *ahsA* gene sequence, containing 450 residues/molecule with a 46% content of hydrophobic amino acids. The predicted hydrophobic amino acid content for the truncated S-protein was found to be 48%, an increase of 2% compared to the full size protein, and the content of basic residues was decreased relative to the wild type protein from 12.0% to 11.2%.

Molecular weight determination of the truncated S-protein

Attempts to determine the solution molecular weight by sedimentation analysis had previously been made for the wild-type S-protein

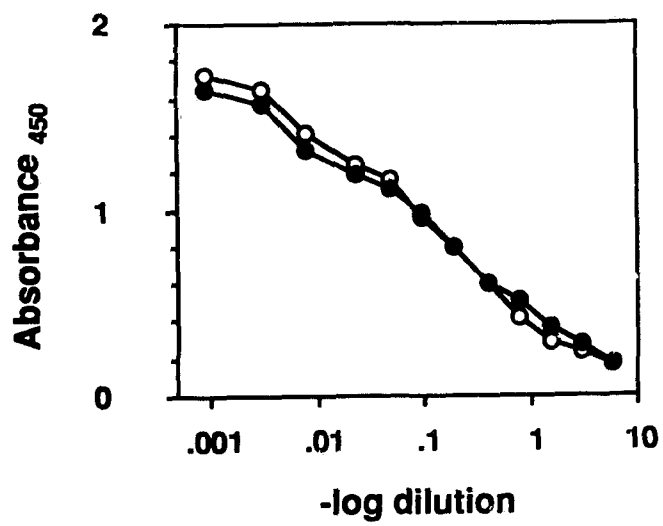


Figure 24. Inhibition ELISA of purified native *A. hydrophila* TF7 S-protein (●) and truncated S-protein produced by Tn5 insertion mutant TF7-ST1 (○).

Table 6. Comparison of the measured amino acid composition of the wild type S-layer proteins of *A. hydrophila* TF7 and the 38 kDa truncated S-protein of mutant TF7-ST1, to the predicted amino acid composition from the *ahsA* gene sequence.

Amino acid	52 kDa wild type S-protein (mol %)		38 kDa truncated S-protein (mol %)	
	Measured(a)	Predicted	Measured(b)	Predicted
Asx	12.2	14.4	12.9	14.7
Thr	8.0	7.6	6.7	7.8
Ser	6.7	6.1	5.4	6.3
Glx	6.1	6.0	5.2	4.9
Pro	2.3	2.4	1.6	2.8
Gly	12.4	5.0	10.6	5.1
Ala	16.5	12.4	19.8	12.4
Val	9.3	11.3	12.7	12.3
Met	1.0	1.4	0.8	1.0
Ile	3.0	4.7	4.1	5.2
Leu	8.6	9.0	8.8	8.2
Tyr	2.1	3.2	1.3	2.9
Phe	3.4	4.5	2.8	4.9
His	0.4	0.0	0.3	0.0
Lys	6.6	9.9	5.9	9.2
Arg	1.5	2.1	1.0	2.0
Trp	ND	0.0	ND	0.0
Cys	0.0	0.0	0.0	0.0
Total # residues	526	450	387	387
Mr (X 1000)	52(c)	45.4	38.6(d)	38.7
Hydrophobic residues (%) (V, M, I, L, A, F, W, and P)	44.1	46.0	51.0	48.0
Basic residues	8.7	12.0	7.1	11.2
Acidic residues	ND	10.8	ND	10.7
pI	4.6	6.72	4.6	5.22

(a) Mean of 7 determinations

(b) Mean of 9 determinations

(c) Estimated by SDS-PAGE

(d) Estimated by analytical ultracentrifugation

ND Not determined

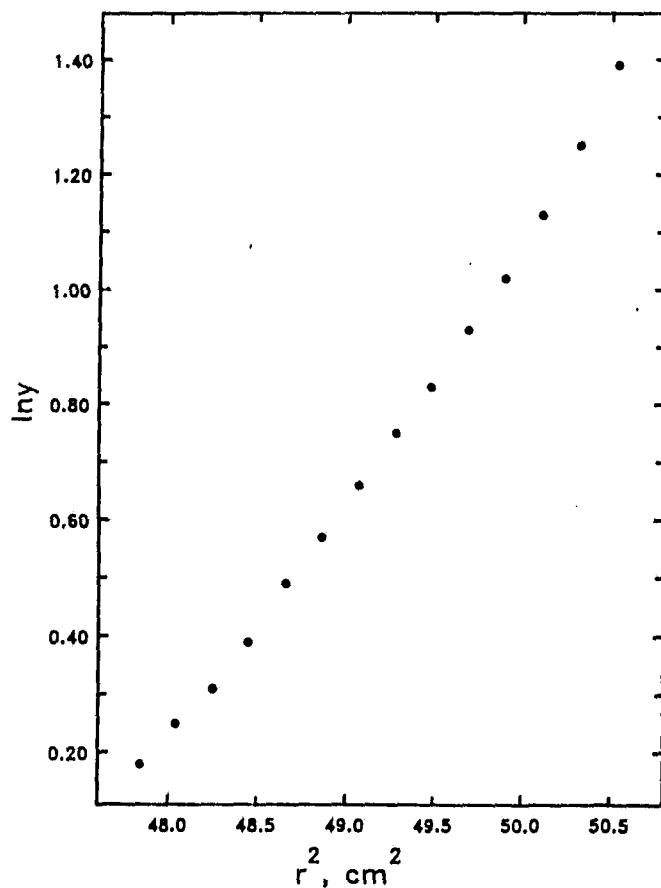


Figure 25. Sedimentation analysis of the truncated S-protein from mutant TF7-ST1. Plot of $\ln Y$ (estimate of protein concentration in absorbance units) vs r^2 (the distance from the center of rotation squared). Solvent was 100 mM-NaCl, 50 mM-Hepes, (pH 7.4). Temperature 20°C, speed 16,000 revs/min. Initial protein concentration was 1.33 mg/ml.

at both pH 2, and pH 7.4. however due to aggregation problems, these efforts were unsuccessful (72). Sedimentation analysis was also attempted for the truncated S-protein, and although no result was achieved at pH 2.1 due to aggregation of the monomer, a satisfactory trace was obtained on the absorption scanner at the higher pH of 7.4. A plot of $\ln y$ versus r^2 (figure 25) showed negative deviation from linearity. Weight average molecular weights ranged from 20,170 near the meniscus, to 38,650 at the cell bottom. All protein samples used in the sedimentation analyses were analyzed by SDS-PAGE prior to the run, and little degradation was found. Therefore, the conditions of the study (40 h at 20°C) were likely responsible for the generation of the range of products found at the conclusion of the sedimentation equilibrium experiment.

Secondary structure of the truncated S-layer protein

CD measurements were made to estimate secondary structure in the purified truncated S-protein. Similar studies had been performed previously on the wild-type S-protein, and so comparisons could be made between the two systems (72). These spectra are presented in figure 26A, and are summarized in table 6. The wild-type protein showed approximately 19% α -helix at either pH 7.4 or pH 2.0. This compares with approximately 10% α -helix at pH 7.4, and 21% at pH 2.1 for the truncated protein. The truncated protein had a large amount of β -sheet, approximately 42%, which did not vary between the two pH values tested. At a pH of 7.4, the β -turn content of the 38,600 protein was higher at approximately 18%, than at the lower pH of 2.1, which gave a value of 6%. In the earlier work performed on the intact protein, the effect of SDS was studied on the protein structure, both at pH 2.1 and 7.4. These conditions were chosen to imitate a hydrophobic

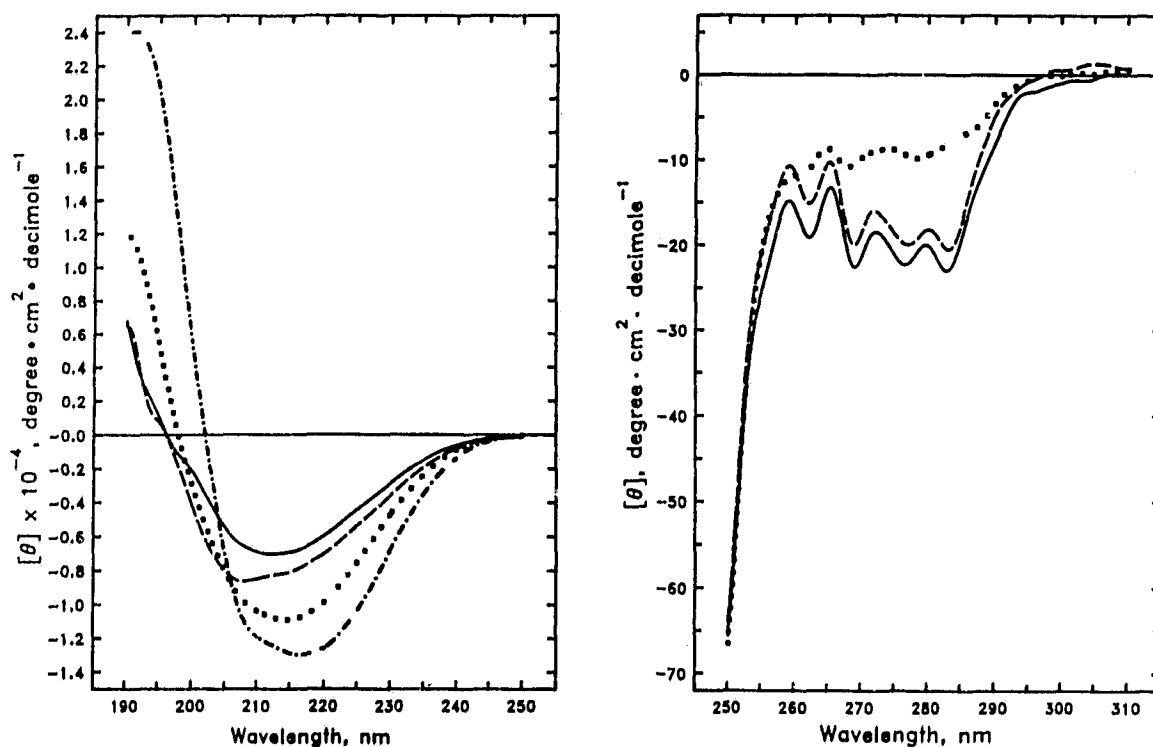


Figure 26. Far (A) and near (B) u.v. c.d. spectra of the truncated S-layer protein from *A. hydrophila* TF7-ST1. The buffer used for the study was 100 mM-NaCl, 50 mM-Hepes (pH 7.4) or 100 mM-NaCl, 0.1% TFA (pH 2.1). (—) S-protein at pH 7.4; (---) S-protein plus 0.1% (w/v) SDS at pH 7.4; (·····) S-protein at pH 2.1; and (-·-·-) S-protein plus 0.1% SDS at pH 2.1. At pH 7.4 the protein concentration was 1.77 mg/ml, and at pH 2.1, the protein concentration was 1.26 mg/ml.

Table 7. Secondary structure of the 38 kDa truncated S-protein produced by *A. hydrophila* Tn5 insertion mutant TF7-ST1 by analysis of solution c.d. spectra using the Provencher-Glockner program

Solvent	Secondary structure content			
	α -helix	β -sheet	β -turn	remainder
100 mM-NaCl, 0.1% (v/v) TFA, pH 2.1	0.21	0.42	0.06	0.31
pH 2.1 solvent + 0.1% (v/v) SDS	0.33	0.42	0.03	0.22
100 mM-NaCl, 50 mM-Hepes, pH 7.4	0.08	0.35	0.19	0.39
pH 7.4, run no 2	0.11	0.46	0.18	0.25
pH 7.4 solvent + 0.1% (v/v) SDS	0.19	0.32	0.18	0.31

environment at neutral pH, and at the acid pH used to extract the *Aeromonas* S-proteins from the cell surface. At a pH of 7.4, the α -helix was raised from 10% to 19%, while at a pH of 2.1, the acidic detergent raised the α -helical content from 21% to 33%.

Figure 26B shows a near uv spectra, which is the spectral region where contributions are found from asymmetrically situated aromatic amino acids. In the region from 270 to 255 nm at pH 7.4, some fine structure is evident for the truncated protein, probably reflecting signals from phenylalanine residues. The effect of 0.1% SDS was to slightly reduce the magnitude of the bands, but the fine structure was not altered. This was not the case when a pH of 2.1 was used, where the fine structure evident under previous conditions of the study at a pH of 7.4, was essentially lost.

Acknowledgments

Dr. John W. Austin is acknowledged for the electron microscopy in figure 21A, and figure 22. Dr. William D. McCubbin, Dr. Cyril M. Kay, and for technical assistance, Kimio Oikawa, are acknowledged for the characterization of the truncated S-protein with respect to the solution molecular weight studies, and the secondary structure analysis using c.d.

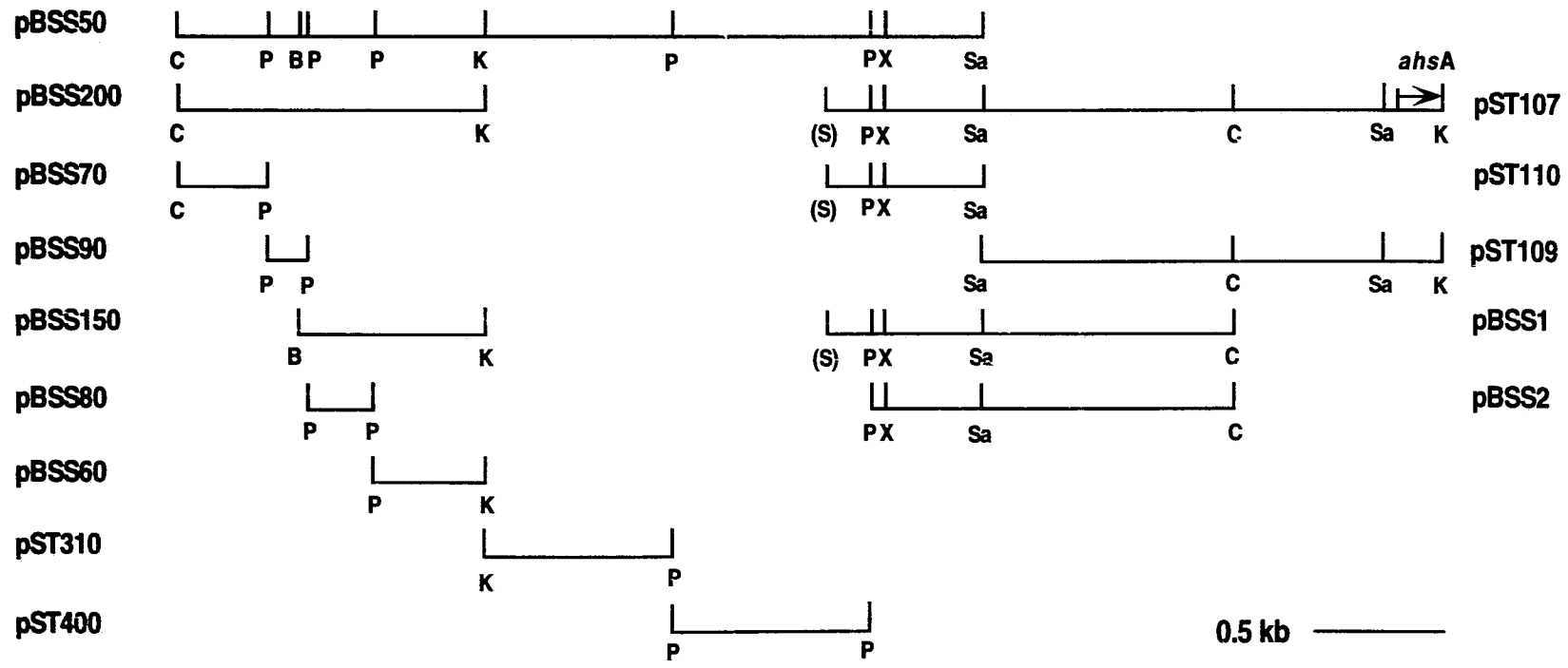
IV. Isolation and Characterization of a Gene Whose Product Is Responsible for the Specific Transport of the S-Protein Across The Outer Membrane in *A. hydrophila*

Continued sequencing in the 5' direction revealed a large rho independent terminator approximately 700 bp upstream from the *ahsA* gene. With the generation and sequencing of more sub-clones from this region, it was found that the ORF ending in this terminator encoded a protein that showed high sequence identity with PulD, a member of the pullulanase secretory system from *Klebsiella* spp. PulD is known to be located to the outer membrane in *K. pneumoniae*, and during the secretion of the pullulanase enzyme, PulD probably plays a part in the final movement of pullulanase to the cell exterior (64). Therefore, this region, the so called *sps* (S-protein secretion) locus was studied further in order to determine whether it encoded a protein specifically involved in S-protein secretion in *A. hydrophila*.

Cloning and sequencing of the region containing the *sps D* gene

The endonuclease sites used for the sub-cloning of small fragments from plasmids pBSS50 and pST107 for the sequencing of the *sps* region are shown in figure 27. All of the sub-clones generated for the sequencing of the region are shown, as well as the position of the S-layer protein gene *ahsA* relative to the DNA sequenced in this study. Synthetic oligonucleotides were used for complete sequencing in both directions. Nucleotide sequence analysis of the approximately 4.1 kb region of DNA bounded by the *Cla*I endonuclease sites revealed the presence of three ORF's, with a fourth incomplete ORF extending from upstream (figure 28 and 29). ORF1 contains 594 nucleotides and a G/C content of 52%, ORF2 contains 498 nucleotides, with a G/C content of 55%, and the ORF describing the *spsD* gene contains 2,211 nucleotides with a

Figure 27. Endonuclease restriction map of the constructs used in the cloning, and nucleotide sequencing of the *A. hydrophila* TF7 *sps* region. The position of *ahsA* gene relative to the region sequenced in this study is shown. The terminator for the *spsD* gene is located approximately 700 bp upstream of the *ahsA* gene, and immediately 5' of the *ClaI* site in plasmid pST107. All subclones were generated from the pBSS50 and pST107 plasmids which were originally cloned from the EMBL 3S lambda vector containing the *A. hydrophila* *ahsA* gene. B = *Bam*HI, Bg = *Bgl*II, C = *Cla*I, H = *Hind*III, K = *Kpn*I, P = *Pst*I, Sa = *Sac*I, S = *Sal*I, and X = *Xho*I. Restriction sites contained within the multiple cloning sites of the vectors are indicated by parentheses.



G/C content of 51.4%. The incomplete ORFJ originating from upstream, contains 615 nucleotides. The start site for ORF1 overlaps the final amino acid codon of the ORF extending from upstream, the initiation codon for ORF2 overlaps the terminal amino acid codon for ORF1, and the initiation codon for the *spsD* gene is separated from the stop codon of ORF2 by 24 nucleotides.

Each of the potential coding regions shows a possible ribosome binding site the expected distance upstream of their respective ATG start codons (figure 28). The Shine-Dalgarno site for the *spsD* gene is located in the intervening 24 nucleotides between its initiation codon and ORF2, while the ribosome binding sites for ORF1 and ORF2 are contained within the coding region of the preceding gene. At position 308, there is a palindromic sequence of 22 bp in length, with a 2 bp mismatch that resides within the coding region of the ORFJ sequence. Directly following the stop codon for this ORF and overlapping the start of the ORF1 reading frame (spanning 23 bp and beginning at position 620 (figure 29)) there is a direct repeat sequence (GTTGCAGC X⁷ GTTGCAGC), and two palindromes, GTTGCAGCAAC, and GCAGCAACAATGGTTGCAGC (figure 29). This final palindrome is followed by a poly T sequence of four nucleotides and could act as a weak transcriptional terminator if the central AT bases were part of a loop structure. Within the coding sequence of ORF1 at base pair 926, there is a third palindrome of 23 bp with a 1 bp mismatch at the center (GCAGAGTCGCATTGCGACTCTGC). Also beginning at position 3032 and within the *spsD* gene, there is a possible stem loop structure with a stem length of 12, a 2 bp mismatch, and a loop size of 6 bp. 3' to the *spsD* gene is a predicted rho independent terminator followed by a pyrimidine rich region with a stem length of 13, a loop size of 4 bp, and a GC content of 62% displaying a free energy of -32 kJ/mol.

Figure 28. Nucleotide sequence of a 4.09 kb portion of the putative *sps* operon from *A. hydrophila* strain TF7 sequenced in this study. Stop codons for each ORF are represented by an asterisk, and various palindromes contained within the coding sequences are underlined. Putative Shine-Dalgarno ribosomal binding sites are double underlined, and a potential rho independent terminator palindrome of the *spsD* gene is dotted underlined. Also indicated is the conserved signal peptidase II cleavage site (▼) in the SpsD protein. Following the incomplete ORF (ORFJ) from upstream, and beginning at position 620, is a further palindrome (overlined) that overlaps a possible rho independent terminator (dotted underlined), and a direct repeat sequence (bold). Included are the endonuclease restriction sites for the enzymes *Cla*I, *Bam*HI, *Hind*III, *Kpn*I, *Sac*I, and *Xho*I. The deduced amino acid sequences are shown below the nucleotide sequence.

