

A mannose-sensitive haemagglutinin (MSHA)-like pilus promotes attachment of *Pseudoalteromonas tunicata* cells to the surface of the green alga *Ulva australis*

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This study demonstrates that attachment of the marine bacterium *Pseudoalteromonas tunicata* to the cellulose-containing surface of the green alga *Ulva australis* is mediated by a mannose-sensitive haemagglutinin (MSHA-like) pilus. We have identified an MSHA pilus biogenesis gene locus in *P. tunicata*, termed *msh112JKLMNEGFACDOPQ*, which shows significant homology, with respect to its genetic characteristics and organization, to the MSHA pilus biogenesis gene locus of *Vibrio cholerae*. Electron microscopy studies revealed that *P. tunicata* wild-type cells express flexible pili peritrichously arranged on the cell surface. A *P. tunicata* mutant (SM5) with a transposon insertion in the *mshJ* region displayed a non-piliated phenotype. Using SM5, it has been demonstrated that the MSHA pilus promotes attachment of *P. tunicata* wild-type cells in polystyrene microtitre plates, as well as to microcrystalline cellulose and to the living surface of *U. australis*. *P. tunicata* also demonstrated increased pilus production in response to cellulose and its monomer constituent cellobiose. The MSHA pilus thus functions as a determinant of attachment in *P. tunicata*, and it is proposed that an understanding of surface sensing mechanisms displayed by *P. tunicata* will provide insight into specific ecological interactions that occur between this bacterium and higher marine organisms.

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INTRODUCTION

Marine surfaces are colonized by a diversity of micro-organisms and sessile marine organisms collectively known as biofouling communities. The biofouling process is initiated by the attachment of bacteria to a surface followed by the settlement and adherence of diatoms, free-swimming algal spores and invertebrate larvae (Bryers & Characklis, 1982). Some sessile higher organisms employ chemical defences against biofouling through the production of

secondary metabolites that inhibit the development and formation of a biofouling community (Harrison, 1992; Mary *et al.*, 1993; Maximilien *et al.*, 1995). For example, furanones produced by the red alga *Delisea pulchra* have been reported to inhibit the settlement of common fouling organisms (de Nys *et al.*, 1995). For marine organisms without intrinsic defence mechanisms, it has been proposed that protection against fouling is maintained by the secondary metabolites produced by surface-associated bacteria (Egan *et al.*, 2001a, b; Holmström *et al.*, 1992, 1996, 1998; Holmström & Kjelleberg, 1999; James *et al.*, 1996).

Many species of the genus *Pseudoalteromonas* have been found to produce bioactive compounds against different classes of fouling organisms and are frequently found in association with the surfaces of living marine eukaryotes (Holmström & Kjelleberg, 1999). A well-studied surface-associated bacterium is *Pseudoalteromonas tunicata*. This

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Abbreviations: CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein; MSHA, mannose-sensitive haemagglutinin; RFP, red fluorescent protein; TEM, transmission electron microscopy.

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green-pigmented bacterium was first isolated from the surface of a tunicate, *Ciona intestinalis*, in Sweden (Holmström *et al.*, 1998) and later from a green alga, *Ulva australis* (formally *Ulva lactuca*), in Australian waters (Egan *et al.*, 2001a). *P. tunicata* produces a number of extracellular bioactive compounds, each of which has a specific inhibitory activity against target organisms such as algal spores, fungi, invertebrate larvae or bacteria (Egan *et al.*, 2001b; Holmström *et al.*, 1992, 2002; James *et al.*, 1996). While several studies have addressed the production of bioactive compounds, no data have so far been reported on the means by which *P. tunicata* colonizes surfaces, including those of higher marine organisms. Investigation of such mechanisms will contribute to a greater understanding of the ecology of *P. tunicata* and its interaction with marine surfaces.

The successful colonization of surfaces by bacteria is often mediated by cell-surface appendages such as pili and flagella. For example, the attachment of *Escherichia coli* to abiotic surfaces is promoted by the presence of both type 1 pili and flagella (O'Toole & Kolter, 1998). In *Pseudomonas aeruginosa*, flagella are believed to be important for initial attachment to a surface, while type 4 pili promote the formation of microcolonies on the surface (O'Toole & Kolter, 1998). It has also been reported that type 4 pili mediate attachment of pathogenic bacteria such as *Neisseria gonorrhoeae* (Morand *et al.*, 2001) and *Pseudomonas aeruginosa* (Zolfaghar *et al.*, 2003) to host epithelial cells. The adherence of *Vibrio cholerae* to environmental surfaces is directly associated with the presence of the mannose-sensitive haemagglutinin (MSHA) pilus, which belongs to a family of type 4 pili (Marsh & Taylor, 1999). In *V. cholerae*, the MSHA pilus has been demonstrated to play a direct role in both colonization and subsequent biofilm formation on abiotic as well as biotic surfaces (Chiavelli *et al.*, 2001; Watnick *et al.*, 1999; Watnick & Kolter, 1999).