Clal ORFJ
 1 ATCGATGGATGTGAATGGCAGCTATGGCGTATGGCGTCTGGCCGAGAGCCGCTTGAGCGAATGTCGGGAGAAAGGGCTCATCGTCTGT 90
 I D G C E W Q L W R D G V L A E S R L S E C R E K G L I V C
 91 GATTACACTCGAGACTCCCGTTGCAAGGGACTGAACGTACATGGCTGGTATGTGTTCCGGCGCGCAGTGGTATGTTGCTGCTCGCG 180
 D Y T R R L P L Q G T E R T W L V M C S A A A V V M L L L A
 181 AGCCTCATGTTGCAAGGAGGCATGGCATTTGGTCAITGGCAGACTTTGACACAGTTGGAAGAGCAGCTTCTGCTCAGGATGAGTCCGCC 270
 S L M L Q G G M A L R H W Q T L T Q L E E Q L S A Q D E S A
 271 GTTATGGAGCAGAAAGCCGCTTAAGAGCCGAAAGGCTTGGCCAGCGGCTCTGGCACAAGGTCGCTGCTGCGCAGCAAGAGAACATCCCA 360
 V M E Q K A R L R A E R L R Q R L L A Q R S L L Q Q E N I F
 361 TTACTTAACAGATATTGGAGCTGATGCCTGGCAGCAGTGAATCTCCAGCAAGTGGTATCCAGCCAGGTCGTATCGAGATGATATTA 450
 L L N Q I L E L M P A T V S N L Q Q V V I Q P G R I E M I L
 BamHI
 451 AGTGTGGTCAACCGGATCCTAGAGGCTATGTGACACGCTTTGATGGCCTGAGGTGGGGCCATGGTACTGCGTAATGTACAGATCCAA 540
 S D G Q P D P R G Y V T R F D G L Q V G A M V L R N V Q I Q
 ORF1
 541 CTCAATAGTAAATGGCAAGGCGTTCGTTTTATCGCAGAGATAAAGAAAGTATCCCGCCAGGGAGAGCACGCTCATGACCTTGGCAAGC 630
 L N S N G Q G V R F I A E I K K V I P A G R A R S *
 M T L Q Q Q
 631 AATGCTTTCAGCTTTTGGCTGAAAGTAGAGCAGTGGGTTACCAACGCTAGGGTTGTTATGGAACCTTGGCGATCCGCCCTTATTTTTT 720
 W L Q L L A E S R E Q W V T N A R V R Y G T L A I G L I F L
 721 TGCTATGGCTTAATCTGGTAAATGAGCGATCTGCGTGCAGACCTGCAGAGCCGTTGAAACCTCGGCCCTCAACCAGCTGGCTGAATATCAGC 810
 L W L N L V M S D L R L D L Q S R E T S A L N Q L A E Y Q Q
 HindIII
 811 AAGCTTCTGGCAAGAAAGCTGGCAGCAGTGGCTCACTCAAGTGGTCAAGTGGTGAATGCAACATCCCGCCACTTTGGCAGTGTCTAGA 900
 A S G K E S W H D R L T Q V R Q V L D A T S R H F G S A Q S
 901 GTGAGGCATTTGGCTCGTCTGACTTGCAGAGTGCATTTGGCAGTCTGCTCAAGGAAAATGGGCTGGCCAGGGCCAAATGAGGTCTCAA 990
 E A L A R A D L Q S R I A T L L K E N G L A Q G Q I E V S N
 991 ACGCCCAAATGCTGATGCGGCAACAGCACTGACACCCGCTCCAACTGCGGATCAGTGGCAGGGCAAGGCGTTGGGGTTGCTTAAGGTGA 1080
 A P N A D A A T A L T P L Q L R I S G R G K A L G L L K V I
 1081 TTAATCAGCTCGAACCTACGGACCCCTGATTTGGCAGTGTGAAAATTTGACCATCAGCAATCAAGTAGGGGACGAGTTGATTTATAACCTCA 1170
 N Q L E R T D P V L R V E N L T I S N Q V G D E L I Y N L I
 KpnI ORF2
 1171 TCGCTACCGTTTGGTACCACCCCTTCCGAGCAACACCATGATAGTCAGGCAGGAGCAGCTGATCGTATTGGGTTGACCTTCTCTGTTTC 1260
 A T V W Y H P P G A T P *
 M I V R Q K Q L I V L G L T F L F S
 1261 TGCTTTGCCACTGGGTTATTTACAGCCGCGAAAGCGGAAATAGAGAATAATGTGGCGCACCTCGCTGGCAGTTACCCACGGCGTGGCC 1350
 A L P L G Y F T A P K A E I E N N V G D T R W Q L P T A L P
 1351 CGATCTTGGCGTGGTTAAACAGATCCACAGCGCGTATCGCGATTCTGGCCCGTCGAAGCCTTGGCAGGCGCAGCTGATGGCGCGCAAAA 1440
 D L A V V K P D P Q R V S R F W P V E A L A G A A D G A Q K
 1441 AGAGCGCCGGAAACCAAGTCTTGGAGTAGAGGCTACCGAATGGCGTGTGGTTCGCGAGTATCCGCCAGGGACGTACCCCCAGGCGTGGT 1530
 E R R E P S L G V E A T E W R L V A V I R Q G R H P Q A L V
 1531 GCTGAGTCCGAAAACAAGTTGTTGACGGTGTGAGTGGGAAGTGAAC T GACCCACAGCAACAGGTCACGGGCAATTTGGCCGDRATCGTGT 1620
 L S P K N L L T V S V G S E L D P Q Q V T G I G P D R V
 -35 -10
 1621 GAAATGGATAACAAGTAAAGGCAACAGGGGTTTGGTTCCTTACCCCAAGCCACAGGAGCTCAGGCTAATACAACACACCTAATT 1710
 K W I T S K G K Q G V L V L Y P K P Q A A Q A N T T Q P *
 spsD
 1711 CATGGCATATAAACACAATATGGATCTCTTGTTTAAATCCGACTCTCGTGTGATCATCATCTTTGAAAGTGTGGACCCCGCTTCAACT 1800
 M D L L F K S D S R A D H H L L K C G P R F T L
 1801 ACTCGCCCTGTCCCTTGGGTATGTTGGCAGGCTGTACGATCAATAACAACCCGACCCGCTTGGCCAAACCGATCCGCGAGCCGAGTCCGA 1890
 L A L S L G M L A G C T I N T N P T A L P K P I R E P Q S Q
 1891 ATCGGTATCAATGAGACTCTGCAGATGAGCAGGCGCAAGACCTCGCCCAAGTAAAGGAGATCGCTTCGGCATTACTCTTAACCAACG 1980
 S V I N E T L Q N E Q A Q D P A Q R K R D R F G I T P N Q R
 1981 TCAGAGTGTACGAGTTATAACCCCTGACGACTCAACTCTGGCGGAGGGCCCTTAGGGGGATGCCATCAGCATGAACGTGAATAACTTTCC 2070
 Q S V R V I T F D D S T L G E G L R G D A I S M N V N N F P
 2071 GCTGCTGCTTTTATCAACGAAGTGTGGAACCCGCTTGGCGCTCTGTTTACCATGACTCCCGAGCTACAGAAGAAGACGACCTAGT 2160
 L P A F I N E V F G N R L G L S F T M T P E L Q K K S D L V
 2161 GACGCTCCGTATGAGTATCCCAACCGCATCGGTATTATTCAATACGCAAGCAATGACTTTCTGATTATGGGGTGGAAAGTCAAGTT 2250
 T L R M S D P Q T P S V L F N T A R N V L S D Y G V E V K L
 2251 GCGTGTAGCCCTCTACACCTTCAATGTGGCCAGAGCGTGTGCTAATGACGCCCTTACCCATCATGGTAAAGCGGTGGCGCCTTACCTGATGT 2340
 R D G L Y T F N V A Q T V A N D A L P I M V S G G A L P D V
 2341 ACCTATGTCCTATCGCCCGTATTTTCAATAGTACCGATGAAGGTTGTTGCGAGTGTGATGAGTGGCTGATGCTGCGCGGAGATGTTTGA 2430
 P M S H R P V F Q I V P H K V V R S D Q M A A W L A E M F E

2431 AAATTCGCAGCTTAAGATAAAGGATGACCGTGTCAATTAATGCCCTGATGCTACGAGGTCCGGTGGAGCTGGTCAAACAGGCTAGCGAGGC 2520
 N S Q L K I K D D R V I N A L M L R G P V E L V K Q A S E A

2521 CATTTCCTCTTTTCGATCAACCTGGCTAAAAAGTAGCCACAGCCTGGCCATCAGTCCCTGTTTATTCAGATGCCGAGCAACTGGGCAATGC 2610
 I S L F D Q P A L K S S H S L A I S P V Y S D A E Q L G N A

2611 CCTCATGAAAGTGTGCAAACCGAAGGGTATGATGTCAGATTACCCACCGTTTGGTGGCGTATGGTACTGAAGATGAAGAGCTGCA 2700
 L M K V L Q S E G Y D V S D S P P F G G V L V L K M K E L Q

2701 GCGTATCATCGTTTTTCCCGCCGAGCCTCGAGTACTCAGTACATTCGTCATGGGTCGAAGTGTGGATCGTGAATCCAGGAGAAGGT 2790
 R I I V F A A E P R V L S H I R Q W V H V L D R E S Q E K V
XhoI

2791 GGAGAATGGCCTCTTCATCTATCAGTGGCAATACCCAGGCGCTGCCATCGCCACCATCTGGGAGCGCTTGGGTATGGGGCTAATAT 2880
 E N G L F I Y Q V R N T Q A A A I A T M L G A L G Y G A N I

2881 TCCGGCAACAGGGGTTAATAGTACCAATACCGTACTGCAACTGGCGAACAGAGCGGGGCTGGTACTTACTGCGGCTTCCGCGGCCAT 2970
 P A T G V N S T N T V T A T G E Q S G A G S L T A A S P A T

2971 ATCGGCACCGACCAGCATAGCCTTCAAGGGAGAGCAGGGAAGCGTTGTGGTAGACGGGAATCCCATGCCCTTATCTCAAGGCACTGG 3060
 S A P T S I A P K G E Q G S V V V D G N R N A L I F K G S G

3061 GCGTGAGTGGGTAACGTTACGTCGTTGCTAGACGAGCTCGACAAGCCGGTCCCTTCTGTGATGATCGATGTGTGTGGCCGAAAGTGA 3150
 R E W V T L R P L L D E L D K P V P S V M I D V L L A E V S
SacI

3151 TCTGAATGATAAAGAGGGGCTTGGTGTGATGGAGTAATATCCACACCGATTTGGGTGCTAAAGATTTGATCATGACTGCCAACCG 3240
 L N D K E G L G V D W S N I H T D L G A K D L I M S T A N G

3241 GATTGGTAACAGTGGTCTTAATCTGACGCTTAATAGTGTGGCCAGACTCGTGCCAAGCTCAACGCATTTTATGAGAACAAGCAAGCTGT 3330
 I G N S G L N L T L N S A G Q T R A K L N A F Y E N K Q A V

3331 TATCCGCTCAAGTCCCAAGTTGATGGTGGGAAGCGGTGAAGAGGCTCGTATTGAGGTGGGAATGAAATTCGGTGTGCACAGGTACGAG 3420
 I R S S P K L M V R S G E E A R I E V G N E I P V V T G T S

3421 CCAGTCAACAGATAACCCCGACGCCCCATCAACAAGACAGTGAATATCGTAAGACCGGTGTGATCCTGACCATTAAACCAACGGTACA 3510
 Q S T D N P D A P I N K T V Q Y R K T G V I L T I K P T V Q

3511 GGCAGTGGGGTGTGTGATCTGACAATTAGTCAAGAGCTTCTGAAACAGCGGATACTTCAACCAGCACCGAGCGGTGAGCCCAACGAT 3600
 A S G V V D L T I S Q E L S E Q A D T S T S T D A L S P T I

3601 CATGAATCGTAAGGTCAATACTGCTTTGACCCCTGCGTGTGAGGGGTCGGTGTGTTGGCGGACTTATCTCAGTACTAAGGGTGAAGG 3690
 M N R K V N T A L T L R D G G S V M L A G L I S S T K G E G

3691 GGATACCGGGTCCCTTGTAGGGGATATCCCTGGATAGGTAGCTTGTTCAAATCGAAAAGCAATAGTCAGAAATCGCACCGAGCTGT 3780
 D T G V P L L G D I P W I G S L F K S K S N S Q N R T E L V

3781 AGTGATGATCATCCCTTATGTTCTTCGTGACTTTCATGAGGCCAAAAGTCTGACCGAACGTTATCAGCAGCAACTGGAATGAACAATGC 3870
 V M I I P Y V L R D F H E A Q S L T E R Y Q Q Q L E L N N A

3871 ACCGACACTGAAGCGTCCGTTTTTACCAGGGATGGAACCGAAAAGAGACAACCCCTCCCGAGTAGAGCAGTAAACAGAGTCATGTTCTTG 3960
 P T L K R R F L P G M E P K E T T P S R V E Q *

3961 ACATTATGTAAGTACATAGCATTAGGGCGCTCACCTTGTATATGATGAAAGACCCCTCCATTACTGAGGGGGTCTTTTTTCCCTGT 4050
 C*laI*

4051 ATTGATCTGGCTATACGATATGGCGGTGCTACATCGAT 4090

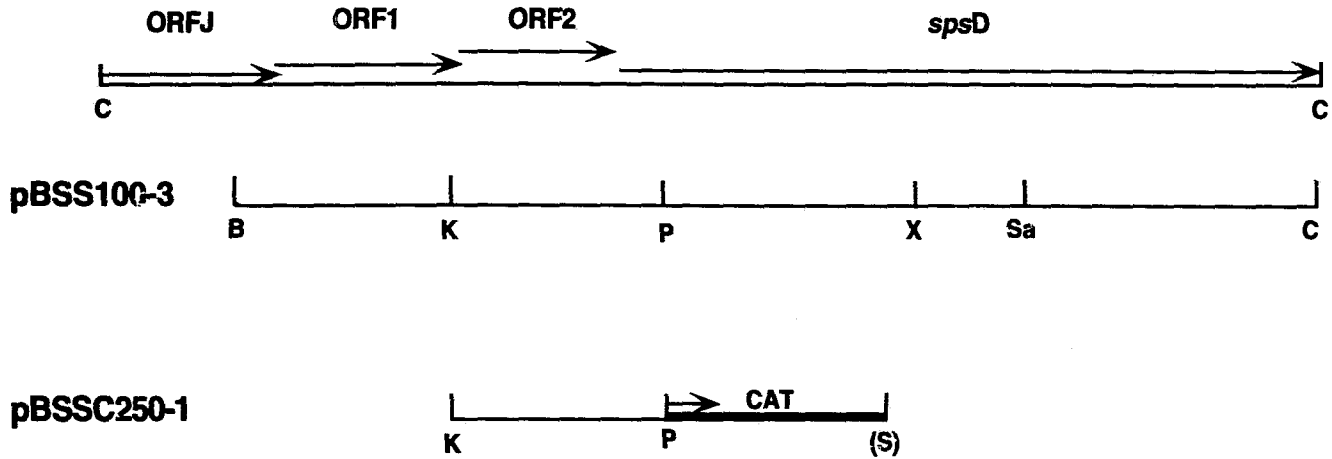
The *spsD* gene contains endogenous promoter activity

The presence of the palindrome that concludes with four T nucleotides at the end of the incomplete ORFJ can be seen in figure 29. Included in the sequence shown is the stop codon for ORFJ (TGA), which is overlapped by the translational initiation start site for ORF1 (underlined). The ribosome binding site (GGAGAG) for ORF1 is indicated (underlined), as well as both the direct repeat sequence (bold lettering) and a smaller palindrome (over lined) which overlap the possible terminator. The presence of this palindrome, which is potentially capable of acting as a terminator prompted us to determine whether or not the *spsD* gene contained its own promoter. The insertion of the promoterless CAT gene into the *Pst*I site beginning at position 1910 (figure 28 and 29) immediately downstream of the initiation site for the *spsD* gene, and subsequent growth of *E. coli* containing the construct in selective media, demonstrated that the *spsD* gene did indeed contain its own promoter.

Due to the complexity of the DNA sequence in the region upstream of the *spsD* gene where there are a number of palindromes, especially around the possible ORFJ terminator itself, regulated transcriptional read through from upstream may be occurring. This would be in addition to a basic constitutive level of expression from the promoter specific to the *spsD* gene. We initiated a study based on this observation, in order to determine whether or not the palindrome at the end of ORFJ (figure 29) was functional. The insert from plasmid pBSS100-3 was sub-cloned into the suicide vector pGK2003 (figure 30) (105) which contains the *oriT* sequence inserted into the *Eco*RI site thereby halting any effect of the β -galactosidase promoter contained in pUC18 on the transcription of *spsD*. This allowed us to; (i) selectively remove the *oriT* fragment by *Eco*RI digestion followed by intra-molecular ligation leaving the palindrome intact; and (ii) remove the palindrome on a *Kpn*I fragment using

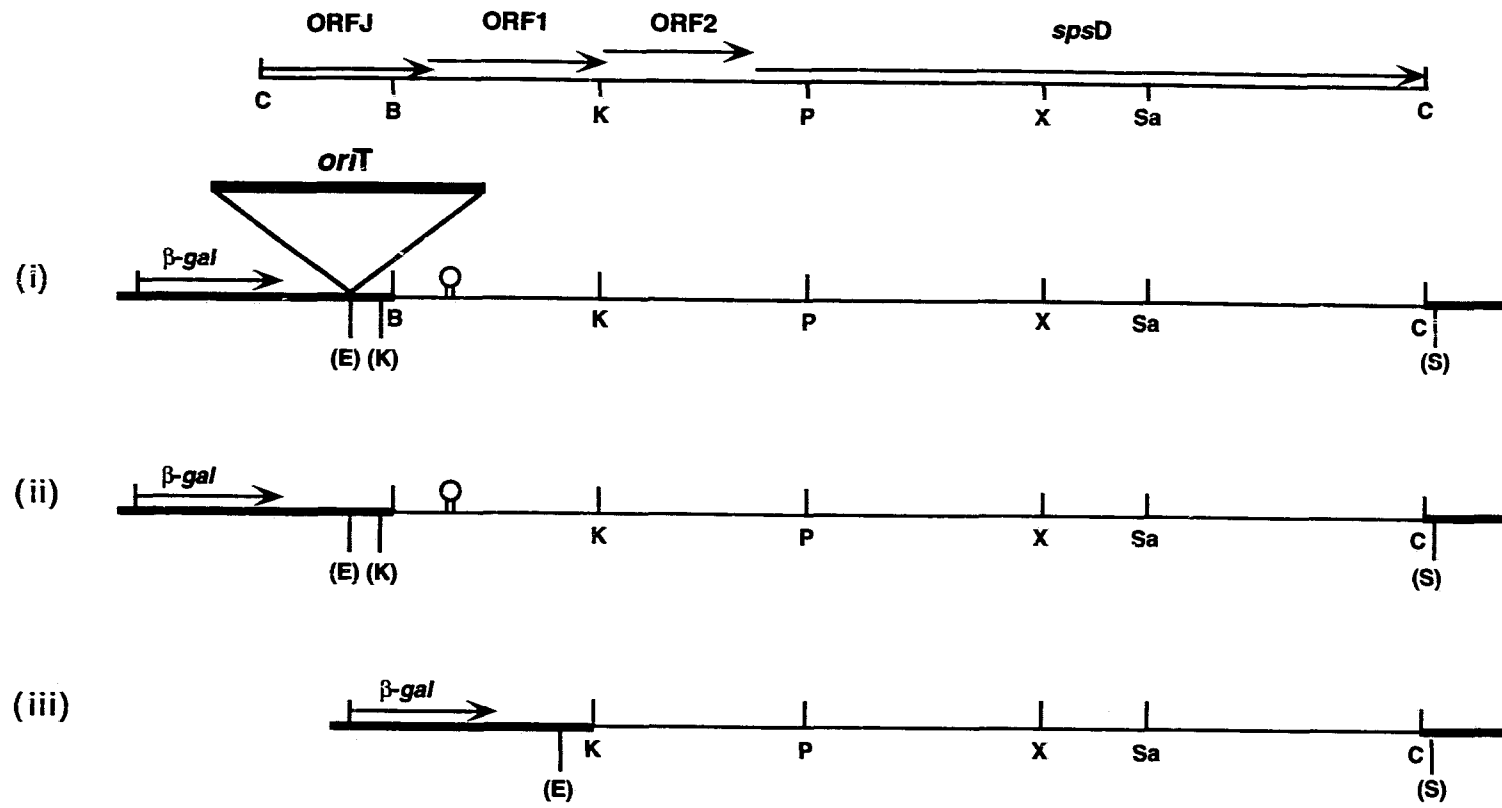
Figure 29. The presence of the nucleotide sequence in the inset at the top of figure 29 encompassing the end of ORFJ and the beginning of ORF1 indicated the possibility that the *spsD* gene contained endogenous promoter activity. The sequence shows the initiation codon and ribosome binding site for ORF1 (underlined). The ATG initiation codon overlaps the stop codon for ORFJ (TGA overlined). There is a small palindrome (GTTGCAGCAAC) (overlined) overlapping a direct repeat sequence (GTTGCAGC X⁷ GTTGCAGC) (bold lettering) and a second palindrome (underlined) with a one base pair mismatch (GCAGCAACAATGGTTCAGC) followed by four T nucleotides that is capable of acting as a rho independent terminator. The promoterless CAT gene was inserted downstream of the *KpnI-PstI* fragment from plasmid pBSS100-3 generating plasmid pBSSC250-1 and was used to prove the presence of promoter activity contained within this fragment.

GGGAGAGCACGCTCATGACGTTGCAGCAACAATGGTTGCAGCTTTTGGCTGAAAAGTAGAG



0.5 kb _____

Figure 30. Physical map describing the method used to test whether the palindrome at the end of ORFJ was functional. The insert from plasmid pBSS100-3 was cloned into the suicide vector pGK2003 (i) on a *Bam*HI-*Sal*I fragment, and *ori*T was removed using an *Eco*RI digest followed by intramolecular ligation (ii). Following the removal of *ori*T, the internal palindrome was removed on a *kpn*I fragment again followed by intramolecular ligation (iii). Results were as described in the text. B = *Bam*HI, C = *Cla*I, E = *Eco*RI, K = *Kpn*I, P = *Pst*I, S = *Sal*I, and X = *Xho*I. Parentheses show restriction sites from the vector.



0.5 kb _____

the site for this enzyme contained in the vector and the site located upstream of the *spsD* gene in the insert. Following the removal of each of these fragments the effect of the β -galactosidase promoter on the resulting construct could be monitored by the loss of integrity of the *E. coli* cells containing the plasmids. We found that removal of *oriT* alone led to unstable growth, presumably because of the added effect from the exogenous β -galactosidase promoter, however, the removal of the palindrome on the *KpnI* fragment resulted in extremely weak growth of *E. coli*, and the complete inability to re-isolate the plasmid containing the *spsD* gene from these cells. This effect was presumably due to loss of function of the terminator followed by an increase in expression of the SpsD protein leading to cell death.

Primary amino acid sequence of the *sps* region

Four polypeptides are predicted to be encoded by the cloned DNA (figure 29), including the carboxy terminal of the incomplete ORFJ extending from upstream. A search of the data bases of both EMBL and Genbank showed significant sequence similarity between portions of the proteins encoded by this region, and proteins contained in the *Xanthomonas campestris* pathovar *campestris* Xps secretory system. The latter pathway is responsible for the secretion of a number of enzymes involved in the pathogenesis of this plant pathogen (79, 124). The protein encoded by *sps* ORFJ (205 C-terminal amino acid residues) displays some similarity to XpsJ (out of 54 amino acid residues, 35% identical and 30% similar at the amino terminal end of XpsJ). In addition, the 198 amino acid residue *sps*ORF1 encoded protein shows 28% identity with 51% similarity to the Xps ORF1 polypeptide over its entire length (figure 31). However, the *sps*ORF2 166 residue protein shows no sequence similarity to the ORF2 encoded polypeptide of the Xps system. The protein encoded by the

final ORF (737 amino acid residues) in the *sps* region, SpsD, displays greatest similarity overall to known polypeptides (see below), including the carboxy terminal of PulD and its homologs, with highest homology over the entire length of the SpsD polypeptide to the *X. campestris* XpsD amino acid sequence.

A possible lipoprotein signal sequence exists at the amino terminal end of the SpsD polypeptide, with the first 34 amino acid residues terminating with a typical LAGC motif characteristic of a LspA or signal peptidase II processing site (240). Lipoprotein signal sequences however, are normally shorter than signal peptides recognized by signal peptidase I, with a more hydrophobic helical region (240). In the case of SpsD, the hydrophilic amino end is 21 residues long, with a net positive charge of 3, and a hydrophobic segment consisting of 11 residues including the leucine at position -3. Processing at this predicted site would leave a mature SpsD protein of 76.1 kDa. Interestingly, the *xpsD* gene of *X. campestris* also contains a lipoprotein processing site, but its signal sequence is somewhat shorter (124).

The *spsD* gene encodes an unprocessed protein of Mr 79,811, with an α -helical content of 28%, a β -strand content of 21.3%, and a loop content of 50.7%, as predicted by the secondary structure prediction algorithm developed by Rost and Sander (252). This is in contrast to outer membrane proteins such as the porins, where the predominant form of secondary structure is β -strand, with a content of 65 +/- 8% (22, 61, 134, 152, 312). SpsD does however have other properties consistent with a membrane-associated protein. For example, the average length of anti-parallel pleated strands required to traverse the membrane is thought to be between 10 and 12 residues, and there are potentially five regions capable of forming β -strands of this length in the SpsD protein. Also, as is typical for outer membrane located proteins, SpsD contains

ORF1 A. hydrophila	MPL-----QQQW-LQL- ----LAES--	RECAWLNARV	RYGTLAIGLI	34		
ORF1 X. campestris	MPAATWTASP SPPNWPVPMP RRPMPFSSVK	RDFAL-----	ALGLL	40		
Consensus	M.....W.....S.....E.....W.....		A...G...	50		
ORF1 A. hydrophila	HLWLNLVMS DLRADLQRE TSALNGLAEY	QQASGKESWH	-DRLTQVRFV	83		
ORF1 X. campestris	LLVMGVAYLV LVHPWFTQPM LAVQDELQSL	RERELRVRVQ	LQQAFQVRSR	90		
Consensus	L.....L.....L.....L.....		Q...V...C...	100		
ORF1 A. hydrophila	LDAT-----SRH-FGSACSE	ALARAALQSR	IATLLKENGL	AQQQIEVSN-	126	
ORF1 X. campestris	LQARQTLCS	REGFLPESSA	ELASAGLQCR	LERAVVDASP	GNRSCAISNR	140
Consensus	L.....S...E...E...S...L...A...A...L...R.....				S...N...	150
ORF1 A. hydrophila	AFNADAATAL	TPQLQRISCR	GKALGILKVI	NCLERTIPML	FVENLTI---	173
ORF1 X. campestris	SFLQPETKRF	TRVAVQVRLR	CGTPELASVL	YSLENGTIPFL	FVDNLNVMAQ	190
Consensus	E.....D.....R.....L...M...L...E...E...L...L...N...L.....					200
ORF1 A. hydrophila	-----SNQV	DEL-TL-YNLI	ATVW-----YH	FF--GA-----	TP	198
ORF1 X. campestris	RYQLSENEGG	NGLDIAFELA	GYLKPGSNAG	FVNTGAAPAA	GEASNAP	237
ConsensusN...C...L...L...L.....E...GA.....E					247

Figure 31. Amino acid sequence alignment between the ORF1 encoded polypeptides from *A. hydrophila* and *X. campestris*.

very little α -helical content capable of anchoring the polypeptide in the cytoplasmic membrane during its movement to its final position.

Homology of SpsD with other outer membrane PulD proteins

A super family of proteins based on their homology to PulD has previously been identified (97). The amino acid sequence homology of the SpsD protein compared to the PulD polypeptide of *K. oxytoca* is limited to a region of 181 residues in the C-terminal, with 39% identity and 24% similarity (figure 32). The *A. hydrophila* ExeD protein (GenBank accession number X66504) shows homology with SpsD over a region of 291 amino acid residues, with an approximate identity of 34% and a similarity of 55%, the majority of which lies at the C-terminal end (figure 32). The greatest similarity that SpsD shows compared to known PulD homologs is to the *X. campestris* XpsD protein. In this case, the similarity spans various regions covering a total of 420 residues over the entire polypeptide, with approximately 30% overall identity and 55% similarity. Within the C-terminal region of SpsD there is a sequence VPLLGDIPWIGSLF that conforms closely to a secretion protein motif common to all the PulD homologs, VP(L/F)LXXIPXIGXL(F/L) (125). There are also many other regions of homology to this family of protein secretors that are matched closely to those published by Genin and Boucher (97).

Conservation of the *spsD* gene amongst *Aeromonas* species

All strains of *A. hydrophila* displaying high virulence for fish contain S-layers on their cell surfaces, we were therefore interested in determining the extent of conservation of the *spsD* gene amongst a number of strains. We had shown previously (289) that the S-protein gene *ahsA* was not conserved amongst all S-layer producing strains of *A. hydrophila* and *A. veronii* biotype

Figure 32. Amino acid sequence alignment showing the C-terminal of SpsD and its homologs. Proteins included in the alignment include ExeD of both *A. hydrophila* and *A. salmonicida*, PulD of *K. pneumoniae*, OutD of *E. chrysanthemi*, and XpsD of *X. campestris*.

SpsD	-----QTRAK	LNAFYENKQA	VIRSSPKLMV	RSGEARIEV	GNEIPVVTGT	569
XpsD	TFLGKNAAI	ISALDQVTNL	RLLOTPSVFV	RNNAEATLNV	GERIPIINSTS	594
OutD	-----SML	LIALSSDGKN	DVLATPSIVT	LDNMEATFNV	GQEVFVLTGS	520
Pu1D	GFYQGNWAML	LIALSSSTKN	DILATPSIVT	LDNMEATFNV	GQEVFVLTGS	467
ExeD A. hydrophila	-----AML	VIALSTNTKS	DILSTPSIVT	MDNKEASFNV	GQEVFVQTGT	473
ExeD A. salmonicida	-----AAL	VIALSTSTKS	DILSTPSIVT	MDNKEASFNV	GQEVFVQSGS	472
Consensus	-----	.A.L .TALS..TK.	DIL.TESIVT	.DN.EA.FNV	GQEVV.TGS	600
SpsD	SQSTINPDAP	INKTVQYRKT	GVLITIKPTV	QASGVLLLI	EQEISSQAD-	618
XpsD	INTGLGSDSS	FS-SVQYIDT	GVLIKVPERV	TKDGMVELLI	EQEISSPPGAR	643
OutD	QTTVGSQDNI	FN-TVERKTV	GILKLVKPOI	NEGDSVLLCI	EQEASSVAE-	568
Pu1D	QTT--SGDNI	FN-TVERKTV	GILKLVKPOI	NEGDSVLLCI	EQEASSVAD-	513
ExeD A. hydrophila	QNSTSG-DTT	FS-TIERKTV	GPKLVVTPQI	NEGDSVLLLI	EQEASSVKG-	520
ExeD A. salmonicida	QSSTSDQ-V	FN-TIERKTV	GPKLVVTPQI	NEGDSVLLNI	EQEASSVAQK	520
Consensus	Q.....D..	FN-TVERKTV	G.KL.V.TQI	NEGDSVLL.I	EQEASSVA.-	650
SpsD	----TSTSTD	ALSPT----	IMNRKVTAL	TLRDGGSVML	AGLISSTKGE	659
XpsD	PAACTAAAT	TVNSAACNVD	INIRRVKTEA	AVQNGDTIML	AGLIDDDTTD	693
OutD	---GNGSSNS	SLGVT----	FNIRTVNNAV	MVTNRETVVV	GGLLDKTAIE	610
Pu1D	---AASSTSS	DLGAT----	FNIRTVNNAV	LVGSGETVVV	GGLLDKSVSD	555
ExeD A. hydrophila	----QATGTD	GLGPT----	FDIRTVKNAV	LVKSGETVVL	GGLMDEQYKE	561
ExeD A. salmonicida	----QATGTA	DLGPT----	FDIRTIKNAV	LVKSGETVVL	GGLMDEQYQE	561
Consensus	----.....T.	.LG.T----	F.TEIV.NAV	.V..GETVVL	GGL.D....E	700
SpsD	GDTGVFLLGD	IPVIGLFLKS	KSNSQNRTEL	VVMIFPVIR	DFHEAQLSTE	709
XpsD	GSNGIFPLSK	IPVIGLFLFR	KTQNSDRREV	IVLITPSIVR	NPQDARDLTD	743
OutD	TNNKVFLLGD	IPVIGLFLRS	KTQTMKRNL	MLFLFPTIIR	DPQQYQOASI	660
Pu1D	TADKVFLLGD	IPVIGLFLRS	TSKKVSKRNL	MLFIRPTVIR	DRDEYRQASS	605
ExeD A. hydrophila	EVSKVFLLGD	IPVIGLFLRS	TSNNYTKRNL	MVFIRPTIIR	DANVYSGISS	611
ExeD A. salmonicida	KVSKVFLLGD	IPVIGLFLRS	TNNYTKRNL	MVFIRPTIIR	DAHVYSGISS	611
Consensus	...KVFLLGD	IPVIGLFLRSSKRNL	MVFIRPTIIR	D...Y...S.	750
SpsD	RYQQQLELNN	APTLKRFLP	GMEPKETTPS	RVEQXTECS	XHYVTSTLGR	759
XpsD	EYG-----	SKF-K	SMRP-----	-----	-----	754
OutD	SKY-----	NSFNN	EQQQ-----	---QRQG--	---NSVLDN	683
Pu1D	GQY-----	TAFND	AQSK-----	---QRGKE--	--NNDAMLNQ	630
ExeD A. hydrophila	NKY-----	TLFRA	QQLD-----	--AVAQEGYA	TSPDRQVLPE	641
ExeD A. salmonicida	NKY-----	TMFRA	EQLD-----	--AAAQESYL	TSPKRQVLPE	641
Consensus	..Y-----	.E..	.Q.-----	-----L..	800

sobria. Using the *Pst*I fragment from the *spsD* gene (figure 28) as a probe we found that unlike the *ahsA* gene which is conserved at least partially through strains Ah598, Ah77-115, Ah80-140, Ah80-160, As701, and As702 (289), the *spsD* gene was only conserved in strains Ah77-115, Ah80-140, and Ah80-160 (figure 33 lanes 6-8). Weak hybridization was also obtained with DNA from strain Ah598 (lane 5), and no hybridization was found between *spsD* and the DNA of the related S-layer producing organism *A. salmonicida* strain A450 (lane 12), or with DNA from an S-protein negative strain of *A. hydrophila* (Ah300), (lane 11).

Isolation and localization of the S-protein in the marker exchange mutant TF7-D2

The isolation of a marker exchange mutant of *spsD* proved to be difficult, even though the phenotypic alteration due to the loss of the S-layer from the cell surface (loss of auto-aggregation in static broth) allows for a strong enrichment process (figure 34). Eventually *SpsD* mutant TF7-D2 was isolated that was auto-aggregating negative. Compared to parent TF7, the growth of mutant TF7-D2 was restricted at 37°C. We predicted that the difficulty experienced in isolating this *SpsD* mutant was because of the normal high expression level of the S-protein, which was subsequently localized to the cell interior. To confirm that this was the case, a two gene mutation was constructed by deleting the 3' region of the *spsD* gene and the 5' region of the *ahsA* gene (figure 31 inset), and tested to determine if such two gene mutants were more easily isolated. Following the enrichment process of serially sub-culturing the upper phase from stationary grown cells three times into fresh broth, 100% of colonies tested proved to be S-protein negative. Southern blotting of a representative "double mutant" TF7-DS (figure 35A), showed that

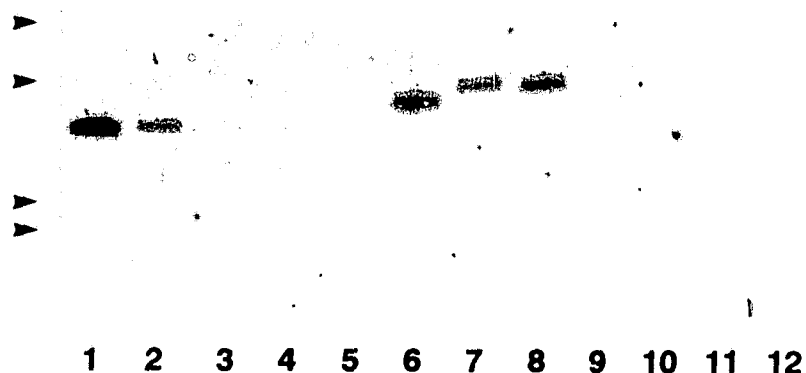


Figure 33. Southern blot analysis of *kpnI* chromosomal DNA digests showing the presence of the *spsD* gene amongst various *A. hydrophila*, and *A. veronii* biotype *sobria* and *A. salmonicida* strains. Lane 1, EMBL-3S; lane 2, TF7; lane 3, Ah 274; lane 4, Ah 423; lane 5, Ah 598; lane 6, Ah 77-115, lane 7, Ah 80-140; lane 8, Ah 80-160; lane 9, As 701; lane 10, As 702; lane 11, Ah 300; and lane 12, *A. salmonicida* A450. The internal *Pst*I fragment from the *spsD* gene was isolated and labeled with ^{32}P using nick translation. This was then used to probe the immobilized DNA. Arrow heads on the left indicate the lambda standards in kilobases from the top, 6.68, 4.36, 2.32, and 2.03.

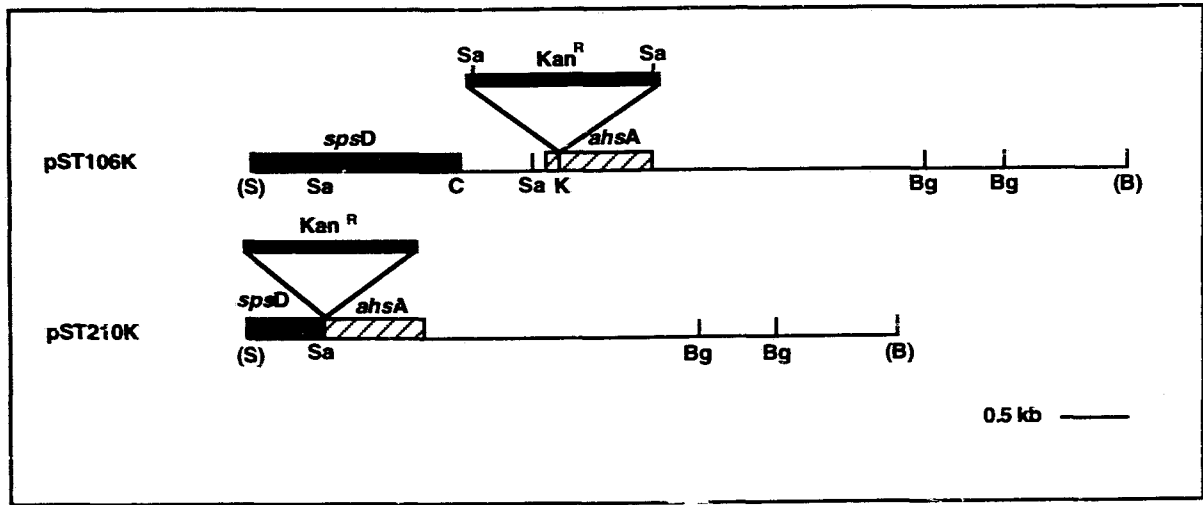
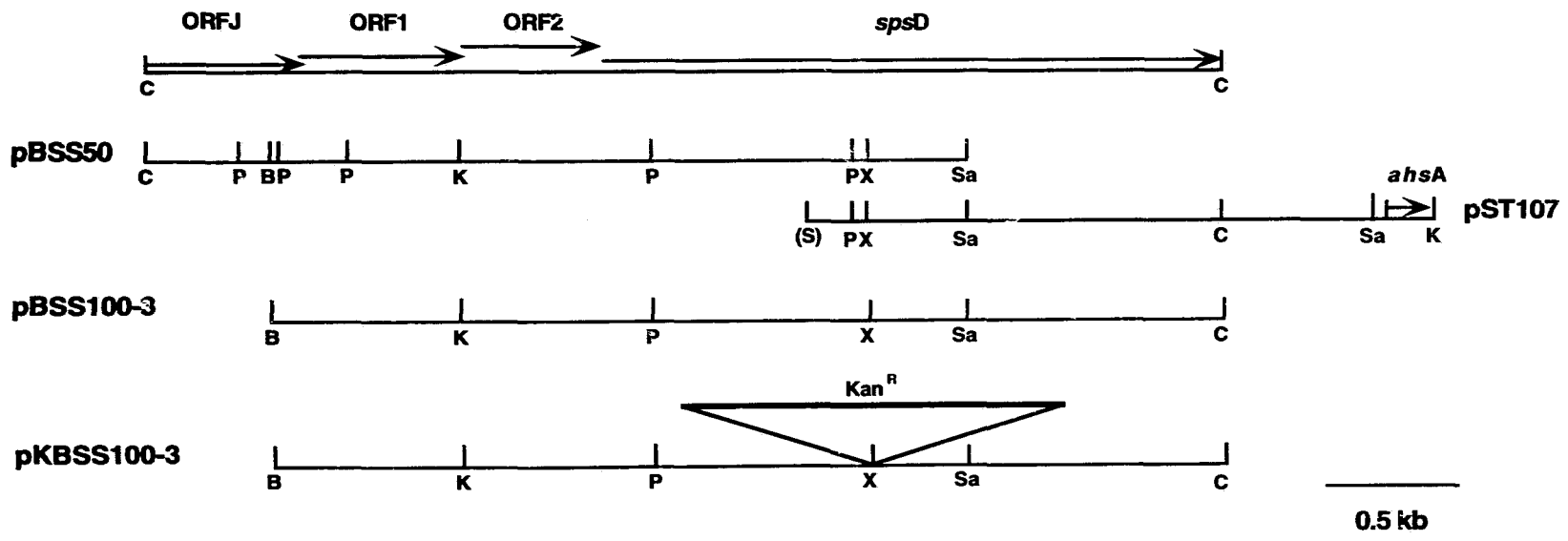
a correct recombinational event had occurred, deleting regions of both genes, and effectively halting expression of both *SpsD* and *AhsA*.

SDS-PAGE analysis of parent TF7 and marker exchange mutant TF7-D2 showed no differences in the quantity of S-protein present in whole cell lysates (data not shown). In the case of S-layer producing aeromonads, the majority of S-protein normally co-purifies with the outer membrane fraction (figure 37A, lane 1), and can be extracted from the cell surface by treatment with 0.2 M glycine HCl, pH 3.0 (figure 37A, lane 7). However, when the outer membrane fraction was isolated from mutant TF7-D2, S-protein was absent (figure 37A, lane 2), and S-protein could also not be extracted from the cell surface with glycine HCl (figure 37A, lane 8). Further fractionation of the cells of mutant TF7-D2 showed that the majority of S-protein was located in the periplasmic fraction (figure 37A, lane 6). This finding was similar to previous studies with certain A-protein secretion mutants of *A. salmonicida* (20). Figure 37A also shows the absence of *AhsA* in the outer membrane and glycine extract fractions isolated from double mutant TF7-DS (figure 37A lanes 4 and 10).

The presence of a functional extracellular enzyme secretory system in the TF7-D2 S-protein secretion mutant

The mutational analysis of the *spsD* gene was especially interesting, not only because of its location immediately upstream of the *ahsA* S-protein gene, but also because of the previous finding of *exeD*, a further *pulD* gene homologue in *A. hydrophila* (136). The *exeD* gene has been shown to be part of an operon with high overall homology to the general secretory system, and is required for the export of a number of extracellular enzymes in *A. hydrophila*. In the marker exchange mutant TF7-D2, a number of other secreted proteins were therefore examined in order to ensure that their export

Figure 34. A representation of the complete region sequenced in this study showing the position of the *sps* ORFs, as well as plasmid pBSS100-3 generated from plasmids pBSS50 and pST107. The Kan^R marker was inserted into the *Xho*I site in pBSS100-3, disrupting the *spsD* ORF and generating pKBSS100-3. Plasmid pKBSS100-3 was conjugated into *A. hydrophila* TF7 generating the marker exchange mutant TF7-D2. The inset shows the previously described pST106K plasmid used for the generation of a S-protein negative mutant of *A. hydrophila* TF7, TF7S (figure 12), and pST210K. For the generation of pST210K, the *Sac*I fragment from pST106K was removed, and a Kan^R marker was inserted in its place. pST210K was then used for the generation of a double *spsD*, *ahsA* marker exchange mutant, TF7-DS. B = *Bam*HI, Bg = *Bgl*II, C = *Cla*I, H = *Hind*III, K = *Kpn*I, P = *Pst*I, Sa = *Sac*I, S = *Sal*I, and X = *Xho*I. Restriction sites contained within the multiple cloning sites of the vectors are indicated by parentheses.



had not been affected by the loss of expression of the SpsD protein. Negative staining of whole cells of both TF7 and TF7-D2 followed by visualization by electron microscopy showed no difference in the presence of pili and flagella in these strains (not shown), indicating no effect on the secretion and assembly of these structures by mutation of the *spsD* gene. Of more interest however, was the observation that secretion of extracellular enzymes by *A. hydrophila* was not affected by this mutation. Using cultures grown on agar plates containing the substrates for the enzymes aerolysin, protease, and amylase, there was no visual indication that the secretion of these proteins was impaired by the loss of expression of *spsD* as determined by the comparison of colony size and zones of clearing (figure 36).

This ability of the *spsD* mutant TF7-D2 to secrete normal levels of the extracellular enzymes was consistent with the presence of an *exeD* homologue in strain TF7. To confirm the presence of this second *pulD* homologue, PCR was used to generate a fragment of approximately 500 bp from the *exeD* gene of *A. hydrophila* Ah65. This *exeD* DNA was radioactively labeled and used as a probe in Southern blot studies against a chromosomal digest of TF7. The results in figure 35B show the presence of a homologue of *exeD* in strain TF7 on a *KpnI* fragment of approximately 1.7 kb (figure 35B). In comparison, the *spsD* gene was present on a 3.2 kb *KpnI* fragment (figure 33 lanes 1 and 2). This result was consistent with the conditions of high stringency used in this study, and the low level of DNA identity between *spsD* and *exeD*.

Complementation of the TF7-D2 marker exchange mutant with the *spsD* gene

Plasmid pBSS100-3 (figure 34) was initially transformed into *E. coli*, an event that led to a large amount of cell lysis and instability within this foreign host. From this result we concluded that the *spsD* gene was active.

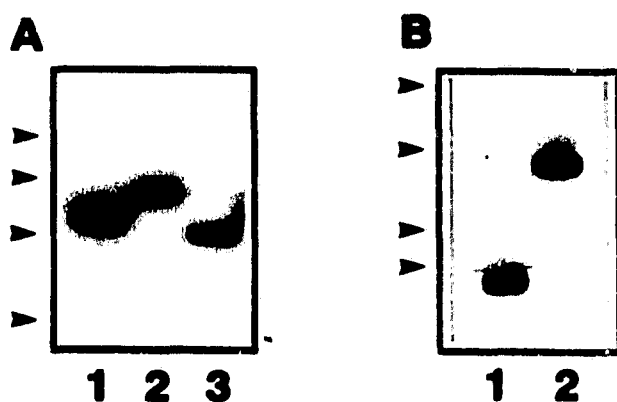


Figure 35. (A) Southern blot of a *Bam*HI-*Bgl*III double digest of *A. hydrophila* chromosomal DNA, showing the correct insertion of the kanamycin resistance cassette during the generation of marker exchange mutants. *spsD* mutant TF7-D2 (lane 2) shows an increase in size compared to the TF7 parent (lane 1) due to the addition of the 1.3 kb insert. The double mutant TF7-DS (lane 3) shows an overall decrease in size compared to TF7 resulting from the addition of the 1.3 kb kanamycin resistance cassette, but the deletion of a 2.44 kb fragment that includes the 3' end of the *spsD* gene and 5' end of the *ahsA* gene. (B) Southern blot of a *Kpn*I digest showing the presence of a second *pulD* homologue in *A. hydrophila* strain TF7 (lane 1), and the positive control containing chromosomal DNA from *A. hydrophila* strain Ah65 (lane 2). A fragment from the *exeD* gene of *A. hydrophila* strain Ah65 was generated by PCR amplification and labeled using nick translation (see text). Arrow heads on the left indicate the Lambda standards in kilobases, from the top, (A) 9.42, 6.68, 4.36, and 2.32, and (B) 6.68, 4.36, 2.32, and 2.03.

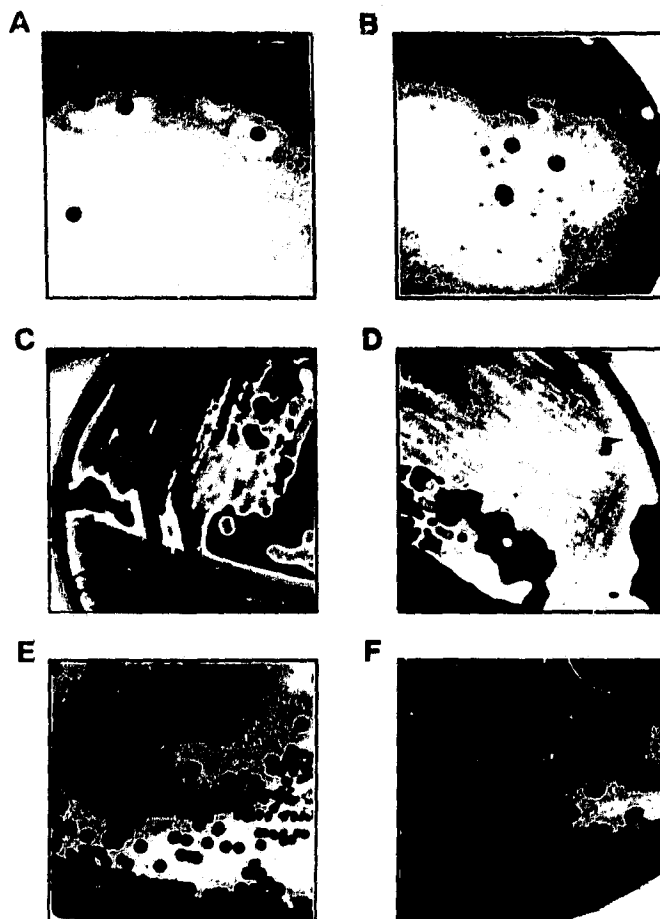


Figure 36. Detection of extracellular enzyme activity from the parent *A. hydrophila* TF7 and the marker exchange mutant TF7-D2 to show that secretion of these enzymes was not affected by mutation of the *spsD* gene. Comparisons were made between colony size and zones of clearing on LBA plates containing the required substrates for extracellular enzyme activity. Boxes A, C, and E show the parent cell, whereas boxes B, D, and F show the mutant TF7-D2. Plates containing the substrates were, blood (A and B), starch (C and D) and casein (E and F).

Complementation of the *A. hydrophila* marker exchange mutant TF7-D2 was then achieved using the fragment from plasmid pBSS100-3 inserted into the broad host range vector pAT19 (Table 3), and conjugated back into the parent strain. Following conjugation of the *spsD* gene into the TF7-D2 mutant, phenotypic alterations included; (i) a return to the parent auto-aggregation positive phenotype; (ii) reinstatement of normal growth at 37°C; (iii) the presence of large amounts of S-protein in samples isolated by the low pH glycine extraction of whole cells (figure 37A lane 9), and in outer membrane fractions (figure 37A lane 4). Figure 37B shows a Coomassie blue stained SDS-PAGE gel of outer membrane fractions of both *E. coli* harboring the vector alone (lane 1), and the complete pBSS100-3 construct (lane 2). A protein band is present in both lanes 1 and 2 at the same position of approximately 80.1 kDa, which is close to the Mr predicted by the nucleotide sequence of the *spsD* gene (79.8 kDa). However, a clear quantitative difference can be seen in lane 2 containing the pBSS100-3 sample. Indeed, there are a number of lower molecular weight products located below the major band of 80.1 kDa, that are not present in lane 1. These lower molecular weight bands may represent degradation products from the overexpressed SpsD polypeptide.