Here we describe a non-piliated mutant of *P. tunicata* (SM5) that carries a transposon insertion in an ORF termed *mshJ*, with high homology to the *V. cholerae mshJ*, a gene encoding a MSHA pilus biogenesis protein. DNA sequencing upstream and downstream of *mshJ* revealed the presence of a gene locus with 17 ORFs that are homologous to the MSHA pilus biogenesis gene locus of *V. cholerae*, and we propose that this gene locus is involved in the assembly and transport of an MSHA pilus in *P. tunicata*. Here we demonstrate a role for the *P. tunicata* MSHA pilus in attachment to abiotic, as well as biotic (*U. australis*) surfaces, and propose that *P. tunicata* demonstrates surface sensing mechanisms as revealed by increased pilus production in the presence of cellulose, one of the major surface polymers of *U. australis*.

METHODS

Bacterial strains, plasmids and culture conditions. *P. tunicata* wild-type was maintained on the complex marine medium VNSS (Marden *et al.*, 1985). For attachment assays, *P. tunicata* was grown

in nine salt solution (NSS), pH 7.0, or Marine Minimal Medium (3M) (Neidhardt *et al.*, 1974), each medium containing either 0.05% (w/v) glucose, 0.25% microcrystalline cellulose (Avicel; Fluka) or 0.1% cellobiose (Sigma) as the sole source of carbon. *P. tunicata* SM5 was isolated from a *P. tunicata* mini-Tn10 transposon library (generated according to the method of Egan *et al.*, 2002a) and was selected on the basis of reduced attachment to polystyrene microtitre plates according to the method of O'Toole & Kolter (1998). The *P. tunicata* SM5 strain was grown on VNSS agar plates containing 100 µg streptomycin ml⁻¹ and 85 µg kanamycin ml⁻¹.

P. tunicata wild-type cells were provided with a green fluorescent protein (GFP) colour tag by transconjugation using the constitutive GFP expression plasmid pCJS10. This plasmid contains the *gfpmut3* gene (Cormack *et al.*, 1996) on an RSF1010 backbone from broad-host-range vector pHRP304 (Bagdasarian *et al.*, 1981). In addition, a red fluorescent protein (RFP) colour tag was provided to SM5 cells using the pCJS10-derived plasmid pCJS10R. This plasmid contains the RFP gene *dsred* (Clontech) in place of *gfpmut3* on pCJS10 (Rao *et al.*, 2005). Triparental conjugations were carried out as described previously for *P. tunicata* (Egan *et al.*, 2002a) and labelled transconjugants were grown on VNSS agar plates containing 15 µg chloramphenicol ml⁻¹ and 100 µg streptomycin ml⁻¹. GFP- and RFP-labelled strains showed bright fluorescence after overnight culture and we observed no differences in the growth rate or surface attachment properties of the labelled strains versus the unlabelled parent strain (data not shown).

Panhandle PCR, DNA sequencing and sequence analysis. To obtain sequence information from the genes disrupted by the mini-Tn10 transposon in the SM5 mutant, panhandle PCR was carried out as described previously using adaptor-specific primer AP1 (5'-GGATCCTAATACGACTCACTATAGGGC-3') and transposon-specific primers Tn10C (5'-GCTGACTTGACGGGACGGCG-3') and Tn10D (5'-CCTCGAGCAAGACGTTTCCCG-3') (Egan *et al.*, 2002a, b). Panhandle PCR products were visualized in 1% agarose gel and purified using a PCR purification kit (Qiagen), according to the manufacturer's instructions. PCR products were sequenced using transposon-specific primers Tn10C and Tn10D and a primer walking strategy. For sequencing, between 50 and 100 ng double-stranded template DNA, 1 µl specific primer (10 pmol), 4 µl CSA buffer and 4 µl BigDye terminator cycle sequencing reaction mix (Applied Biosystems) were mixed in a final volume of 20 µl. Amplification of DNA was conducted using the following parameters: 94 °C for the initial denaturation step which was followed by 25 cycles of 94 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. After cycling, the sequencing mixture was cleaned and purified using a butanol purification protocol (Tillett & Neilan, 1999). Separation of sequencing products was performed on an ABI 377 DNA sequencing system at the Sydney University Prince Alfred Macromolecular Analysis Centre (SUPAMAC). The DNA sequence electropherograms were analysed with ABI-PRISM software. Multiple sequence alignments were performed using the Staden Package System (Medical Research Council – Laboratory of Molecular Biology, University of Cambridge). The complete consensus DNA sequence was compared with sequences in the GenBank database using the BLAST-search algorithm (Altschul *et al.*, 1990) and ORFs were defined using the ORF finder program made available through the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Promoter prediction was performed using the neural network promoter prediction tool (Reese, 2001) available through the Berkley *Drosophila* genome project website (www.fruitfly.org/seq_tools/promoter.html). The GCG software package provided by the Australian National Genomic Information Service (ANGIS) website (www.angis.org.au/WebANGIS/) and the ExpASY (Expert Protein Analysis System) site (<http://expasy.proteome.org.au/index.html>) of the Swiss Institute of Bioinformatics (SIB)