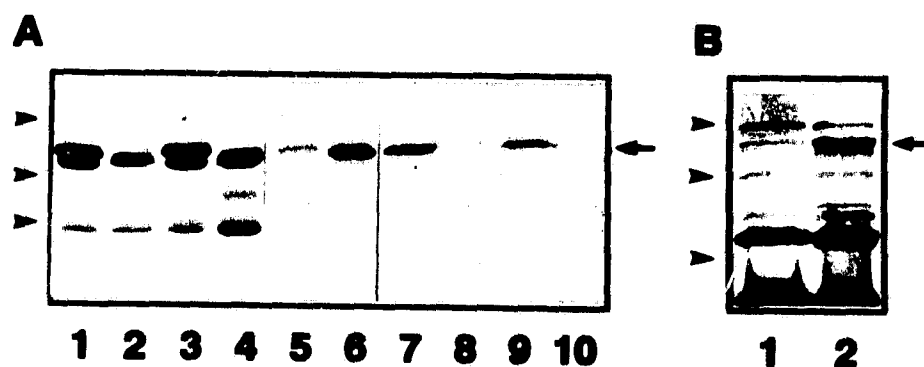


Figure 37. Coomassie blue stain of samples separated by SDS-PAGE showing the localization of the S-protein (arrow) from the parent strain TF7, marker exchange mutants TF7-D2 and TF7-DS, and *spsD* complemented TF7-D2D. (A) Outer membrane protein profiles of TF7, TF7-D2, TF7-D2D, and TF7-DS lanes 1 to 4 respectively. Lanes 5 and 6, contain periplasmic fractions from TF7 and TF7-D2, respectively. Lanes 7 to 10 show glycine extraction's from the same strains and in the same order as lanes 1 to 4. (B) Coomassie blue stained outer membrane fractions from *E. coli* DH5 α expressing SpsD from plasmid pBSS100-3 (lane 2) (arrow) and *E. coli* harboring the vector alone (lane 1). The samples shown in panel A were separated on a 12.5% SDS-PAGE gel, and the samples in panel B were separated on a 10% SDS-PAGE gel. Indicated on the left are the positions of the molecular weight markers from the top (A) 66,200, 45,000, and 31,000, and (B) 97,400, 66,200, and 45,000.

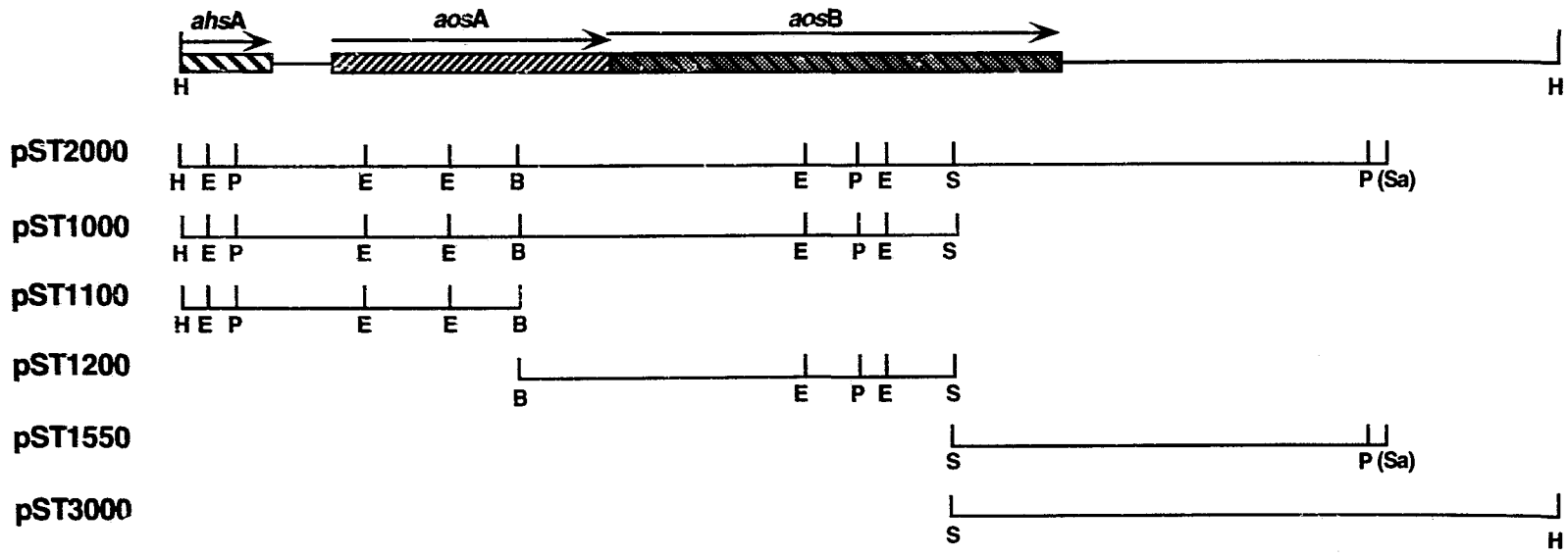
V. Identification and Characterization Of Two Genes Involved In O-Polysaccharide Side Chain Secretion

The homogeneous LPS O-polysaccharide side chain phenotype has been found in conjunction with the presence of an S-layer in all high virulence strains of *A. hydrophila*. Wild type O-polysaccharide side chains have been shown previously to be important for the anchoring of the S-layer to the cell surface, and so both these macromolecular structures are potentially important virulence factors in the pathogenicity of *A. hydrophila*. With continued sequencing in the 3' direction from the *ahsA* S-protein gene, we have identified a region containing two ORF's. These ORF's encode proteins showing high homology to a family of ATP-binding cassette (ABC)-type transporters termed the ABC-2 subfamily (242). Bacterial ATP-exporters typically consist of two components; (i) hydrophobic, polytopic integral membrane proteins that share similar hydropathy profiles; and (ii) more hydrophilic proteins, that contain a consensus sequence for nucleotide binding (84). We describe here the cloning and sequencing of two genes from *A. hydrophila* TF7 encoding both components of the ATP-exporters, and show that they are essential for the normal secretion of the O-polysaccharide side chains, and hence, in the anchoring of the S-layer to the cell surface.

Nucleotide sequence analysis of the *aosA/B* region

The strategy used for the complete sequencing of the *aosA/B* (*Aeromonas* O-polysaccharide secretion A and B) region in both directions is outlined in figure 38. The DNA sequenced in this study was carried on a *HindIII-SalI* fragment derived from clone EMBL 3S which contained the S-protein gene (*ahsA*) as described above. The *HindIII-SalI* insert in plasmid pST2000 carried a portion of *ahsA* and flanking DNA 3' to *ahsA* (figure 38).

Figure 38. Constructs used in the cloning and nucleotide sequencing of the *A. hydrophila* *aosA* and *aosB* genes. The position and organization of the *aos* genes is indicated relative to the S-protein *ahsA* gene, which contains a *Hind*III endonuclease site within its 3' region. Plasmid pST2000 was isolated from EMBL 3S as indicated in the text, and was used to generate the sub-clones as shown. When it was found that a rearrangement had occurred at the 3' region of pST2000, plasmid pST3000 was isolated for further sequencing. All of the endonuclease sites shown were used in the generation of sub-clones in the sequencing of the region. B = *Bgl*II, E = *Eco*RV, H = *Hind*III, P = *Pst*I, S = *Sac*I, and Sa = *Sal*I. Endonuclease sites in parentheses originate from vector multiple cloning sites.



0.5 kb 

For sequencing, smaller fragments of this *HindIII-SalI* insert were subcloned using the restriction sites shown in figure 38. In regions where there were no restriction sites present e.g., the large *BamHI-EcoRV* fragment, synthetic oligonucleotides were used as primers for sequencing. To verify that no small DNA fragments were lost during the sub-cloning process, the endonuclease sites that were used were also sequenced through using synthetic oligonucleotides. Sequence analysis of the 2315 bp region beginning at the poly-T sequence following the rho independent terminator of the *ahsA* gene revealed the presence of two complete ORF's, with the beginning of a third possible ORF extending downstream from the second of these ORF's. The nucleotide sequence of this region was verified in both directions, and is presented in figure 39.

The *aosA* ORF contains 816 nucleotides with a G/C content of 49.6%, while the *aosB* ORF contains 1314 nucleotides, with a G/C content of 50.4%. The predicted initiation site for *aosA* occurs 203 nucleotides downstream of the stop codon for *ahsA*, a region which includes the putative rho independent terminator for this gene. Included within this flanking DNA 5' to the *aosA* gene is a conserved -10/-35 sigma 70 like promoter sequence, and a typical purine rich Shine-Dalgarno ribosome binding site (figure 39). The second ORF in this *aos* region, *aosB*, also contains a potential Shine-Dalgarno ribosome binding site, and an initiation codon located 8 nucleotides 5' to the stop codon for the *aosA* gene. The absence of an identifiable palindrome capable of forming a stem loop structure at the end of the *aosB* ORF, and the presence of a purine rich region located the appropriate distance upstream from an initiation codon suggested the possibility of a third ORF following *aosB* (figure 39). However, continued sequencing in the 3' direction from the end of the *aosB* gene identified a possible DNA rearrangement that had

Figure 39. The nucleotide sequence of the *aos* region from *A. hydrophila* strain TF7 including the poly T sequence of the *ahsA* gene transcriptional terminator, to 37 nucleotides 3' of the *aosB* gene stop codon. Conserved sigma 70 promoter -10, -35 regions are shown (underlined). The poly-T region following the terminator sequence from the *ahsA* gene is dotted underlined. Initiation codons for each ORF are shown in bold type, and Stop codons are represented by an asterisk. Putative Shine-Dalgarno ribosomal binding sites can be seen upstream of each ORF and are double underlined. The putative ribosome binding site and initiation codon for the third ORF downstream of the *aosB* gene is shown in bold type.

-35

1 TTTTATTTGGCGATTTCACCATCGTCAGTGGATGTCGCACCCCGTGATGGGTGAAGGGATGCTGCGCAAGGATGGATGCTCTCTCT 90

-10

91 GGCAGGTTATGTTATTTATCGCGCCGCTCGGTTTCAGCCGGGGGCGAGTTTTTTAAAGGAGTCTCATGATCCGAGATGACAGCATTTCAT 180
M I R D D S I H

181 CCGCGTCGTATACTTCCCTGGTGGTCCCAGCGCCATCTTATCGCTACCCTGGTGGCAGCTGAGSTGCCAGCCGATATCGTGGTTCACCTG 270
P R R I L A C W S Q R H L I A T L V R R E V A S R Y R G S L

271 TTTGGAAATTTATGGCGTGTTCACGCCACTTTTATGTTGGCGGTATATACCTTTATTTTCAGTGGTATTTAAGCCCGTTGGACC 360
F G N L W A L F T P L F M L A V Y T F I F S V V F K A R W T

361 GGCGGAGTGATTCTCGCAGTGAATTTGCCCTGGTGGTGTGTTGCCGGCTTGATGGTTTCAACCTCTTTCCGAGTGTTTAACCGGGCT 450
G G S D S R S E F A L V L P A G L M V F N L F S E C F N R A

451 CCTCGTCTGATCTGGAGAATGTCAACTACGTCAAGAAAGTGGTATTCCTCTCGATATCTGCCATGGGTTGCATTAGGCAGTGCCATG 540
P R L I L E N V N Y V K K V V F P L D I L P W V A L G S A M

541 ACCAATTTGCTGATCAGTTTCTAGTGTGGATTGCATTTTATATGATTGCCTTTGGCATGCCGTCTGTACCCTATTGCTTTTCCCCATT 630
T N L L I S F L V W I A F Y M I A P G M P S V T V L L F P I

631 GTGATTTTCCCGCTGGTATTTTGGTTCATGGGCATATCATGGCGCTGGCTGCAATGGGGTCTATTTGCGAGATCTCTCACAACCTGTA 720
V I L P L V F L V M G I S W A L A A M G V Y L R D L S Q L V

721 GGGGTGATCAGCACCATCTCTGATGTTCCCTCGCCAAATTTCTACCTGTTGAGGCGCTGCCGGAAGAGTATCGCCATTTGGTGAACGCC 810
G V I S T I S T C I L M F L S P I F Y P V E A L P E E Y R H W L N A

811 AATCCACTAACCCCGTATGTCACAGGTGCCGGATGTGCTCTATTTGGGAAATTCCTTCTGGGAGTCATACGGTATGACATGATT 900
N P L T P V I A Q V R D V L Y W G K L P S W E S Y G M T L I

901 GCCGGTATGGCGGTAATGTATGCCGGATTGGCTTGTCCAGAAAACCGTAAAGGGCTTTGCCGATGCTCTGACAGAGTCCGCGATTGA 980
A G M A V M Y A G F A L F Q K T R K G F A D V L *
M C S D R V A I E

991 AGTGGTGGTTTGGAGCAATGTTATCAGGTTTATGAACAGCCGATGATCGACTCAAGCAGTTTGCATCCCCCGCTTCCAGGATGTG 1080
V R G L S K C Y Q V Y E Q P I D R L K Q F V I P R F S R M C

1081 TCGCCGAGTTGCCAGCAATATTTTCGTGAGTTTGGGCGCTCCGGACATGATTTCACGATTAAGGGTGGAGACCTGTTGGGATTAT 1170
R R V A R E F R W A I D F T I K K G E T C V G I I

1171 CGCCATAACGGGGCGGAAATCCACGCTGCTCCAGCTGATTGCGGCACCCTTAATCCATCTCATGTTGAAGTCAACGTCAATGGCCG 1260
G H N G A G K S T L L Q L I C G T L N P S H G E V N V N G R

1261 TATTGCGGCTTGCTTGAGCTTGGGCTGGATTTAATCCGGAGTTCAGTGGGCGTGATAATGCTATCTCAATGCCAGTGTCTTGGGGTT 1350
I A A L L E L G A G F N P E F S G R D N V Y L N A S V L G L

1351 GAGTAAGAGGATATTGACGCAAGTTTGTGAAATGCCCGTTTTCGGCAGTCCGGGATTTTATGGAGCAACCCGTCAGACCTATTC 1440
S K E D I D A R F D E I A R F A D I G D F M E Q P V K T Y S

1441 CAGTGGCATGTACGTCGCTTGGCGTTTGGCGTACAGGCGTGCATAGATCCTGAAATTTGATCGTCGATGAAGCGCTGCCCGTTGGCGA 1530
S G M I A V R L A F A V Q A C I D P E I L I V D E A L P V G D

1531 TATCGGTTTCCAATACAAGTGTTCAGCGTATGGAGGCTTGAAGCAAGGGTGAACCATTTGATGGTGACCCACTCAACAGGCAG 1620
I G F Q Y K C F K R H E A L K A K G V T I L M V T H S T G S

1621 TATTCTGGAGTATGCCGATCGCTGTCTGGTGTGGAGGGGGCCGCTTATTGGTGATACCCTGATGTGCTGCCTGCAGGGATGGCGTA 1710
I L E Y A D R C L V M E G G R L I G D T T D V L P A G M A Y

1711 TGAGAAAGGCATGATCTTGTCCGAGGAGCAAGCTGATACCTTGGCATTGCCAGATATCGAGAACGATGGTGGTATGCCCATCAGCTAGT 1800
E K G M I L S Q E Q A D T L A L P D I E N D G D C P S Q L V

1801 TCTACTGAAAAGCAGAAAATGAGGCCAATCTGGCGCTGGGTGAGAAGCGATTTGGTCTGCCCGCGGATATCGCTGGCCTGACCAT 1890
L L E K Q K N E A N L A L G E K R F G S A R A I I A G L T I

1891 TGCAAGGCGGATGGTACACCTTTTCATGAAGAACCCTTGTAAATCAGGTGAAACTCTGACGCTGCGTTTTGAGCTCTGGTCCGAGCCA 1980
V K A D G T P F H E E P L V K S G E T L T L R P E L W S S Q

1981 GAGCATTGAAGATGTGGCATTAGGACTTTCATGACCCGGTCCAAGGTAGTGATTTTGGGGTATAGCAATATCGCTGCTGGTCAACC 2070
S I E D V A L G L S L S R V Q G S D I W G D S N I A A G H P

2071 CATAACCTTGAACCCGGTCCGCAACTGGTGACTTATCAAGTACCTTGCCAAATTAATAGCGGCGATTACTTGTTCGCTGTGGTGGC 2160
I T L K P G R Q L V T Y Q V T L P I N S G D Y L L H C G L A

2161 CTCTTTAAGGGAGGGAGCGTGAAGAAGTTCATCAACGTAGCCGATGCAGGCGATAAATCTGGTCTTCCAGAGAGCTGGGTGGCCT 2250
F L K G G E R E E L D Q R R P M Q A I K F W S S R E L G G V

2251 CGTACACGCGCTATTACTGTTTTGGCCTGAATTTTCGAAATTATCAATGCTGGCTGCTTTTCT 2315
V H A P I T V L A *

occurred approximately 200 bp's downstream of *aosB*. By comparing products generated by PCR from the *A. hydrophila* TF7 chromosome, and the EMBL 3S clone, we were able to verify that a rearrangement had indeed occurred (data not shown). A *HindIII-SacI* fragment was therefore cloned from the *A. hydrophila* TF7 chromosome (pST3000), and the first 300 bp of the ORF extending downstream from *aosB* was determined in one direction to check for homology to any previously identified polypeptides (see below).

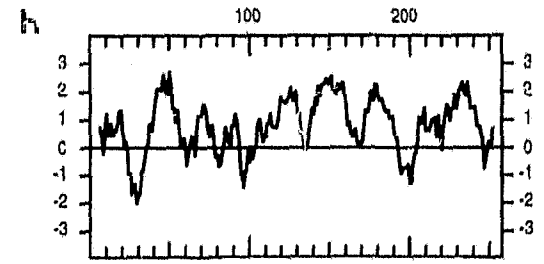
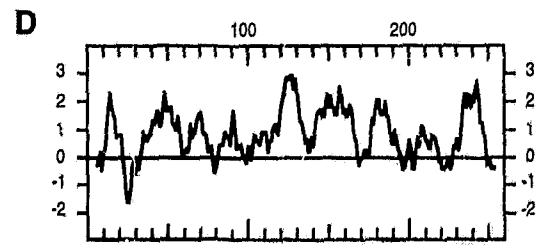
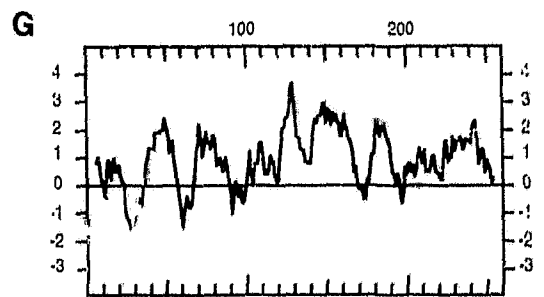
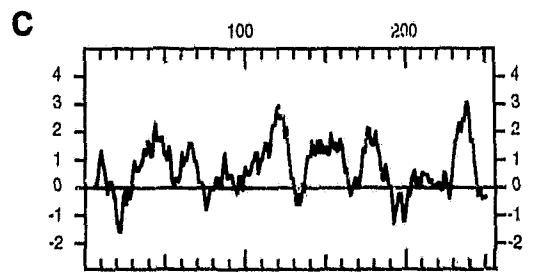
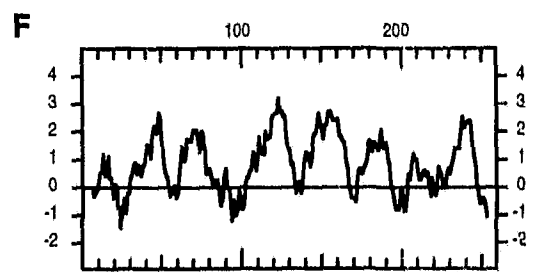
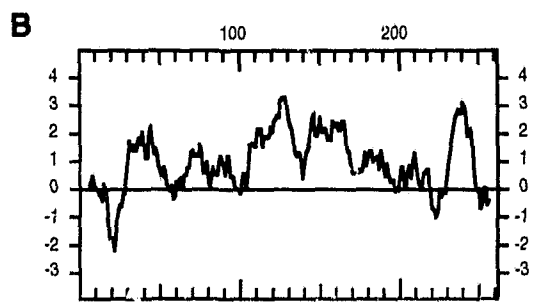
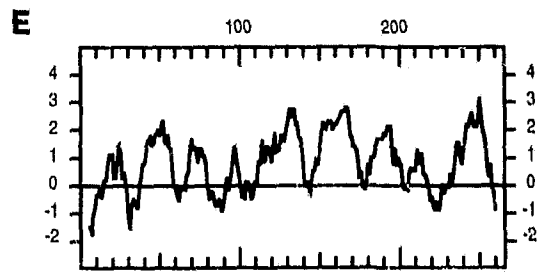
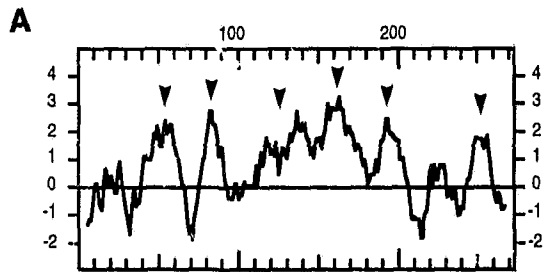
Predicted structure of Aosa

The *aosA* gene encodes a 272 amino acid protein of Mr 30,912 which is lacking an N-terminal signal sequence (figure 39). Aosa is hydrophobic with a non-polar amino acid residue content of 60.6%, and a predicted basic isoelectric point (pI) of 9.76. The Kyte and Doolittle (172) hydrophobic index using an interval of nine amino acids gives a grand average hydrophobicity score of 7.52. Analysis of the deduced sequence by the method of Klein *et al.* (153) classified Aosa as an integral membrane protein, and examination of the Kyte and Doolittle hydrophobicity plot showed the presence of clusters of hydrophobic residues consistent with a polytopic membrane protein (figure 40). Furthermore, the Rost and Sander algorithm (252) predicts a high α -helical trans-membrane content of 54.8%, capable of forming six or seven membrane spanning regions. The algorithm of Argos and Rao (11), also predicts six membrane spanning regions within Aosa, while the procedure of Eisenberg *et al.* predicts seven membrane spanning domains (81). The regions most likely to form the six membrane spanning domains are arrowed in figure 40.

Predicted structure of AosaB

The *aosB* gene encodes a predicted 438 residue protein of Mr 48,142

Figure 40. Comparison of Kyte-Doolittle hydropathy profiles of the integral membrane components from various polysaccharide exporters. AosaA homologs include ORF261 of *E. coli* 09 (B), RfbA of *S. marcescens* (C), RfbA of *K. pneumoniae* (D), BexB of *H. influenzae* (E), KpsM of *E. coli* K1 (F), RfbD of *Y. enterocolitica* (G), and RfbH of *V. cholerae* (H) (EMBL accession #S28474) (39, 167, 168, 224, 225, 284, 328). Putative membrane spanning domains contained in the primary sequence of AosaA are indicated by arrows. All the homologs contain similar hydrophobic regions in their primary sequences which are indicated by positive values. The x axis indicates the residue number, and the y axis indicates relative hydrophobicity.



lacking an N-terminal signal secretion sequence. AosB has a predicted acidic pI of 5.32 and contains 43.6% hydrophobic residues. The Kyte and Doolittle index for an interval of nine amino acids gives a grand average hydropathicity score of -0.54. By the method of Rost and Sander AosB has a predicted α -helical content of 26.9% and contains two regions capable of forming membrane spanning domains located close together, between positions 132 and 174 (252). However, application of other protein structural prediction algorithms (Argos, Eisenberg, Klein) predict that the protein is membrane associated rather than being an integral membrane protein. The Ratio 3 value (17) of 0.96 is also consistent with AosB being a peripheral membrane protein. AosB contains the conserved sequences 70-GXXGXGKST and 148-ILIVD which comprise the consensus Walker box and Rossman fold motif that form an ATP-binding pocket (figure 41 and 42) (251, 315).

Similarity to bacterial ABC polysaccharide transporters

The tandem localization of genes encoding a polytopic membrane protein and an apparent ATP-binding protein was similar to the arrangement of genes for a number of ATP-driven bacterial transport systems. Not surprisingly searches of the SwissProt database using the algorithm of Smith and Waterman (277), and Genbank using Blast (6), identified significant sequence similarity between the proteins encoded by the *aos* region, and proteins involved in bacterial polysaccharide transport across the cytoplasmic membrane. Examples displaying the highest homology to the Aos A/B polypeptides include the ORF261 and ORF431 proteins of *E. coli* O9 (284), the RfbA/B proteins of *K. pneumoniae* (39), and the RfbA/B proteins from *S. marcescens* (39). In the case of the integral membrane components, the *E. coli* ORF261 protein which is thought to be a component of a putative ABC

transporter, shows approximately 27% identity and 50% similarity over the entire length of Aosa, while the proteins of both *S. marcescens* and *K. pneumoniae* display approximately 25% identity and 50% similarity. The Kyte and Doolittle hydrophobicity profiles of Aosa, and a number of other proteins involved in polysaccharide export, including the ORF261 encoded protein and *S. marcescens* RfbA all display strong similarity (figure 40).

In the case of the ATP-binding components, AosB at 438 residues is similar in length to the *E. coli* ORF431 encoded protein, and both are considerably longer than the 200-250 residue length of other ATP-binding proteins in this group of exporters. The RfbB proteins of *Serratia* and *Klebsiella* display 42% identity and 63% similarity over 230 residues with the N-terminal segment of the AosB sequence. The 308 residue AbcA protein of *A. salmonicida* shows the greatest homology to AosB, with a 51% identity and 69% similarity. but this homology is also limited to the first 230 amino acid residues of AbcA. The 431 encoded protein of *E. coli* shows intermediate homology in this ATP-binding region of the protein (47% identity and 65% similarity). The Kyte and Doolittle plots of AosB, AbcA, and the ORF431 protein are very similar over the N-terminal 230 residues, presumably because of the ATP-binding function of this region of the various proteins (figure 41). However, AbcA has a putative leucine zipper followed by a highly basic sequence from residues 210 to 260, distinguishing this protein from both AosB and the ORF431 proteins.

Interestingly, although the Kyte and Doolittle hydrophobicity profiles of AosB and the ORF431 protein of *E. coli* are similar over their entire length, the C-terminal sequence homology is limited to a region of 20 amino acid residues between residue number 381 and 400 of AosB. In this region of the protein there is 40% identity and 75% similarity. Within this region, there is a short

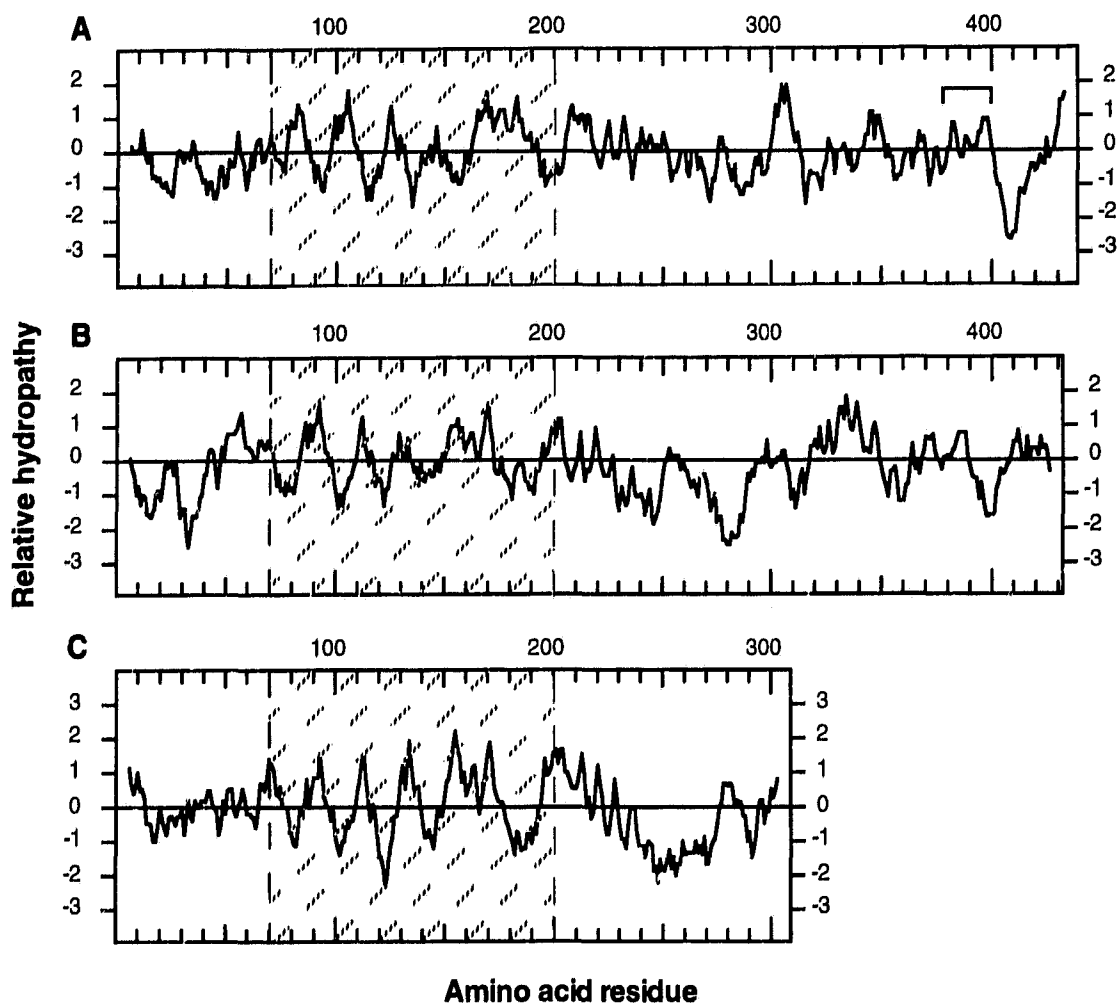


Figure 41. Comparison of Kyte-Doolittle hydropathy plots for AOsB (A), ORF 431 of *E. coli* 09 (B), and AbcA of *A. salmonicida* (C). There is some C-terminal sequence homology between AOsB and ORF431 that is limited to 20 amino acid residues (40% identity and 75% similarity) located between residues 380 to 400 of AOsB. Within this 20 amino acid region, there is a short sequence (NSGDYLL) which shows 100% identity between AOsB and ORF431. The region from approximate amino acid residue 70 to 200 contains the ABC-cassette of these proteins (shaded). Positive values indicate hydrophobicity.

Figure 42. Primary amino acid sequence alignment of the ATP binding region from AosB of *A. hydrophila*, AbcA of *A. salmonicida*, ORF431 of *E. coli* ¹⁰, RfbB of *S. marcescens*, RfbB of *K. pneumoniae*, BexA of *H. influenzae*, KpsT of *E. coli* K1, RfbE of *Y. enterocolitica*, and RfbI of *V. cholerae*. The ATP binding pocket or Walker box (315) consists of two conserved sites present in a large variety of ATP binding proteins responsible for the transport of a number of different substrates, and are indicated by the consensus sequences, GXNGAGKSTL where X is a basic residue, and ILIIDE.

AosB	MC--SDRVAIE	VRGLSKCYQV	YEQPIDRLKQ	FVIPRFSRMC	RRVARQYFRE	49
ORF 431	M-----SIK	VQHVGVKAYKY	YPSKWSRVIE	KLLP--GDKP	RHSKK-----	37
AbcA	MS-E--PVLA	VSGVVKSFPI	YRSPWQALWH	ALNP---KAD	VKVFO-----	39
KpsT	M-----IK	IENLTKS---	YRTPTGR--H	YV-----	-----	20
BexA	M-----IR	VNNVCKK---	YHTNSGW--K	TV-----	-----	20
RfbE	MRFBEMTSLI	FKNVMTSYPI	YNAHSQSLRN	QLVRVSTGGR	IGGSRGEVVT	50
RfbI	MIELSNVNLH	YPVPGHFSHS	LQTTISSKIG	GVLGSSSAKD	KEMKY-----	45
RfbB K. pneumoniae	MH----PVIN	FSHVTKEYPL	YHHIGSGIKD	LIFHPKRAFQ	LLKGR----K	42
RfbB S. marcescens	MS----VAIE	FKNVTKRYPL	YHHIGSGIYE	LIFNPRRALS	LLSGR----S	42
Consensus	M.....I.	...V.K.Y..	Y.....	50
AosB	FWALRDIDFT	IKKGETVGII	GNGAGKSTL	LQLICGTLNP	SHGEVNVNGR	99
ORF 431	--WVKDLNFS	IEPGEAVGIV	GNGAGKSTL	LKLLTGTTQP	TKGSEIIGGR	86
AbcA	--ALRDIELT	VYRGETIGIV	GNGAGKSTL	LQLITGVMQP	DOGQITRTRGR	87
KpsT	---FKDLNIE	IPSGKSVAFI	GNGAGKSTL	LRMIGGIDRP	DSGKIIITNKT	67
BexA	---LKNINFE	LQKGEKIGIL	GNGAGKSTL	IRLMGVEPP	TSGTIERSMS	67
RfbE	VTALDNISFE	LNSGDSVGLI	GNGAGKSTL	LRTMAGIYPA	SSGIIREGS	100
RfbI	VHALRDINK	LEDSSRLGII	GNGAGKSTL	LRLLSQVYPP	TSGKVTIEGK	95
RfbB K. pneumoniae	YLAIEDVSFT	VKGGEAVALI	GNGAGKSTL	LGLVAGVIKP	TKGIVTTEGR	92
RfbB S. marcescens	YLAIEDICFQ	VQKGESVALI	GNGAGKSTL	LGLVAGVMKP	SSGIVHVQGR	92
Consensus	..AL.DI.F.	...GE.VGII	G.NGAGKSTL	L.L..GV..P	.SG.I...GR	100
AosB	IAALLELGAG	FNPEFSGRDN	VYLNASVJGL	SKEDIDARFD	EIAAFADIGD	149
ORF 431	VAALLELGAG	FHPDFASRON	VYMS...MAGL	GREETERLMP	EIEAFADIGD	136
AbcA	VVGLLELGSG	FNPEFAGREN	IFFNGAILGM	SQREMDRLE	RILSFAAIGD	137
KpsT	ISWPFVGLAG	FOGSLIGREN	VKFVARLYA-	KQEELKEKIE	FVEEFAELGK	116
BexA	ISWPLAFSGA	FOGSLIGMDN	LRFICRLYD-	VDPDYVTRFT	K--EFSELGD	114
RfbE	VATVFELGAG	MPELGSVEN	IMFMILLGN	SVASAKSKIP	EIEEFELIGD	150
RfbI	ISALTDFTLG	MDPNAIGLVN	IEIRLVFMGC	TFKEAQAQVE	EIVAFSELGE	145
RfbB K. pneumoniae	VASMLELGGG	FHPELIGREN	IYLNATLLGL	RRKEVQORME	RIIEFSELGE	142
RfbB S. marcescens	VASMLELGGG	FHPELIGREN	IRLNATLLGL	RRKELKQRLD	KIIEFSELGE	142
Consensus	VA..LELG.G	F.PELIGREN	I.....LLG.	...E...R..	.I.EEL.ELGD	150
AosB	FMECPVKIYS	EGMVRVIFA	VQACIDPELL	IIDEALPVGD	IGFDYKCFKR	199
ORF 431	YIEEPVRIYS	EGMVRVIFA	VATASRPDIL	IIDEALSUGD	SREDAKCYAR	186
AbcA	FIDCPVKIYS	EGMVRVIFA	VIINTDPDVL	IIDEALAVGD	DAEFOKCYAR	187
KpsT	YFDMPVKIYS	EGMVRVIFA	LSMAFKFDYI	IIDEVAVAGD	AREFEKLAQL	166
BexA	YLVEPVKIYS	EGMVRVIFA	LSLSVEFDYI	IIDEVAVAGD	SREFEKCKYE	164
RfbE	FLVIEVRIYS	EGMVRVIFA	VATSMRPEIL	IIDEAFGIGD	AAFEKAEKR	200
RfbI	FINLPVRIYS	EGMVRVIFA	ISTHFTPDIL	IIDEVAVAGD	ETEFREKALS	195
RfbB K. pneumoniae	FIDEPIRVYS	EGMLAMLCFS	VISQVEPDIL	IIDEVAVAGD	LAEFAKCIQT	192
RfbB S. marcescens	FIDEPIRVYS	EGMLAMLCFS	VITQVDPDIL	IIDEVAVAGD	LAEFOKCIKT	192
Consensus	FI..EVR.VS	EGM..RVIFA	V.....PDIL	IIDEVAVAGD	..EFO.KC..R	200
AosB	MEALKARGVT	ILMVVSTGSG	ILEYADRCLV	MEGGRLIGDT	TDVLPAGMAY	249
ORF 431	LDFKEQGT	LLLVSHSAGD	IVKHCORAIF	LKNGDICHMDG	TARDVTNRYL	236
AbcA	LKQLSQGVT	ILLVSHSAGS	VIELCDRAVL	LDRGEVLLQG	EPKAVV-HNY	236
KpsT	FKE-RHKES	FLMVSHSLNS	LKEFCDAIV	FKNSYIIG--	-----YY	205
BexA	LFE-KRKDRS	IILVSHSASA	MKSYCDNAV	LENGIM----	-----HHF	202
RfbE	MRDWIAGSDI	FVFASHDRSL	IKKLCNRIFR	LEHGLIYEE-	-----	239
RfbI	LESLIKKSRM	VVLSSHDLNA	IKQYCDQAV	MEKGEIVFNG	-----T	236
RfbB K. pneumoniae	IRDFKKRGVT	ILFVSHMSD	VEKICDRVIW	IENHRLREVG	-----SA	234
RfbB S. marcescens	INEFKSKGVT	ILFVSHNLED	VEKICDKVIW	IENHKMKAMG	-----DA	234
ConsensusK.KG.T	IL.VSH....CDRAI.	.ENG.....G	-----..	250

sequence (NSGDYLL) which shows 100% identity between AosB and ORF431. The region incorporating this sequence is indicated on the hydropathy profile for AosB (figure 41), and is included in a region that shows high similarity between the two polypeptides. Immediately following this 20 amino acid sequence at the C-terminal, there is a region of high hydrophilicity that is common to both polypeptides. Searches of the Swiss Prot and Genbank databases (6, 277) with the C-terminal 184 amino acids of AosB failed to provide any further insight into the possible function of this region.

Figure 42 shows the primary amino acid sequence conservation between the N-terminal of AosB containing the ATP-binding region, and its polysaccharide exporter homologs.

Preliminary sequencing of the third *aos* ORF

Nucleotide sequencing downstream of *aosB* was continued in one direction for 300 bp's (not shown), and indicated that there is a possibility of there being an ORF in this position. Searches of the data banks identified no homology with any previously characterized polypeptide associated with ATP transport systems. Interestingly though, some homology was found between the encoded amino acid sequence and a hypothetical 30.1 kDa protein from the regulatory region controlling the synthesis of the polysaccharide alginate in *P. aeruginosa* (142).

Phylogenetic analysis of AosA and AosB

To examine the evolutionary relationship between AosA and AosB and other bacterial ATP polysaccharide exporters, a phylogenetic analysis was performed using the method of Nei (214). Analysis of a number of inner membrane components from ABC-type 2 exporters (figure 43) showed that the

phylogenetic tree contains two branches. Aosa is on a branch together with the *E. coli* ORF261 protein and the RfbA proteins of *S. marcescens* and *K. pneumoniae*. Aosa appears however, to have diverged earlier in the evolution of this group of proteins. The other branch contains the membrane component of the LPS O-antigen exporters of *V. cholerae* and *Y. enterocolitica*, and the capsular polysaccharide exporters KpsM of *E. coli* and BexB of *H. influenzae*.

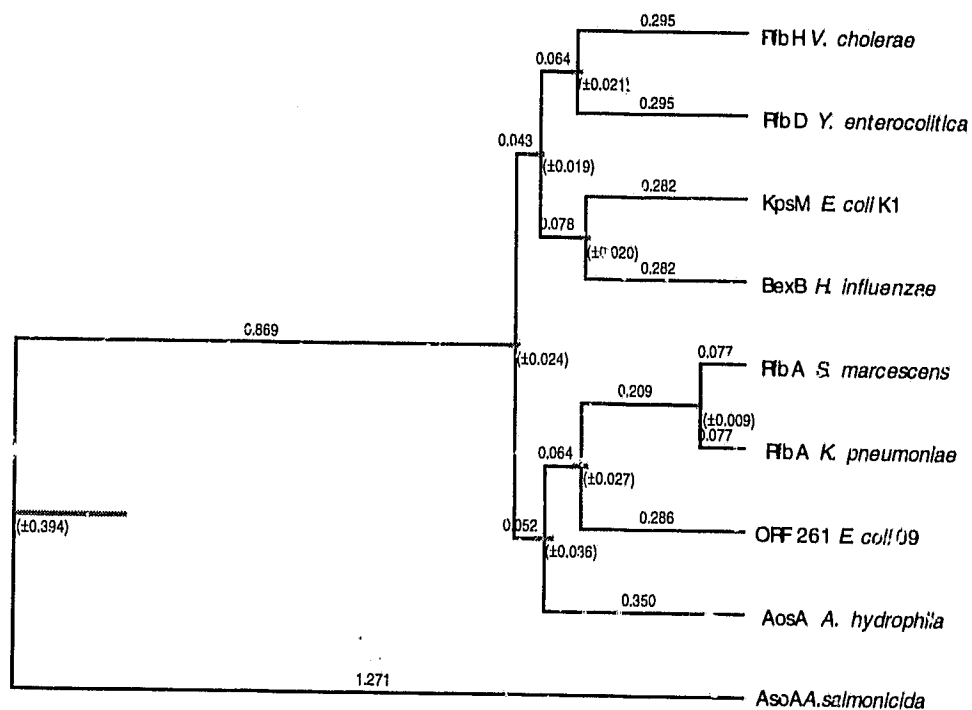
In the case of the putative ATP-binding proteins AosB and the ORF431 protein from the phosphomannomutase and GDP-mannose phosphorylase region from *E. coli* 09, they appear on the same branch of the phylogenetic tree, and both appear to have evolved earlier than the proteins on the other branch (figure 43). The proteins on this second branch include the O-polysaccharide involved proteins AbcA (*A. salmonicida*), RfbI (*V. cholerae*), RfbE (*Y. enterocolitica*), and RfbE (*K. pneumoniae*, *S. marcescens*), and the capsular polysaccharide involved proteins BexA (*H. influenzae*) and KpsT (*E. coli*).

Marker exchange mutagenesis of *aosA*

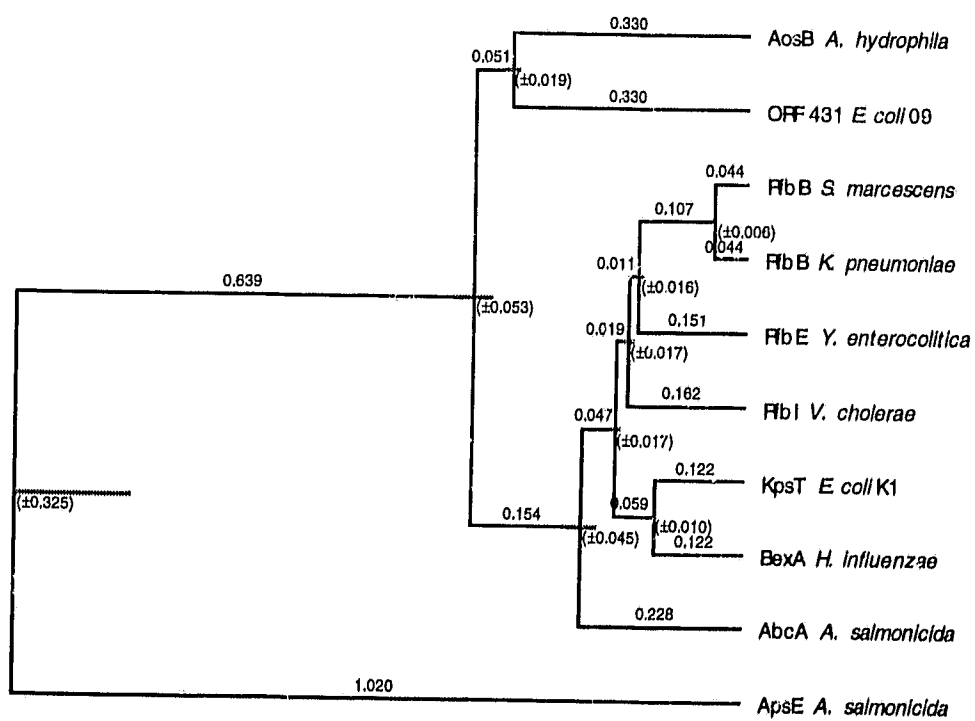
The similarity and evolutionary relatedness of Aosa and AosB to proteins involved in LPS biogenesis, especially to *A. salmonicida* AbcA, prompted us to determine the effect of mutagenesis of the transporter encoding region on the LPS structure of *A. hydrophila* TF7. To disrupt the *aosA* reading frame and halt transcription, a Kan^R cassette was inserted into the unique *Bgl*III site in plasmid pST1000, generating pKST1000 (figure 44) which was inserted into the suicide plasmid pSUP205, and conjugated into *A. hydrophila* TF7. Following isolation of mutant TF7-OS1, a Southern blot of a *Kpn*I-*Bam*HI chromosomal digest of both the wild type TF7 and mutant TF7-OS1 was performed to confirm that a correct double cross-over event had

Figure 43. Phylogenetic tree analysis of Aosa (A) and AosB (B) proteins and their homologs using the unweighted pair group method using arithmetic means (214). Proteins used in the phylogenetic analysis include, RfbH/I of *Y. enterocolitica*; RfbD/E of *V. cholerae*; KpsM/T of *E. coli* K1; BexB of *H. influenzae*; RfbA/B of *S. marcescens*; RfbA/B of *K. pneumoniae*; ORF261/431 of *E. coli* O9; and of AbcA *A. salmonicida*, and for rooting the trees, the ApsE and AsoA proteins of *A. salmonicida* were used (216, 217). Sequences are paired on the basis of homology, while the horizontal length of the lines is an indication of the genetic distance between the sequences. Shaded areas represent error bars of the branch points and numbers in brackets represent the standard errors.

A



B



occurred. In figure 44 (inset), lane 1 shows a strongly reacting band of approximately 4.5 kb for the TF7 parent, while in lane 2, the mutant TF7-OS1 shows a reactive band which has increased in size by approximately 1.3 kb as a result of the insertion of the Kan^R.

The LPS of *A. hydrophila* TF7 is characterized by the presence of O-polysaccharides of constant chain length. In SDS-PAGE analysis, the effect of this is that complete *A. hydrophila* TF7 LPS typically runs as a doublet (figure 42A lane 1). Mutation of *aosA* resulted in the biogenesis of an LPS which was deficient in O-polysaccharide (figure 45A lane 2). This mutation was complemented where plasmid pST2000 subcloned into the broad host range vector pMMB206 was conjugated into *A. hydrophila* TF7-OS1 to provide *A. hydrophila* TF7-OS2. Figure 45B lane 2 shows that mutant TF7-OS2 produces LPS identical to wild type TF7 LPS (lane 1).

Conservation of the *aos* region amongst S-layer producing *Aeromonas* species

Previous studies performed on the LPS profiles from various *Aeromonas* spp. have shown that strain TF7 displays prominent O-antigen side chain bands generally arranged as a doublet (74, 155). Figure 47 is a silver stain of the LPS of a variety of *A. hydrophila* and *A. veronii* biotype *sobria* strains, showing the homogeneous O-polysaccharide chain length characteristic of S-layer producing strains of motile aeromonads. LPS preparations from strains Ah274 and Ah423 (lanes 2 and 3 respectively) display a shorter O-antigen profile when compared to the parent strain TF7 (lane 1), whereas the LPS of *A. sobria* strains As701 and As702 (lanes 8 and 9) have a longer O-antigen chain length. The Southern blot (figure 46) of chromosomal DNA from the same *A. hydrophila* and *A. veronii* biotype *sobria* strains as in figure 47, shows that under the hybridization conditions employed the *aos*

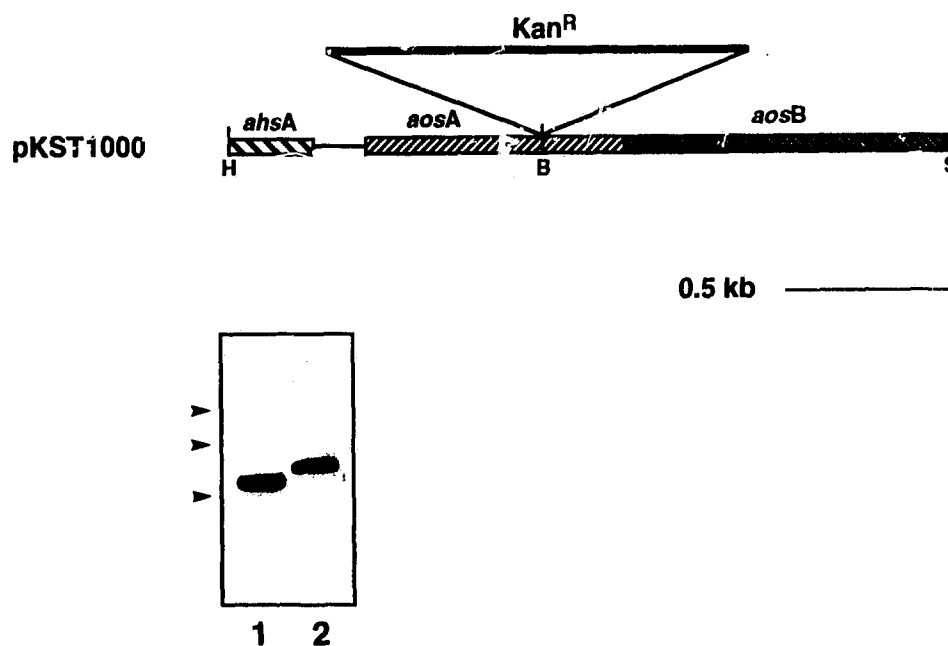


Figure 44. A kanamycin cassette excised on a *Bam*HI fragment from pUC4SAC was inserted into the unique *Bgl*II (B) site of the *aosA* gene generating plasmid pKST1000 as shown. Southern blot analysis of a *kpn*I-*Bam*HI double digest of chromosomal DNA from the parent *A. hydrophila* TF7, and the marker exchange mutant TF7-OS1 shows the correct incorporation of the Kan^R cassette into the TF7 chromosome following marker exchange mutagenesis (inset). Lane 1 containing TF7, shows a reactive band of approximately 4.5 kb, and lane 2 shows the mutant TF7-OS1 displaying the expected increase in size due to the insertion of the Kan^R cassette into its chromosome. DNA size markers on the left of the autoradiogram, from the top, 9.4 kb, 6.7 kb, and 4.3 kb.