were also used for sequence analysis. Additional sequence information was obtained by analysis of the draft genome sequence for *P. tunicata* using the BLAST-search algorithm (Altschul *et al.*, 1990).

Transmission electron microscopy (TEM). Cell surface morphology of *P. tunicata* wild-type and SM5 strains was examined using TEM. Bacterial cells were grown on VNSS plates for 24 h (equivalent to the stationary phase of growth) and colonies were gently resuspended in PBS, pH 7.4 (l^{-1} : 8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4 , 0.24 g KH_2PO_4) to a concentration of 10^6 cells ml^{-1} . Alternatively, bacterial cells were grown in static conditions in NSS liquid medium containing either glucose, cellobiose or cellulose as the sole carbon source. Carbon and Formvar-coated copper grids were placed on a drop of cell suspension for 5 min and then negatively stained with 2% phosphotungstic acid for 30 s. The cells were examined using a Hitachi H7000 transmission electron microscope.

Haemagglutination assay. Haemagglutination assays were performed as described by Gardel & Mekalanos (1996). Bacterial strains were grown in VNSS medium at 28 °C under static conditions and assayed during both exponential and stationary growth phases. Briefly, the cells were washed twice and resuspended in Krebs-Ringer solution (KRT). An initial concentration of 10^{10} cells ml^{-1} was serially diluted in 96-well microtitre plate with each well containing 100 μ l 3% (v/v) washed horse erythrocyte suspension. The mixture was incubated at room temperature for 30 min and scored for haemagglutination. *V. cholerae* strain M1615 was used as a positive control. To determine if pili are sensitive to the presence of mannose during the haemagglutination assay, 50 μ l 1% α -methyl-D-mannoside (a non-metabolizable derivative of mannose) were added to the haemagglutination mixture. The haemagglutination assay for each strain was carried out in three replicates.

Attachment assays. The attachment of the *P. tunicata* wild-type and the SM5 mutant strains to abiotic surfaces was tested by modifying an attachment assay described by O'Toole & Kolter (1998). Bacterial isolates were grown under static conditions at 28 °C on VNSS medium and harvested after 24 h. The cell suspension was centrifuged (6000 g, 5 min), washed twice and resuspended in 10 ml PBS, pH 7.4, to an OD_{600} of 0.6–0.7. Aliquots (1 ml) of cell suspension were added to wells of a 24-well polystyrene microtitre plate. The plate was shaken slowly for 1 h at room temperature. Wells were then washed twice using sterile distilled water and air-dried for 45 min. The attached cells were fixed at 80 °C for 30 min and stained with 0.1% crystal violet for 45 min. The cells were destained with 95% ethanol and quantified by measuring OD_{590} .

We examined attachment of GFP-labelled *P. tunicata* wild-type and RFP-labelled SM5 cells to microcrystalline cellulose. Attachment was monitored essentially as described by Bayer *et al.* (1983) and by direct confocal laser scanning microscopic (CLSM) imaging of fluorescent cells attached to cellulose particles. Cells grown using glucose or cellobiose were harvested after 24 h and cells grown using cellulose were harvested after 48 h. The cells were then gently washed twice with PBS (centrifugation at 5000 g for 10 min) and resuspended in 10 ml PBS solution. The assay mixture consisted of 1 ml cells ($\sim 10^7$ cells ml^{-1}) (pre-grown in glucose, cellobiose or microcrystalline cellulose), 1 ml 20% microcrystalline cellulose in PBS and 1 ml PBS. The mixture was shaken slowly for 1 h and incubated statically for 1 h at room temperature. The OD_{400} of the suspension was measured and compared to a control (identical cell suspension in PBS). To test the effect of mannose in the attachment of cells to cellulose, the attachment assay mixture was added with 100 mM α -methyl-D-mannoside. The attachment assay for each strain was carried out in three replicates. The samples were mounted on a glass microscope slide and transmission fluorescent images were captured using an Olympus LSMGB200 confocal laser scanning microscope.