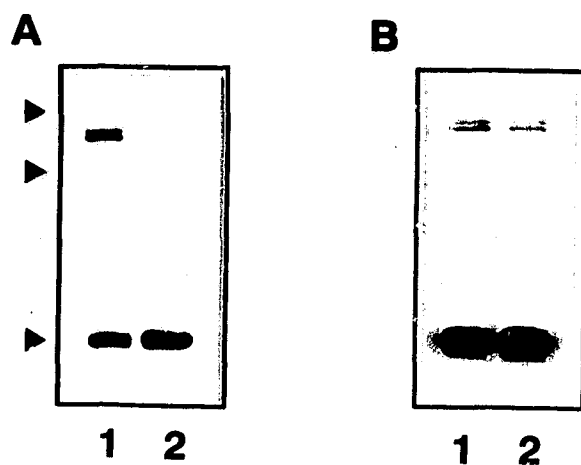


Figure 45. Silver stain of a 12.5% SDS-PAGE of LPS samples of (A) wild type *A. hydrophila* TF7 (lane 1) and the marker exchange mutant TF7-OS1 (lane 2), showing loss of expression of complete LPS from the mutant. (B) Complementation of mutant TF7-OS1 with plasmid pST2000 (figure 35) contained on a broad host range vector, generating strain TF7-OS2. Lane 1 contains LPS from the parent TF7, and lane 2 shows the return of the O-side chain doublet in complemented mutant TF7-OS2. On the left of panel A are protein size markers indicating from the top, 66.2 kDa, 45.0 kDa, and 21.0 kDa.

region is conserved through all isolates studied, other than short O-chain length strains Ah274 and Ah423. When performing the Southern blot to determine the conservation of the *aos* region the insert from plasmid pST1000 was initially used as the probe. The fragment from plasmid pST1200 was also used as a probe, and gave the same result. This ensured that the sequence homology being detected when probing with pST1000 was not from the end of the *ahsA* gene. To determine whether mutation of *aosA* also affected S-protein expression and secretion in *A. hydrophila*, localization studies were performed on the AhsA polypeptide. Figure 48 shows a Western immunoblot which indicates that there is no reduction in S-protein expression overall, but the localization of AhsA relative to wild type has been altered. Lanes 1 and 2 show similar levels of S-protein produced in wild type *A. hydrophila* compared to the marker exchange mutant TF7-OS1. Analysis of the cytoplasmic, the inner membrane, and the periplasmic cell fractions (data not shown) showed that S-protein was not localized to the interior of the cell, indicating that secretion of the S-protein had not been affected by disruption of *aosA*. Analysis of outer membrane fractions and low pH extracts showed that the majority of the S-protein in the parent was localized to the exterior of the cell, whereas low pH extraction of mutant TF7-OS1 released little to no S-protein from its cell surface consistent with an absence of cell surface associated S-layer (figure 48 lane 6). However, a large amount of the S-protein was found in the culture supernatant of the mutant compared to the same fraction of the wild type culture (lanes 7 and 8 figure 48). This was fully consistent with the role played by the LPS polysaccharide in the anchoring of a complete S-layer to the cell surface of *A. hydrophila* (75).

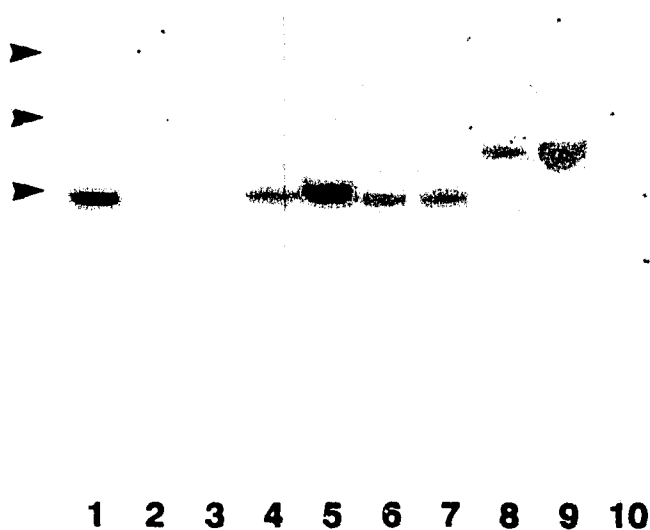


Figure 46. Southern blot of a *Kpn*I-*Bam*HI double digest of chromosomal DNA, showing the conservation of the *aosA/B* region amongst various *A. hydrophila* and *A. veronii* biotype *sobria* strains. Strains Ah274 and Ah423 (lanes 2 and 3), both of which contain homogeneous O-chain phenotypes, and the O-chain negative Ah300 (lane 10), show a lack of conservation of the *aos* region. The other strains examined, all showed a positive reaction when probed with a ^{32}P labeled DNA fragment as described in the text. *Aeromonas* strains are loaded on the gel in the order, TF7, lane 1; Ah274, lane 2; Ah423, lane 3; Ah598, lane 4; Ah77-115; lane 5; Ah80-140, lane 6; Ah80-160, lane 7; As701, lane 8; As702, lane 9; and Ah300, lane 10. DNA size markers, from the top include, 9.4 kb, 6.7 kb, and 4.3 kb.

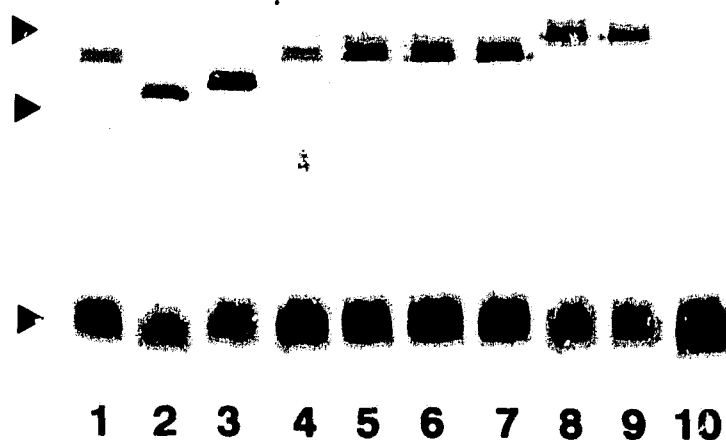


Figure 47. LPS profiles of SDS-PAGE silver stains of various *A. hydrophila* and *A. veronii* biotype *sobria* strains. All strains examined show the homogeneous O-polysaccharide side chains displayed by high virulence aeromonads, other than the S-layer negative *A. hydrophila* strain Ah300 (lane 10). Strains Ah274, and Ah423 contain shorter side chains (lanes 2 and 3), compared to *A. hydrophila* strain TF7 (lane 1). *A. veronii* biotype *sobria* strains As701 and As702 (lanes 8 and 9 respectively), display a larger O-chain phenotype compared to *A. hydrophila* TF7. *Aeromonas* strains are loaded on the gel in the order, TF7, lane 1; Ah274, lane 2; Ah423, lane 3; Ah598, lane 4; Ah77-115; lane 5; Ah80-140, lane 6; Ah80-160, lane 7; As701, lane 8; As702, lane 9; and Ah300, lane 10. Protein Mr markers are included on the left from the top, 66,200, 45,000, and 21,000.

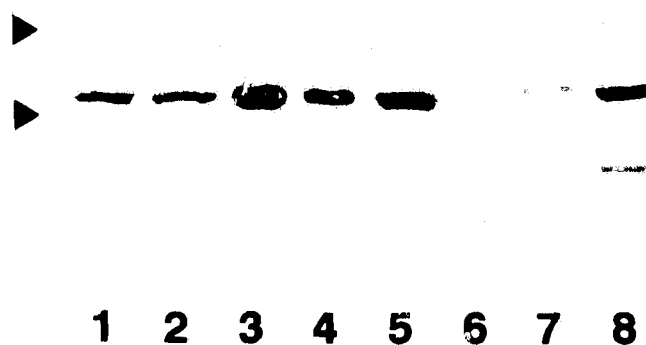


Figure 48. Western immunoblot of SDS-PAGE showing the location of the S-protein in the O-chain negative marker exchange mutant TF7-OS1, compared to the parent *A. hydrophila* TF7. Lanes 1, 3, 5, and 7, contain fractions isolated from the parent TF7, whereas lanes 2, 4, 6, and 8, display samples isolated from the mutant TF7-OS1. Total S-protein synthesized by either strain, is shown in lanes 1 and 2. Outer membrane fractions are contained in lanes 3 and 4, and lanes 5 and 6 show the result of a low pH extraction, indicating large amounts of S-protein released from the cell surface of the wild type TF7, but an almost complete lack of S-protein in the mutant TF7-OS1 sample. The supernatant from broth grown cultures shows that the mutant TF7-OS1 secretes large amounts of S-protein (lane 8) into the medium compared to the parent strain (lane 7). Molecular weight markers from the top are at 66.2 kDa, and 45.0 kDa.

VI. Role of the S-layer and LPS O-Polysaccharide Side Chains in Serum Sensitivity

All highly virulent strains of *A. hydrophila* possess both an S-layer and an LPS which has uniform length O-polysaccharide side chain. What role, if any, either of these molecules play in the pathogenicity of *A. hydrophila* is at present unknown. The S-layer has been shown to be an immunodominant epitope in *A. hydrophila* infections of humans (157). More recently, Janda *et al.* performed a study to determine whether S-layer negative strains of *Aeromonas* spp. were more susceptible to complement-mediated lysis by 65% pooled human serum than those containing a complete paracrystalline array (132). Findings indicated that serum sensitivity generally correlated with the absence of the O-polysaccharide side chains, rather than loss of the S-layer. In this study we performed similar serum sensitivity studies with *A. hydrophila* TF7, and as well as testing rabbit non-immune and immune serum, we also tested non-immune and immune serum collected from rainbow trout.

Western blot analysis of outer membranes to determine presence of the S-layer

In order to ensure the presence or absence of the S-layer on the cell surface of the various mutants used in the study, outer membrane fractions were prepared and analyzed by SDS-PAGE followed by Western immunoblotting with anti S-protein polyclonal antiserum. Figure 49 shows a Western immunoblot of SDS-PAGE analysis of outer membrane preparations from the parent strain *A. hydrophila* TF7 and insertion mutant TF7-ST3 (lane 1 and 3 respectively). Both the parent and the mutant shows the presence of large amounts of S-protein localized to their outer membranes, and insertion mutant TF7-ST1 (figure 49 lane 2) which does not maintain S-protein on its

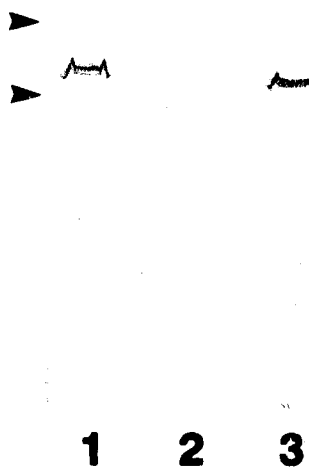


Figure 49. Western blot using antisera JD3 of SDS-PAGE analysis of outer membrane fractions from various strains used in this study. Lane 1 wild type *A. hydrophila* TF7; lane 2 Tn5 insertion mutant TF7-ST1 producing a truncated S-protein that does not assemble or anchor to the cell surface; and lane 3, Tn5 insertion mutant TF7-ST3 which displays an absence of the O-polysaccharide side chain doublet (figure 48), but maintains the presence of S-protein on its cell surface. Molecular weight size markers are shown on the left from the top, 66,200 and 45,000.

cell surface.

Electron microscopy of mutant TF7-ST3

Although the presence of S-layer protein in the outer-membrane fraction of the cell indicates that the monomers may be retained on the cell surface, this finding does not in itself show that these monomers form an assembled paracrystalline array. Figure 50 is an electron micrograph of a negative stain of the outer membrane fraction of mutant TF7-ST3 displaying the typical p-4 symmetry of the *A. hydrophila* S-layer. A number of fields of view of the outer membrane samples were analyzed under the electron microscope to ensure that the S-layer was present uniformly on this membrane. Most outer membrane fragments did in fact contain S-layer, which was in agreement with Dooley and Trust who found that S-layer could be loosely maintained on the cell surface of rough mutants of *A. hydrophila* (75).

Electrophoretic analysis of LPS

We were interested in studying the effect that both the S-layer and the LPS O-polysaccharide side chains had in serum sensitivity. The mutants we were using displayed either wild type S-layer and wild type O-polysaccharide (strain TF7), wild type S-layer but a reduction in the O-antigen (insertion mutant TF7-ST3), or wild type O-polysaccharide, but no S-layer (insertion mutant TF7-ST1) on the cell surface. The electrophoretic characteristics of the LPS profiles prepared by Proteinase K digestion of *A. hydrophila* parent TF7, and the mutants TF7-ST1 and TF7-ST3 were analyzed by SDS-PAGE and silver staining (figure 51). SDS-PAGE analysis displayed the characteristic doublet of the homogeneous pattern for the high virulence group of *A. hydrophila*, as well as a faster migrating band (core) seen in each lane. The complete

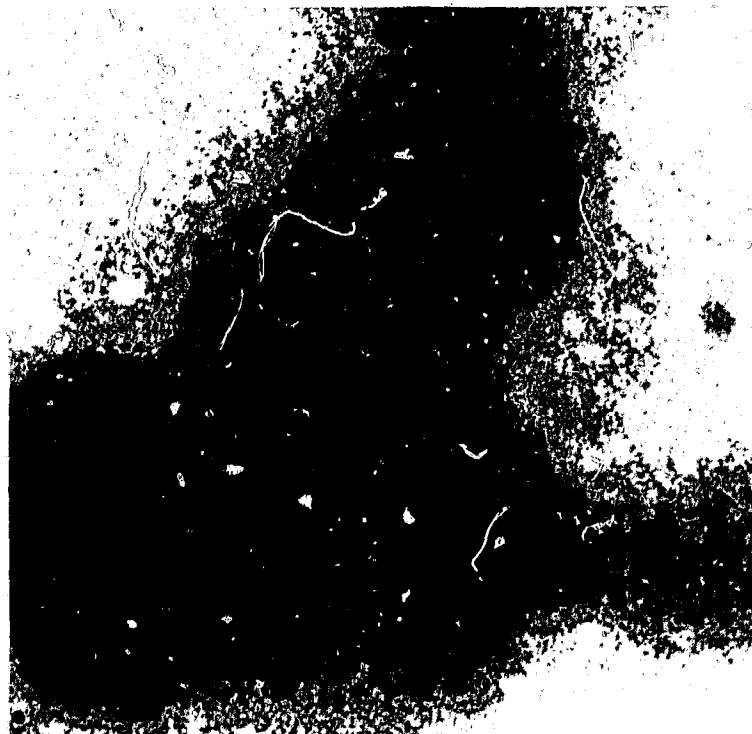


Figure 50. Negative stain of a fragment of outer membrane from Tn5 insertion mutant TF7-ST3 showing the presence of the tetragonal S-layer. The negative stain used was 0.1% ammonium molybdate.

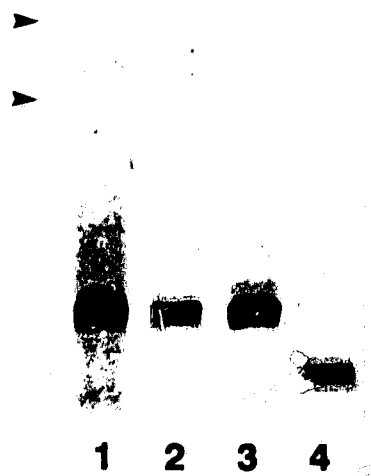


Figure 51. Silver stain of SDS-PAGE of LPS of *A. hydrophila* wild type TF7 (lane 1), S-layer negative Tn5 insertion mutant TF7-ST1 (lane 2), O-polysaccharide negative Tn5 insertion mutant TF7-ST3 (lane 3), and u.v. induced deep rough mutant TF7/B (lane 4). Protein molecular weight markers are on the left from the top, 66.0 kDa, and 45.0 kDa.

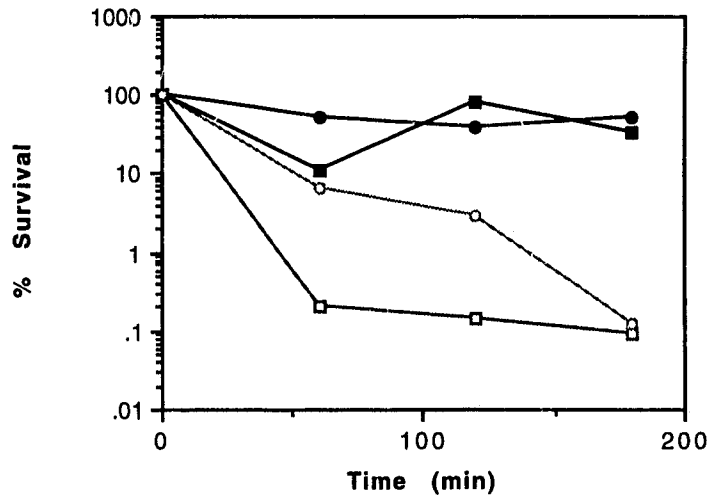
lipopolysaccharides of this group, as seen in lanes 1 and 2 (TF7 and TF7-ST1) includes the homogeneous O-polysaccharide side chains, whereas lane 3 shows the absence of O-polysaccharide chains but normal core oligosaccharide for mutant TF7-ST3. In lane 4, the LPS profile for the deep rough mutant TF7/B is shown, which displays a shortened core oligosaccharide. Mutant TF7/B was used as a control in the serum sensitivity studies due to its deep rough LPS phenotype, and concomitant loss of the S-layer from the cell surface (75).

Serum sensitivity (rainbow trout)

We examined the parent *A. hydrophila* TF7, Tn5 insertion mutants TF7-ST1 and TF7-ST3, as well as the u.v. induced deep rough mutant TF7/B, for their susceptibility to complement mediated lysis. Figure 49 shows the results obtained for the percent survival of the number of cells in the initial inoculum for the various *A. hydrophila* strains in fresh non-immune, or immune rainbow trout serum. In both non-immune and immune serum the parent strain TF7 and the S-layer deficient mutant TF7-ST1 displayed similar survival curves, being reduced to approximately 90% of the initial number of cells used. Following 1 h exposure the survival rate of mutant TF7-ST3 in fresh non-immune serum was approximately 6.7% of the initial inoculum, whereas that of TF7/B was 0.2%. Over the time course of the study both LPS mutants were reduced to approximately 0.1% of the initial number of cells (figure 52).

In immune rainbow trout serum susceptibility studies, both TF7/B and TF7-ST3 were completely killed within 1 h, whereas wild type TF7 and TF7-ST1 showed no obvious difference in survival rate compared to their survival in fresh non-immune serum. Control tubes containing either heat

A



B

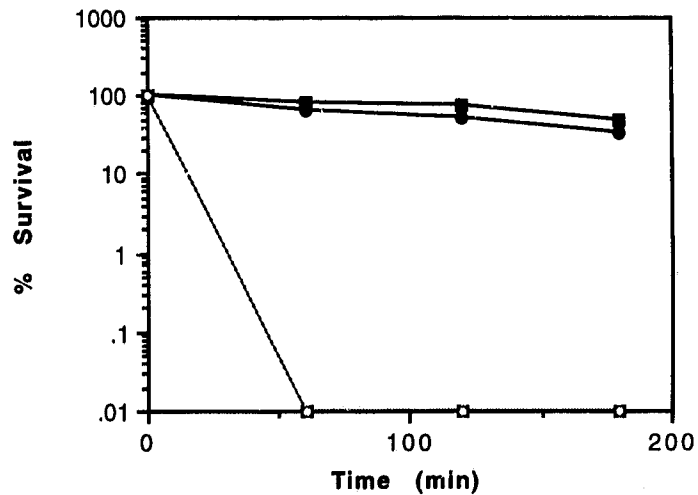


Figure 52. (A) % survival of *A. hydrophila* strains TF7, TF7-ST1, TF7-ST3, and TF7/B in fresh non-immune rainbow trout serum, (■) TF7, (●) TF7-ST1, (○) TF7-ST3 and (□) TF7/B. (B), % survival of TF7 (■), TF7-ST1 (●), TF7-ST3 (○), and TF7/B (□) in immune trout serum.

inactivated serum or nutrient broth alone showed similar growth for all strains, increasing to 156% of initial numbers over the same time course (data not shown).

Serum sensitivity (rabbit)

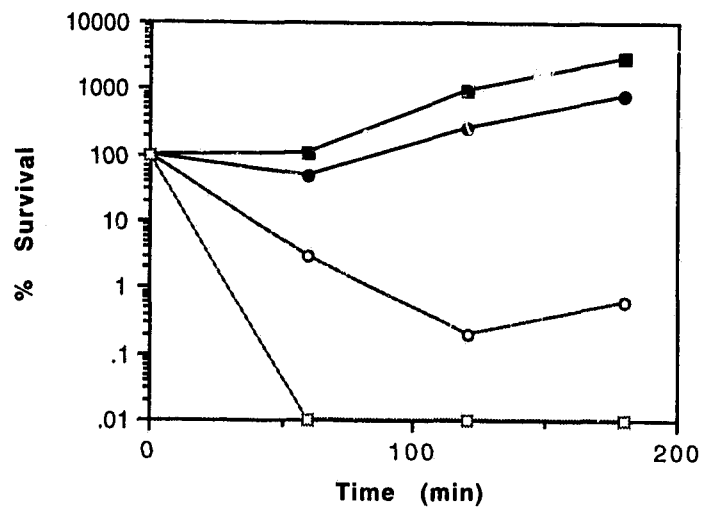
The results of serum killing studies of the *A. hydrophila* strains when using rabbit serum can be seen in figure 53. In fresh non-immune rabbit serum, deep rough mutant TF7/B was most sensitive to complement mediated lysis, and was killed completely within 1 h. On the other hand, the rough mutant TF7-ST3 was less sensitive, and although it was reduced to 0.2% of initial viable cells following a 2 h incubation, it eventually began to increase in numbers. Neither parent strain TF7 nor insertion mutant TF7-ST1 were significantly affected in the fresh non-immune rabbit serum.

When immune rabbit serum was used the same general trends of sensitivity were found for all strains tested as when using non-immune serum. The numbers of viable cells of deep rough mutant TF7-B decreased to 0.003% of initial viable cells over 1 h and were most sensitive, whereas TF7-ST3 was decreased to 15.3% of initial viable numbers over the same time period. Neither wild type strain TF7 nor mutant TF7-ST1 showed any killing over the time course of this assay.

Antibody titers for the immune serum used in the study

To show that the immune anti-serum that we produced for this study was capable of reacting against surface components of *A. hydrophila* cells, we determined agglutinating antibody titers using the slide-agglutination method. Agglutinating antibody titers for non-immune rainbow trout serum against the wild type TF7, were between 8 and 16, while titers for immune

A



B

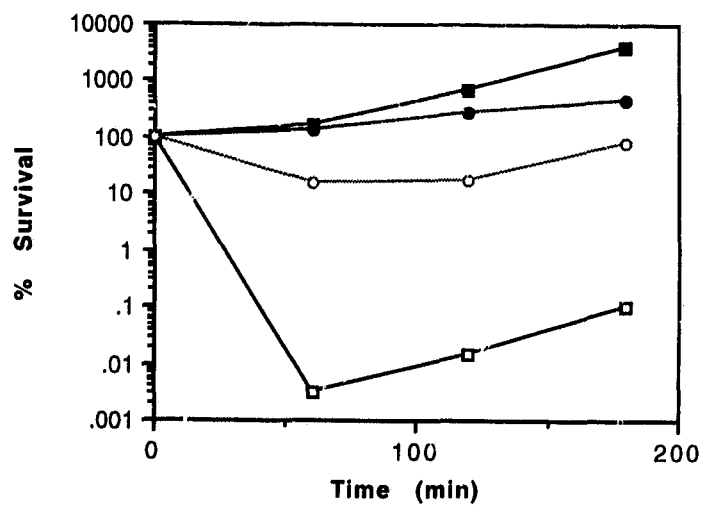


Figure 53. (A) % survival of *A. hydrophila* strains TF7, TF7-ST1, TF7-ST3, and TF7/B in fresh non-immune rabbit serum. (■) TF7, (●) TF7-ST1, (○) TF7-ST3, and TF7/B (□). (B), % survival of TF7 (■), TF7-ST1 (●), TF7-ST3 (○), and TF7/B (□) in immune rabbit serum.

Table 8. Slide agglutination using fresh non-immune and immune rainbow trout and rabbit antiserum

	<u>Dilution</u>							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Rainbow trout								
Normal	++	+	+/-	-	-	-	-	-
Immune	++	++	++	++	+	+	+/-	-
Rabbit								
Normal	-	-	-	-	-	-	-	-
Immune	++	++	++	++	++	++	+/-	-

(++) strongly agglutinating within 1 min

(+) strongly agglutinating within 3 min

(+/-) weakly agglutinating within 3 min

(-) non agglutinating following 3 min

rainbow trout serum were between 128, and 256. Agglutinating antibody titers for non-immune rabbit serum were below 2, and for immune rabbit serum titers were between 128, and 256.

DISCUSSION

I. Cloning and sequencing of the *A. hydrophila* S-protein gene

The molecular cloning and characterization of the genes encoding S-layer proteins is a necessary prerequisite for detailed elucidation of the properties of these macromolecules and the mechanisms involved in their biosynthesis, secretion, and their assembly and anchoring on the outer cell envelope. Unfortunately, the expression of S-proteins at a high level in the commonly used host *E. coli* has posed many problems in the past.

After attempting a number of different methods of cloning the S-protein gene of *A. hydrophila*, the lambda replacement vector EMBL 3 proved to be a successful cloning vehicle for *ahsA*. Following the cloning of the *ahsA* gene into this vector, *E. coli* strain LE392 was initially used for phage propagation, however, a homogeneous phage population expressing the S-protein could not be isolated. The high level of expression of the S-protein in phage lysates suggested that we had probably isolated the *ahsA* gene under control by its native promoter, and this presumably was causing the stability problems. Although cells infected with phage eventually lyse, the high expression of S-protein may have affected maturity of the phage particles leading to instability. Because other studies have shown that some recombinants cannot grow or are biased in a *rec*⁺ background such as LE392 (212), the *rec*⁻ *E. coli* strain KW251 was used. This alleviated the initial instability problem. Using KW251 we were able to isolate large amounts of phage DNA containing the *ahsA* gene (EMBL 3S), and limited restriction enzyme analysis allowed us to localize the gene to a large *Sal*I-*Bgl*II fragment. Further difficulties were experienced when attempting to sub-clone the *ahsA* gene on the *Sal*I-*Bgl*II insert for expression from plasmids in *E. coli*,

presumably due to the presence of the native *S*-gene promoter. To remove the native promoter, and therefore reduce the level of expression, *ahsA* was sub-cloned on a *Sau3A* fragment into pUC18, and this led to the isolation of plasmid pST100, which surprisingly maintained a high level of expression of the recombinant *S*-protein in *E. coli*. The isolation of plasmid pST53 was achieved at the same time as pST100 from the *Sau3A* sub-cloning studies, and was found to express a truncated *S*-protein of Mr 32,000.

During sub-cloning in preparation for sequencing using the internal *Bgl*III and *Bam*H1 sites in plasmid pST100, it was noticed that re-ligation following removal of the region downstream of the *Bgl*III site created a construct that again could not be stably maintained. This downstream segment was confirmed to be essential for the stable maintenance of the *ahsA* gene in *E. coli*. Limited sequencing of this region showed the presence of lambda DNA from the EMBL 3 vector, generated by the re-ligation of *Sau3A* digestion products. This lambda DNA was found to carry the genes encoding the lambda anti-terminator N protein, the repressor Cro protein, and the transcriptional activator CII protein, as well as the O protein and part of the P protein. One or more of these proteins which are required for regulation of the lambda integration event leading to either lysogeny or cell lysis and initiation of DNA replication (137, 219, 269) apparently stabilize *A. hydrophila* *S*-protein expression in *E. coli*, possibly by transcriptional repression of the *ahsA* promoter. An explanation for this observation, is that the region immediately 5' of the start codon for the *ahsA* gene contains a sequence with homology to the Cro binding site (not shown). This may lead to a limited amount of binding by the Cro protein, resulting in a decrease in transcription from the *ahsA* gene.

Plasmid pST107 was generated for the sequencing of the *ahsA* gene by

utilizing the 5' *KpnI* site internal to the *ahsA* ORF. This created initial problems, perhaps because it allowed for the expression of the hydrophobic amino terminal sequence from the *ahsA* gene which may have been targeted by the *sec* system to the cytoplasmic membrane in *E. coli*. Due to the truncation of the AhsA protein from the pST107 construct, the polypeptide may not be processed correctly and so could concentrate in, and disrupt the membrane. A similar effect is seen in the hydrophobic delta toxin of *Staphylococcus aureus* which functions by inserting into, and solubilizing membranes (89). When a stable form of plasmid pST107 was isolated and sequenced, it was found that a nucleotide had been deleted near the beginning of the sequence encoding the signal peptide. This led to a frame shift mutation halting expression from the portion of *ahsA* present on the plasmid.

The S-protein primary sequence was used to search the SwissProt and the GenBank databases using the algorithm of Smith and Waterman (277), and Altschul *et al.* (6). Sequence similarity to a number of outer membrane associated proteins from various bacteria was found. These included the 190 kDa antigen precursor and 120 kDa surface exposed protein of *R. rickettsii* (55.4% over a stretch of 272 amino acid residues, and 53.5% extending 148 residues respectively), the outer wall protein precursor (OWP) of *B. brevis* (69% homology over a length of 54 amino acids), and the S-proteins of *C. fetus* (40% over 168 amino acids), and *Ca. crescentus* (35% over a length of 232 amino acids) (9, 28, 98, 99, 274, 304). The significance of these similarities is not known at the present time.

In terms of amino acid sequence, earlier studies had shown strain to strain differences in N-terminal sequence, peptide polymorphism upon one-dimensional endoproteinase Glu-C mapping, as well as antigenic differences among the S-proteins of *A. hydrophila* and *A. veronii* biotype *sobria*,

consistent with considerable strain-to-strain sequence diversity (160). This sequence diversity was confirmed at the DNA level here. Using the cloned *ahsA* gene of strain TF7 as a probe, strains that produce S-proteins with strong antigenic cross-reactivity to the TF7 AhsA protein gave strong hybridization signals with the TF7 *ahsA* gene, strains producing weaker antigenically cross-reactive proteins showed lower homology at the genetic level, and strains with S-proteins which showed no antigenic cross-reactivity with TF7 AhsA showed little or no hybridization with the TF7 *ahsA* probe. Variation of S-protein antigenicity, both inter- and intra-strain, has also been seen in the case of the S-protein of *C. fetus*. In the intra-strain variation, the antigenically different S-proteins have conserved and variable regions within the same primary structure framework and are generated by homologous recombination between expressed and silent copies of the S-protein gene (306). This is clearly not the case here because the S-layer producing aeromonads have only one copy of the S-protein gene.

Not surprisingly, given the sequence diversity among the S-proteins of the motile aeromonads, the primary sequence of the AhsA protein of *A. hydrophila* TF7 showed low relatedness to the primary sequence of the VapA protein of *A. salmonicida*, despite the morphological similarity displayed by the two *Aeromonas* surface arrays. Direct alignment showed 10.6% identity, and even when aligned for maximal matching using the algorithm of Needleman and Wunsch (213), the two *Aeromonas* S-proteins shared only 33% identity.

The complete sequencing of the *ahsA* gene allowed for the generation of an isogenic mutant of *A. hydrophila* deficient in the expression of S-protein. The generation of such an isogenic S-protein mutant of *A. hydrophila* was important for two purposes; (i) to study the role that the S-protein plays in the

pathogenesis of *A. hydrophila* disease; and (ii) for the eventual generation and secretion of chimeric proteins, the *ahsA* gene must be expressed from a plasmid and secreted in its native host environment. The isogenic S-protein mutant TF7S was generated by mutant allele replacement using homologous recombination.

When the non-disrupted *ahsA* gene was conjugated into the S-layer deficient TF7S marker exchange mutant of *A. hydrophila* TF7, the majority of AhsA co-purified with the outer membrane fraction, with only trace amounts in the other cell compartments. When outer membrane fragments of this TF7SS transconjugant were negatively stained and examined by electron microscopy, assembled sheets of S-layer could be seen, which was consistent with the native secretion pathway being used. The high-expressing configuration provided by the plasmid encoded *ahsA* gene also allowed S-protein secretion to be examined in different host backgrounds. *A. salmonicida* which contains both a general secretory system for the secretion of extracellular enzymes (112, 248, 294), and a secretion system for surface array VapA protein subunits (20), and *E. coli* which lacks these secretion pathways were compared with the native *A. hydrophila* host. In contrast to *A. hydrophila*, when *ahsA* was conjugated into *E. coli* and *A. salmonicida* A449-TM4 which carries a Tn5 insertion in the surface array subunit gene *vapA* (56), the *A. hydrophila* S-protein was detected in each of the cell compartments examined. The largest quantity was present in the periplasm, consistent with the *A. hydrophila* signal sequence being functional in both *A. salmonicida* and *E. coli*. However after passage across the periplasm, the *A. hydrophila* S-protein appeared not to be actively translocated across the outer membrane of either foreign host, but rather inserted into the membrane. The quantity of AhsA detected in the *E. coli* and *A. salmonicida* culture

supernatants was insufficient to allow for the assembly of sheets of S-protein, but was compatible with leakage resulting from the high level of *ahsA* expression in these foreign hosts. These findings suggested strongly that active translocation of *Aeromonas* S-protein across the outer membrane is a highly specific process, and in the case of *Aeromonas* is apparently restricted to the homologous S-protein. This was subsequently confirmed in the course of this study during characterization of the *sps* region which is located immediately upstream of the *ahsA* gene.

II. Characterization of the post-translational modification of the *A. hydrophila* S-protein

The primary sequence of the *ahsA* gene was predicted to encode a protein of Mr 45,400, with a pI of 6.72, both values being in marked contrast to the experimentally determined values of Mr 52,000 and a pI of 4.6 for the mature AhsA protein. Often, post-translational modification of proteins can account for differences between measured and calculated molecular weight and isoelectric point values. Indeed, many S-proteins of gram-positive bacteria and archae are glycosylated and therefore predicted values often do not correspond to those determined experimentally. We therefore examined the possibility that the S-protein of *A. hydrophila* was post-translationally modified.

In vivo labeling of cells with ^{32}P showed that the S-layer protein *A. hydrophila* TF7 was phosphorylated. To our knowledge, this is first demonstration of phosphorylation in this important group of proteins. Phosphorylation increases the negative charge of the S-protein, and the relevance of charged groups for the integrity of S-layers has been discussed previously. Sara and Sleytr showed that the close proximity of amino and

carboxyl groups in the native S-layer of *B. coagulans* lead to a stabilizing effect due to electrostatic interactions between these charged groups (260, 261). However, this usually involves the amino and carboxyl moieties already present in the protein subunits, and not on a negative charge due to modification.

Using ascending thin layer chromatography of acid hydrolyzed S-protein and immunoreactivity with anti-phosphotyrosine monoclonal antibody, we were able to show that the phosphorylation involved tyrosine residues. Indeed all of the S-layer producing strains of *A. hydrophila* and *A. veronii* biotype *sobria* examined gave a positive signal with this monoclonal antibody suggesting that tyrosine phosphorylation of S-layer protein is a common occurrence in the motile aeromonads. While protein phosphorylation on histidine residues is of major importance in bacterial physiology and has been studied intensively (186, 256, 282), phosphorylation of serine, threonine and tyrosine, once considered characteristic of eukaryotic cells, has received much less attention. Indeed until recently, the existence of tyrosine phosphorylation in prokaryotes was in doubt (90). One of the first bacterial proteins of known function, shown to contain phosphorylated tyrosines was the flagellin of *P. aeruginosa*, and it appears that the phosphorylation of this motility organelle may be a common occurrence in *Pseudomonas*. The *Pseudomonas* flagella is the only other supra-assembled bacterial surface protein other than the S-layer of *A. hydrophila* which has been reported to have phosphorylated subunits.

A number of other proteins phosphorylated at tyrosine residues have now been identified in prokaryotes, e.g., *Streptococcus pyogenes* (53), *P. aeruginosa* (147), *Acinetobacter calcoaceticus* (65), and *E. coli* (60). However, a physiological role for such a modification has yet to be identified. In

eukaryotes, reversible tyrosine phosphorylation performs a crucial role in cell development and various other intracellular signaling systems. In contrast, signaling systems in bacteria have so far involved phosphorylation at histidine and aspartic acid residues in the two component signal transduction system, and histidine and serine residues in the phosphotransferase system (38, 243, 283).

In *Pseudomonas*, the tyrosine kinase activity appears to be localized in the cell envelope fraction (14, 90, 147, 148), and a number of the bacterial phosphotyrosine-containing proteins are cell envelope proteins (278), leading to the suggestion that some of these may represent primitive forms that evolved to become a class of eukaryotic tyrosine phosphorylated membrane proteins that serve in signaling pathways at cell surfaces (26). In the case of *Pseudomonas* flagella where there are two tyrosines per flagellin, only one of which appears to be phosphorylated, a role has also been proposed for this phosphotyrosine in cell signaling related to flagellin export (278).

The S-layer of *A. hydrophila* is clearly well sited to participate in signaling events related to the cell's environment, and being a secreted protein there is also a potential need for signaling related to protein export and secretion. The technical difficulties described earlier have precluded determination of how many of the nine tyrosine residues contained in AhsA are phosphorylated. Analysis by automated Edman degradation has ruled out phosphorylation of Y289 and Y403, leaving the phosphorylation status of seven tyrosine residues unaccounted for. The phosphotyrosine(s) in AhsA are not exposed on the S-layer surface and, in contrast to the phosphotyrosine in *Pseudomonas* flagellin, appears to be quite inaccessible to phosphatases, suggesting the S-protein phosphotyrosine(s) may have a structural rather than a signaling role.

Interestingly, the *A. hydrophila* S-protein expressed from plasmid pST100 was phosphorylated to the same extent as wild-type when expressed in either *E. coli* or *A. salmonicida* backgrounds. In contrast, when the type A flagellin gene from *P. aeruginosa* PAK was cloned and expressed in *E. coli*, the foreign host did not phosphorylate the expressed product (295). This suggests that endogenous protein tyrosine kinase activity is activated in *E. coli* by the *A. hydrophila* S-protein.

Release of phosphate by enzymatic methods is an important criteria for the determination of a phosphomonoester bond. In our studies we successfully reduced the molecular weight of a limited amount of the mature S-protein using acid phosphatase to the correct size as specified by the gene. Previous work involving the removal of S-layers from the cell surface, and disassembly into its constituent subunits indicate that the bonds holding the crystalline array together may be more resistant to disruption than those holding the protein sheets on the cell envelope (72). We have no indication to what extent the S-layer is assembled under any particular condition, and so it is feasible that the phosphate moieties are inaccessible to the enzyme due to self-assembly products. In order to determine whether or not the phosphotyrosine residue was surface exposed, we used immuno-gold labeling with the anti phosphotyrosine monoclonal antibody, however, no specific signal was detected. Problems in the removal of the phosphate moiety from phosphorylated proteins have been experienced previously (267). Protease digestion of the S-protein in non-denaturing conditions produces a resistant core, even though there are many sites for these enzymes in the mature protein, adding to the evidence that inaccessibility of certain sites is a common problem when using enzymes on the S-protein.

In summary, the studies described have for the first time identified

phosphorylation as a form of post translational modification of an S-layer protein. The exact role of this modification is unknown at the present time, and elucidation of the role, if any, may require the construction of a tyrosine phosphate negative mutant of the AhsA protein.

III. Roles of structural domains in the morphology and surface anchoring of the S-layer, and biochemical characterization of the major structural domain

Many S-layer proteins have now been identified and characterized, and much is known about S-layer ultrastructure at a morphological level (19, 263). However, despite the information gathered concerning the ultrastructural analyses, there has been little communication dealing with the biochemistry of the separate morphological domains of the S-layers.

Earlier work using protease digestion of purified *A. hydrophila* TF7 S-protein has provided biochemical evidence for the presence of two major structural domains (160). Treatment with trypsin, chymotrypsin, or endoproteinase Glu-C produced an N-terminal peptide of approximate Mr 38,000, which was refractile to further proteolytic cleavage under nondenaturing conditions. The carboxy terminal, however, was readily susceptible to each of the proteases used. The isolation of the Tn5 transposon mutant TF7-ST1 in the current study and analysis of the S-protein produced by this mutant has provided important new information on the structural domains of the S-layer of *A. hydrophila*. Mutant TF7-ST1 expressed a stable, exported, truncated S-layer protein identical in size to the trypsin resistant core of the native *A. hydrophila* S-protein by SDS-PAGE analysis.

The N-terminal sequence of the truncated S-protein from mutant TF7-ST1 was identical to that of the wild type S-protein, and sedimentation analysis suggested a molecular weight of 38,650. Electron microscopy

following negative staining of samples further showed that the monomers were able to associate with each other at one end of the molecule. The association of these monomers provides for a cup-shaped assembly with parallel sides when viewed from the side. When viewed from the top, the assembly appears as a ring with a diameter of 6.5 nm, which is in close agreement with previous measurements for the major morphological tetramer of both the *A. hydrophila* and *A. salmonicida* S-layers (3, 73, 211, 280).

Amino acid compositional analysis of the truncated S-protein suggested an increase in the relative hydrophobic amino acid content relative to the wild type protein from approximately 44% to 51%. The higher content of hydrophobic amino acids at the N-terminal would be consistent with the functional requirements of a structural domain which presumably associates hydrophobically with other monomers in order to produce the major protein mass of the tetragonal surface array. The amino acid content of the *A. hydrophila* S-layer protein as deduced from the gene sequence can be compared to the estimates obtained earlier by Dooley *et al.* (72, 157). Compositional analysis had predicted S-proteins of Mr 52,000 containing 498-526 residues, and 44 - 46 % hydrophobic residues, compared to the mature TF7 S-protein deduced from the nucleotide sequence which contains 450 residues, 45.7% hydrophobic residues and a Mr of 45,413. In the case of the truncated S-protein secreted by mutant TF7-ST1, sedimentation analysis combined with amino acid compositional analysis indicated that this truncated protein contains 387 residues, and a Mr of 38,650. This Mr value is in excellent agreement with the deduced Mr of 38,745 obtained for the N-terminal 387 residues of AhsA, containing 48% hydrophobic residues.

Comparison of secondary structure predictions from the CD measurements for the mature Mr 52,000 protein versus the Mr 38,650 polypeptide gave similar values. S-layer proteins typically have a relatively high β -sheet structure with only a small amount of α -helix and β -turn conformations (161, 272). This is also true for the S-layer protein of *A. hydrophila*, which contains approximately 44% β -sheet, 19% α -helix, and 12% β -turn. Although the content of β -sheet for the truncated protein of 42% did not vary between the two pH values studied, and was found to be similar in value to the β -sheet content of wild type protein, the two polypeptides did vary in their α -helix and β -turn estimates when the effects of pH and SDS were studied. The α -helix content of the truncated protein was increased at pH 2.1 compared to pH 7.4 (8% to 21%), whereas the β -turn value decreased at pH 2.1 compared to pH 7.4 (19% to 6%). For the wild type protein, variation in the pH had no effect on the α -helix content, but the β -turn content varied from 19% at the higher pH to 12% at pH 2.1. When CD analysis was performed in the presence of 0.1 % SDS to model the hydrophobic environment predicted to exist in an assembled S-layer, the results comparing the wild type protein to the truncated protein were far more significant. In the presence of the detergent at pH 2.1 the α -helix content of the truncated protein increased from 21% to 33%, similarly, with added SDS and at a pH of 7.4, the helical content rose from 10% to 19%. These results are similar to those achieved for the A-protein of *A. salmonicida*. When CD measurements were made for the Mr 39,439 trypsin resistant core, an overall increase in α -helix was found at the expense of β -structure (70). For the wild type S-protein of *A. hydrophila*, the α -helical content dropped from 19% to 11% in the presence of SDS, with a concomitant increase in β -structure (72). In contrast, in the case of the wild-type A-protein of *A.*

salmonicida, the effect of the SDS was to double the α -helical structure at the expense of β -structure (70).

The fact that the hydrophobic environment provided by the SDS has different effects on the secondary structure of the wild-type protein compared to the truncated polypeptide emphasizes the role played by the C-terminal domain in determining S-layer conformation. This is important considering that in the assembly of the native S-layer, hydrophobic interactions are thought to be a major stabilizing force. Alterations in conformation and stabilization also occur when flagellin is polymerized into the flagella filament (313, 314).

The truncated protein produced by mutant TF7-ST1 also carries virtually all of the epitopic reactivity of the wild-type protein. Inhibition ELISA results confirm earlier data regarding the localization of the major surface epitopic activity of the S-protein of *A. hydrophila* (160). This is in contrast to the A-layer protein of *A. salmonicida*, where the C-terminal 111 residues comprise 22.4% of the total mass of the polypeptide, but carry 57% of the polyclonal epitopic activity exposed on the surface of the A-layer (70). At the present time it is unknown why there should be such a major difference in antigenic conformation between the two S-layers at the C-terminal, considering their morphological similarity.

The C-terminal region which is missing from the truncated protein studied here appears to contribute to the lesser morphological unit of the assembled array. This morphological unit is connected by linker projections to the primary morphological unit (211). Three dimensional image reconstruction's of *Aeromonas* S-layers indicate that this lesser domain of the S-protein is located towards the outer surface of the layer (3, 73), while the N-terminal comprises the primary unit forming the major tetragonal core of the

surface array, and is located towards the inside of the layer. The minor C-terminal domain allows for correct spatial positioning of the major structural domains by contributing the molecular size and structure essential for the subunit interactions which provide connectivity within the layer, and perhaps influencing the degree to which the tetramers are open.

The C-terminal domain also appears to participate in the anchoring of the S-layer to the cell surface, presumably *via* interactions with LPS carbohydrate (72) (figure 54). Although previous studies have shown that deep rough mutants of *A. hydrophila* cannot retain an array on their cell surface, rough mutants do have an anchored array (see below, and (75)). In this study, the failure of mutant TF7-ST1 to anchor the truncated S-protein monomers to the cell surface seemed to be caused by an alteration in the protein structure, as opposed to a change in LPS phenotype.

Previous studies on transposon mutants of *A. salmonicida*, have suggested that *Aeromonas* S-layer proteins are secreted by a specific pathway which is different to other exported proteins, and proceeds through the periplasm (20). Secretion of the TF7-ST1 truncated protein also proceeds via the periplasm, but it is not exported as efficiently as the wild type S-protein, presumably due to a role that the C-terminal plays in the translocation process. This decrease in secretion efficiency can be seen by the accumulation of truncated S-protein in the cytosol and the periplasm, unlike the wild type, where the S-protein was specifically isolated in the outer membrane fraction. Conformation differences may be a possible reason for the decrease in translocation competency, however, comparison of spectra obtained in the near-u.v. region for the 52 kDa wild type protein and the 38.6 kDa truncated protein are very similar (72). The similarity of these spectra indicate that no significant conformational changes had occurred in the tertiary structure of

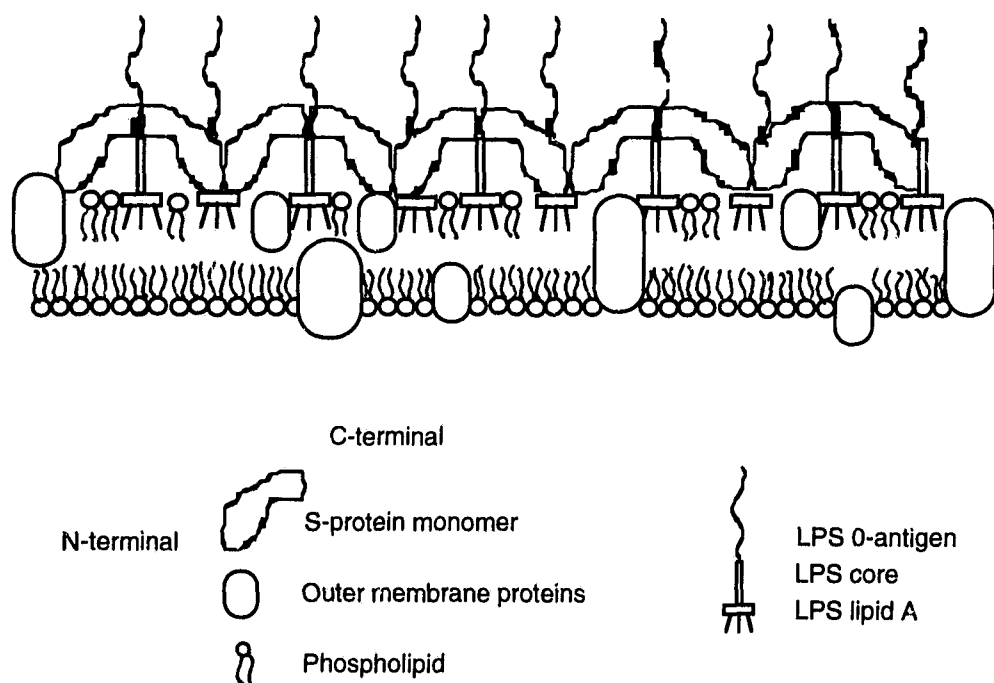


Figure 54. A cartoon depicting the anchoring of the S-layer to the outer membrane of *A. hydrophila*. The N-terminal of the AhsA monomer is anchored to the outer membrane via hydrophobic interactions, while the C-terminal is required for correct array morphology and attachment to the LPS core and O-antigen (211). In the absence of the C-terminal, the N-terminal alone is insufficient for anchoring and the truncated S-protein expressed by mutant TF7-ST1 is lost by gentle washing or low pH extraction.

the N-terminal domain of AhsA as a result of the truncation. This was also seen in the relative stability of the truncated polypeptide to withstand proteolytic degradation in the protease rich cytosol, periplasm, and extracellular compartments.

In summary the C-terminal domain appears to be responsible for a number of aspects in the normal assembly of S-layer and the anchoring of assembled S-layer to the cell surface. Loss of the C-terminal results in the inability of the monomers to form any kind of macromolecular structure resembling the tetragonal paracrystalline array, by virtue of the role this domain plays in the formation of the lesser morphological linking unit. The ring like structures that are formed cannot be retained on the cell surface, presumably indicating the interaction between the missing C-terminal domain and the carbohydrate of the LPS.

IV. Isolation and characterization of a gene whose product is responsible for the specific transport of the S-protein across the outer membrane in *A. hydrophila*

PulD is a membrane-associated protein of *Klebsiella* spp. which is responsible for the transport of the enzyme pullulanase across the outer membrane (64). A superfamily of proteins has now been classified, based on their homology to PulD, which are involved in different secretion pathways in gram-negative bacteria (97). The extent of the homology between these proteins is especially significant at the C-terminal end, which is shared by all of the members of this superfamily. While the N-terminal shows far less overall homology, conservation of this region is shared between different sub-groups of the PulD family, and has been assigned the domain responsible for the substrate binding function (255). Within the PulD superfamily, there are

possibly four sub-groups, with members of each sub-group sharing N-terminal homology (97, 191).

This study has identified a gene encoding a PulD homologue which appears to be specific for the transport of S-layer protein subunits across the outer membrane of *A. hydrophila* TF7. It has been shown previously that S-protein secretion is a highly specific process, due to the inability of the A-protein secretory system of *A. salmonicida* to functionally replace the S-protein secretory system of *A. hydrophila*. Mutation of the *spsD* gene results in loss of S-layer from the cell surface, and periplasmic accumulation of S-protein. A class of Tn5 insertion mutants of *A. salmonicida* unable to secrete the A-protein across the outer membrane (20) accumulates large amounts of the A-protein in the periplasm, indicating a similar secretory system for this organism. Mutation of *spsD* has no apparent effect on the outer membrane protein profile of the organism, or on the secretion of the extracellular enzymes. The secretion of these enzymes in *A. hydrophila*, as well as a normal outer membrane protein composition requires another PulD homologue, ExeD (135, 136). Southern blot analysis confirmed that *A. hydrophila* carried the *exeD* gene in addition to *spsD*. This finding is consistent with S-layer producing strains having separate pathways for S-layer protein subunits, and for extracellular enzymes and outer membrane proteins. Interestingly however, *spsD* was not conserved in all S-layer producing strains of *A. hydrophila* and *A. veronii* biotype *sobria*, suggesting the presence of further PulD encoding gene homologues in this group of bacteria.

In *A. hydrophila* TF7, *spsD* is located 700 bp upstream of *~hsA*, the structural gene for the S-layer protein, and on a different chromosomal fragment to *exeD*. The two genes show little similarity at the nucleotide level, indicating different codon usages, and *spsD* also appears to possess its own

promoter making it unusual among genes encoding PulD homologues. *spsD* appears to be the final gene of a substrate-specific general secretory pathway. In this regard the putative S-protein secretion operon of *A. hydrophila* TF7 shows organizational similarity to the *xps* operon of *X. campestris* pathovar *campestris*, where *xpsD* is also the terminal ORF in the secretion operon (124). Two of the upstream genes, ORFJ and ORF1, in both systems also encode structurally related proteins (79, 124). In contrast, *exeD* and other genes encoding PulD homologues are internally located in their respective gene clusters (240).

Comparison of the predicted sequences of SpsD and ExeD shows that the proteins share approximately 34% identity at their C-termini. This finding is typical for this family of proteins. Interestingly, other than a region of approximately 120 amino acid residues beginning at position 164, identity between XpsD and SpsD is shared over the entire length of the two proteins. Within the PulD family of proteins, there are four highly conserved regions in the C-terminal domain (97, 191). The C-terminus of SpsD contains only three of these regions, with the amino acid sequence contributing to region B being absent. The significance of this is not understood at the present time, especially as the B sequence is predicted to form a strongly amphipathic β -sheet, and could therefore be anchored into, or be a membrane spanning region.

How the PulD-like proteins function in their secretion of various substrates across the outer membrane is unknown. PulD contains 7 stretches of 12 residues which are predicted to form strongly amphipathic β -sheets, which may form some type of β -barrel structure similar to the porins, although the size of the proteins to be transported across the membrane precludes the possibility of any constantly open pore (64). Regarding the likely

method of passage of the secreted proteins across the outer membrane, at least two hypotheses have been postulated. Martin *et al.* have noticed an interesting arrangement of the genes required for the maintenance of the peptidoglycan (PG) layer in *E. coli* (191). The protein PonA is a high molecular weight penicillin binding protein, one of a class of proteins that function in the formation and maintenance of the PG layer. Whereas PilQ is a protein involved in the assembly of Type IV fimbriae, and shares homology at the C-terminal with PulD. Interestingly, there is a *ponA* homologue in *P. aeruginosa* that shares similarity in both sequence and arrangement to genes in the *pilQ* region, suggesting that the remodeling of the PG layer may be involved in the assembly of fimbriae and other surface associated protein complexes. Martin *et al.* suggest that as the fimbriae assemble in the periplasm, PilQ possibly works as a porthole for the passage of the fimbriae through the outer membrane. In contrast, Russel *et al.* have proposed the possibility that the PulD homologue pIV plays a role in the opening of a gated channel from the cytoplasm for the emerging phage particle (254). Considering that SpsD cannot be visualized in Coomassie blue stained outer membrane fractions, the level of this polypeptide in the outer membrane is likely to be low, however, the rate at which S-protein must be secreted and assembled to maintain a complete paracrystalline array in *A. hydrophila* is high. Some pre-assembly therefore, prior to secretion, would be consistent with the idea of a gated channel or porthole as suggested for these other assembled proteins.

In summary, this study has shown that *A. hydrophila* TF7 has (at least) two PulD homologues, SpsD and a homologue of ExeD. In contrast to ExeD, SpsD appears to be specific for S-protein secretion, and does not appear to be involved in exoenzyme secretion. SpsD further appears to belong to an S-protein specific terminal branch of the general secretory pathway, the genes of

which are located immediately upstream of the structural gene for the S-protein subunit AhsA.

V. Identification and characterization of two genes involved in O-polysaccharide side chain secretion

High virulence strains of *A. hydrophila* were first identified because of their enhanced ability to produce disease, and it was found that there was a direct correlation between this capacity and certain cell surface characteristics (207). These high virulence strains as well as producing an S-layer, were shown to auto-aggregate in liquid media, belong to a single *Aeromonas* serogroup (O:11), and to possess homogeneous length O-polysaccharide side chains (74, 155, 156).

In this study, we have identified two genes (*aosA* and *aosB*) whose products display significant homology to previously characterized polysaccharide secretion systems belonging to the ABC-type 2 family of ATP dependent transporters. The *A. hydrophila aosA* ORF encodes the integral membrane component of these transporters, which is typically hydrophobic and commonly consists of up to six trans-membrane domains. On the other hand, *aosB* encodes a polypeptide that is more hydrophilic, and contains the consensus Walker box or Rossmann fold nucleotide-binding pocket (251, 315). The ABC exporter of *E. coli* K1 consists of two proteins, KpsM and KpsT. The KpsM polypeptide is a 258 amino acid 29.6 kDa hydrophobic protein, while KpsT is a 219 amino acid 24.9 kDa ATP-binding protein (225). Insertion mutations in the genes encoding these proteins from *E. coli* K5, *kpsM* or *kpsT*, results in polysaccharide that is cytoplasmic and shorter than surface polymers (169). Mutation of the *aosA* gene resulted in a dramatic reduction

in the amount of whole LPS expressed by mutant TF7-OS1 in this study, and led to release of large amounts of S-layer protein into the culture supernatant. However, the presence of complete LPS could be seen when outer membranes fractions were overloaded and analyzed by SDS-PAGE, indicating that a limited amount of the O-polysaccharide was exported through the cytoplasmic membrane. This finding is consistent with the occurrence of a low level of cross-talk between different, but related ABC transport systems, as seen in other microorganisms. Previous reports have identified similar results, for example, the *nod* regulon of *Rhizobium* and *Bradyrhizobium* strains contain genes whose products are responsible for the biosynthesis and modification of lipo-oligosaccharide molecules. NodI and NodJ are thought to be involved in the export of the lipo-oligosaccharide molecules produced by the biosynthetic genes of the *nod* regulon, but mutation of NodI and NodJ does not lead to a complete Nod⁻ phenotype (83). Vasquez *et al.* suggest that other transport systems may be responsible for the continued secretion of the substrates for these proteins, but less efficiently than normal (311).

Within the *A. hydrophila aos* region, overlapping start and stop codons of *aosA* and *aosB* suggests that (at least) these two genes are translationally coupled. Continued nucleotide sequencing in the 3' direction from the *aosB* gene indicated that there was a third ORF present. Indeed the absence of a transcriptional terminator following the *aosB* gene suggests that *aosA* and *aosB* are transcribed as part of a larger multicistronic transcript. However, the successful complementation of the *aosA* mutant when the *aosA* and *aosB* genes were expressed from a plasmid in this *Aeromonas* host indicated that the third ORF did not encode a product essential for the export of O-polysaccharide side chains in *A. hydrophila*.

In the earlier part of this thesis, describing the cloning and sequencing

of the *ahsA* gene from *A. hydrophila*; we performed Southern blot analysis to determine the level of conservation of the S-protein gene amongst various *Aeromonas* spp. We found that two *A. hydrophila* strains, Ah274 and Ah423, showed no conservation of the *ahsA* gene (figure 11). Southern blot analyses performed in this part of the study have shown that these same two strains show no conservation of the *aos* region (figure 55 summarizes both results). Whether this indicates a possible connection between the expression of a specific S-protein and LPS O-polysaccharide phenotype is unknown. Inhibition of the export of the O-chains by mutation of *aosA* did not affect the secretion of the S-protein, and similarly, the presence of wild type LPS was seen in an isogenic mutant that accumulated a periplasmic pool of the S-layer subunits. Phenotypically, those strains that showed no conservation of the *aos* region or the *ahsA* gene, displayed a shorter O-polysaccharide side chain length. These results extend previous conclusions drawn by Dooley and Trust (74), in that the LPS is not only essential for the normal anchoring the S-layer to the cell surface of *A. hydrophila*, but the connection between the two major surface located antigens also appears to occur at the genetic level.

This clustering of genes on the chromosome of virulent aeromonads required for the presence of the two major antigens has been seen previously. The *AbcA* protein is a bi-functional ATP binding protein responsible for the synthesis and export of the O-polysaccharide side chains produced by the related organism *A. salmonicida* (58). The chromosomal position of the *abcA* gene is similar to that of the *aos* region, in that it lies immediately following *vapA*, the structural gene for the S-layer protein of *A. salmonicida* (58). As with *A. hydrophila*, the presence of both a homogeneous O-polysaccharide side chain in association with an S-layer is a common feature. In contrast to *A. hydrophila*, however, which expresses both components associated with

ABC-type 2 exporter function, *A. salmonicida* does not contain a similarly located *aosA* gene homologue encoding an integral inner membrane protein that is expressed in conjunction with AbcA.

In summary, we have shown that located immediately downstream of the *ahsA* gene of *A. hydrophila*, there is a putative operon whose products exhibit high homology to previously characterized ABC type 2 polysaccharide exporter systems. This primary amino acid similarity and the generation of an *aosA* mutant which was unable to express wild type LPS, suggested that the putative *aos* operon appeared to be specific for the export of the O-polysaccharide side chains in *A. hydrophila* in an energy dependent manner.

VI. Role of the S-layer and LPS O-polysaccharide side chains in serum sensitivity

The bacteriolytic activity of normal serum is one of several natural host defenses active against gram-negative bacteria. This activity is largely dependent upon complement-mediated lysis or killing of intact organisms, and bacteria that are resistant to this bacteriolytic mechanism often have an advantage in the formation of disseminated disease (85). Individuals that are deficient in one or more complement components, most notably C3 and C5, are at an increased risk of developing life threatening infections (85).

Previous work had established that the O:11 serogroup comprising high virulence strains of *A. hydrophila* were resistant to the bactericidal action of fresh normal mammalian serum (207). This serogroup typically consisted of *Aeromonas* isolates that contained an S-layer and homogeneous LPS side chains on their cell surface. For *A. hydrophila* strain TF7, we have now studied the role played by the S-layer and the homogeneous LPS O-polysaccharide side chains in the serum resistance of this organism. In order

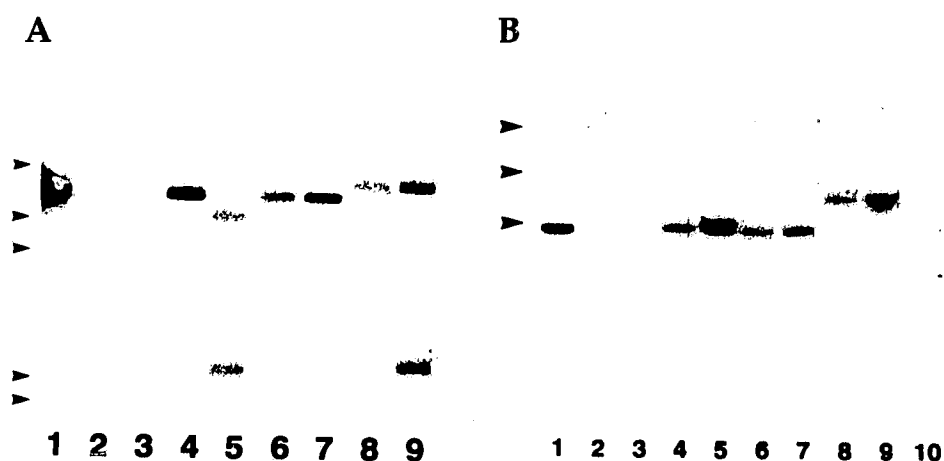


Figure 55. A summary of the results taken from figures 11 (A) and 46 (E) showing the lack of conservation of both the S-protein *ahsA* gene, and the *aosA aosB* genes in *A. hydrophila* strains Ah274 and Ah423. *A. hydrophila* and *A. veronii* biotype *sobria* strains are loaded similarly in both gels. Lane 1, TF7; lane 2, Ah 274; lane 3, Ah 423; lane 4, Ah 598; lane 5, Ah 77-115, lane 6, Ah 80-140; lane 7, Ah 80-160; lane 8, As 701; and lane 9, As 702. Lane 10 of panel B contains an O-antigen-S-protein negative *A. hydrophila* strain Ah300. On the left of A are the DNA lambda size markers in kilobases from the top, 23.13, 9.42, 6.68, 4.36, 2.32, and 2.03, while on the left of B size markers include, from the top, 9.4 kb, 6.7 kb, and 4.3 kb. Chromosomal DNA in panel A was digested using a *SalI-BglII* double digest, while in panel B, DNA was digested using a *BamHI-KpnI* double digest.

to accomplish this, we utilized two isogenic Tn5 insertion mutants, one which failed to anchor an intact S-layer on its cell surface, and a mutant that does not express the LPS side chains but retained an S-layer on its outer membrane. A u.v. induced mutant was also studied, which was unable to anchor S-layer to its cell surface, and produces a deep rough LPS which lacked both its O-polysaccharide side chains and its outer core. Results obtained using the S-layer negative mutant suggest that the S-layer of *A. hydrophila* does not impart any advantage to the parent cell in its resistance to either fresh non-immune or immune rainbow trout or rabbit sera. In contrast, the mutant expressing a rough LPS phenotype TF7-ST3, was more sensitive in all sera tested compared to the wild type parent, but was less sensitive than the deep rough mutant TF7/B. The result showing the partial sensitivity of strain TF7 to the bactericidal action of fresh trout serum was consistent with previously published data regarding the survival of *A. hydrophila* TF7 in this type of serum (181).

Janda *et al.* have compared the serum sensitivity of a group of O:11 strains to those of a control group which were S-layer negative and belonged to serogroups other than O:11 (132). There was a significant difference in these two groups with regard to their ability to resist the normal lytic action of complement. Overall, O:11 S-layer positive strains were more resistant to pooled human serum than non-O:11 strains. Within the non-O:11 group, however, two distinct populations could be distinguished. The larger of these groups was also refractory to complement mediated lysis despite the fact that these strains were S-layer negative, although they did have an intact O-polysaccharide side chain profile. The susceptible group showed no side chain on their LPS. These results would seem to parallel our findings in this study in that the O-polysaccharide side chains are important for the resistance

to serum killing of *A. hydrophila*.

Clearly, the LPS of *A. hydrophila* plays a major role in resistance to serum killing. This is also true for other gram negative bacteria where serum resistance has been attributed to the LPS. For example, Grossman *et al.* (104) found that the survival of *Salmonella montivideo* during serum treatment depended on the presence of O-antigen associated with the LPS molecule, and concluded that the O-antigen side chain protected the cell by sterically hindering the C5b-9 complex, thereby protecting hydrophobic domains of the outer membrane. The O-antigen of *A. hydrophila* may well perform a similar function in serum resistance.

Most of the evidence supporting an important role for S-layers in *Aeromonas* pathogenesis stems from studies conducted on the salmonid pathogen *A. salmonicida* (127). Investigations indicated that *A. salmonicida* strains bearing an S-layer were virulent in fish models while S-layer deficient strains were avirulent. Avirulent isogenic mutants or attenuated derivatives of wild-type *A. salmonicida* lacking the S-layer supported this conclusion. In the case of *A. salmonicida*, possession of an S-layer imparts high-cell surface hydrophobicity to the bacterium, and *in vitro* this property appears to correlate with resistance to lysis in fresh serum and attachment to fish macrophages. Munn *et al.* assessed the bactericidal activity of normal and immune fish sera against *A. salmonicida* and found that the S-layer in conjunction with LPS played an integral part in the overt resistance of the bacterium to complement mediated lysis (209). In similar studies performed with *C. fetus*, unlike their parental counterparts, isogenic mutants lacking the 100 kDa surface array protein which forms the S-layer were found to be serum sensitive (27). Results from the present study do not support a similar role for the *A. hydrophila* S-layer.

The exact role of the *A. hydrophila* S-layer in the pathogenesis of this organism still has to be elucidated. One study performed on mice suggested that neither the S-layer nor the characteristic serogroup O:11 homogeneous LPS side-chain was directly involved in pathogenicity (156). Both a spontaneous mutant, AS-180-1, that was S-layer negative, and an "atypical" strain (AH-77) which synthesized S-layer but failed to anchor it to the cell surface due to an altered LPS profile, were as virulent as the parental S-layer positive strain. Similarly when Proteinase K digestion was used to remove the S-layer from classic O:11 strains prior to inoculation the 50% lethal dose did not alter when compared to non-Proteinase K treated parent cells. Unfortunately, these studies did not address the possible differences in growth rate of the various mutants used, which in turn can affect the quantity of the extracellular enzymes secreted into the environment. In 50% lethal dose challenges involving rainbow trout, the serum sensitive deep rough mutant TF7/B was found to be more virulent than the parent strain TF7 (Thomas and Trust unpublished results), but its growth rate is also significantly higher than the wild type.

In summary, this study has shown that the S-layer of *A. hydrophila* strain TF7 does not impart serum resistance to the parent cell. On the other hand, loss of the O-polysaccharide side chains does make the cell more sensitive to complement-mediated lysis. It is tempting to speculate that a possible role of the *A. hydrophila* S-layer in the pathogenesis of the organism may be two fold; (i) during the infection process, its presence leads to auto-aggregation of the infecting bacteria, and so performs a protective role against the host defenses by virtue of the clumping effect; (ii) it may play a role in the concentration of nutrients or cations around the rapidly multiplying bacterial mass.

VII. Summary

Prior to this study, the *A. hydrophila* S-layer had been characterized morphologically with regard to 3D structure (3) and subunit arrangement (211), and biochemically with respect to the S-layer subunit protein (72). We have continued these studies at a molecular level and have contributed valuable information concerning the synthesis, secretion, primary and tertiary structure, assembly and anchoring of the S-layer protein to the outer cell membrane of *A. hydrophila*. The S-layer protein gene *ahsA* was cloned and the nucleotide sequence determined, and the AhsS protein was shown to be post translationally modified at tyrosine residues. The AhsA protein was shown to be expressed from a single copy chromosomally located gene that was transcribed as a monocistronic unit. Expression at high levels of the AhsA protein in *E. coli* showed that the native *A. hydrophila* S-gene promoter was present and recognized by the foreign host transcriptional machinery.

Secretion of the *A. hydrophila* S-protein was shown to be a highly specific process by virtue of the fact that the A-layer protein transport system of *A. salmonicida* could not replace the native export system. Indeed, a chromosomal locus containing the *spsD* gene was identified which expressed a product that showed high homology to the PulD protein of *Klebsiella* spp. Confirmation of the role of SpsD in the secretion of the *A. hydrophila* S-protein was achieved using insertional mutagenesis and mutant allele replacement. Specificity in the secretion of the S-protein was further established by showing that loss of expression of SpsD did not affect export of the extracellular enzymes from *A. hydrophila*. With nucleotide sequencing in the 3' direction from the *ahsA* gene, a putative operon which expressed protein products with homology to the ABC-type 2 ATP dependent

transporters was found. Mutational analysis of the first ORF of this region, the *aosA* gene, led to a rough LPS phenotype, and a concomitant decrease in the amount of S-layer anchored to the mutant cell surface. With the characterization of this complete region of the *A. hydrophila* TF7 chromosome, both upstream and downstream from the *ahsA* gene (figure 56), we have identified an example of each of the two major groups of dedicated export systems expressed by gram-negative bacteria. These include a terminal pathway of the general secretory system, and an example of the ATP dependent ABC transporters. Together, these secretion systems are responsible for the presence of the principal elements on the cell surface which have been identified in all high virulence strains of the *Aeromonas* O:11 serogroup.

Morphologically, the role of the C-terminal in the assembly and anchoring of the tetragonal array to the *A. hydrophila* cell surface was established. A Tn5 insertion mutant expressed a truncated N-terminal fragment of the S-layer protein which assembled into structures that were similar morphologically to the major mass domain of the mature S-layer. These structures were missing the minor mass domain of the S-layer, and were incapable of self-assembling on, or anchoring to the cell surface. Using two Tn5 insertion mutants, one which failed to anchor S-layer to the cell surface, and one which expressed a rough LPS phenotype, we performed serum sensitivity studies. We found that the absence of the S-layer on the cell surface of *A. hydrophila* Tn5 mutant TF7-ST1 did not significantly affect its survival in serum compared to the parent S-layer positive strain, but the presence of the O-polysaccharide side chains was more crucial to cell viability under the conditions tested.

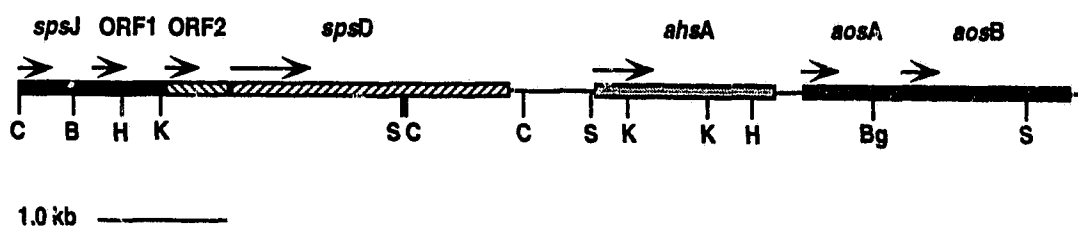


Figure 56. An illustration of the complete region characterized in this study. The structural gene encoding the S-layer protein of *A. hydrophila* TF7, the *ahsA* gene is shown, as well as part of the putative *sps* operon located immediately upstream of *ahsA*. The *spsD* gene was shown to be responsible for the specific secretion of the S-protein across the outer membrane. Located downstream of *ahsA* is the putative *aos* operon, two of the genes *aosA* and *aosB* encode products with high homology to members of the ABC-type 2 family of bacterial transporters. Loss of expression of *aosA* led to a rough LPS phenotype and a large decrease in the amount of S-layer anchored to the cell surface of *A. hydrophila* TF7.

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In Preparation:

1. **Thomas, S. R. and T. J. Trust.** 1995. Characterization of two overlapping genes from *Aeromonas hydrophila* belonging to the family of ABC-transporters that are responsible for the export of the homogeneous LPS O-polysaccharide side chain

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