Preparation of axenic thallus of green alga *U. australis* and culture conditions. Axenic thallus of *U. australis* was obtained following the protocol of Rao *et al.* (2006). Briefly, plants of *U. australis* were collected from rocks at Clovelly Bay, Sydney, Australia. The collected plants were rinsed with 50 ml autoclaved sea water and thallus discs of around 0.6 cm diameter were excised from the lower part of *U. australis* by using a steel punch. To remove other bacteria from the surface of *U. australis*, surfaces of *U. australis* discs were swabbed with sterile cotton tips and exposed to 0.012% (v/v) NaOCl for 5 min. Plant pieces were incubated in an antibiotic mixture containing 300 mg ampicillin l^{-1} , 30 mg polymyxin l^{-1} and 60 mg gentamicin l^{-1} for 24 h followed by 1 h recovery in 20 ml sterile sea water.

Attachment assay with axenic *U. australis*. GFP-tagged *P. tunicata* wild-type and SM5 mutant strains were grown on VNSS medium containing chloramphenicol for 24 h. After 24 h the cell suspensions were centrifuged at 6000 g for 7 min. The bacterial cells were rinsed twice with 10 ml PBS and resuspended in PBS to an OD_{610} of 0.35–0.45. The assay mixture consisted of 1 ml washed cells, one axenic thallus disc of *U. australis* and 1 ml PBS. The mixture was incubated for a period of 2 h with slow shaking and for 1 h without shaking at room temperature. The *U. australis* pieces were rinsed twice with 10 ml PBS to remove unattached cells. For each sample, 10 images were manually analysed by counting the bacteria attached to the surface using CLSM. The attachment assay was repeated four times with three replicates for each bacterial strain.

RESULTS

The *P. tunicata* genome encodes a putative MSHA biogenesis locus

DNA sequencing of the regions flanking the Tn10 insert in the *P. tunicata* SM5 mutant yielded a 4291 bp nucleotide sequence with six potential ORFs (MshI1–MshM). Analysis of this region in the draft genome sequence of *P. tunicata* revealed an additional 11 ORFs predicted to be in two transcriptional units (Fig. 1). Promoter prediction analysis revealed potential transcriptional start sites approximately 19 bp upstream of the *mshI1* start codon (–10 box 'ATTTAAGAT' and –35 box 'TTGCTT') and 34 bp upstream of the *mshB* start codon (–10 box 'CAGTTAAAT' and –35 box 'TTGTAT'). These regions are in agreement with the –10 and –35 regions of the *E. coli* σ^{70} promoter and match closely with other predicted promoter sequences for *P. tunicata* (Egan *et al.*, 2002a). Following the translational stop of ORF17 (*mshQ*) is a region indicative of a transcriptional terminator (92 bp downstream of *mshQ*) (Mathews & van Holde, 1990). The deduced amino acid sequence of the 17 ORFs showed homology to the secretory and structural genes for the MSHA pilus of various bacteria (data not shown). Moreover, the operon structure reflects that of the *V. cholerae* MSHA biogenesis locus with the only major difference being in the genes encoding the MshI protein. In *P. tunicata* there are two genes with similarity to different regions of the *V. cholerae* *mshI* gene and these have been termed *mshI1* and *mshI2*, respectively (Fig. 1).

Electron microscopic detection of cell surface pili

Following the identification of the putative MSHA pilus operon, we examined whether *P. tunicata* expresses

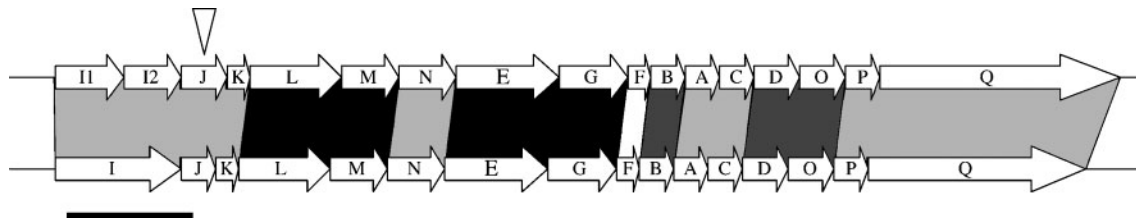


Fig. 1. Schematic representation of the predicted MSHA gene locus in *P. tunicata* (top) and *V. cholerae* El Tor (bottom). The entire locus is 17 525 bp in length and consists of 17 continuous ORFs (*msh112JKLMNEGFACDOPOQ*). The scale bar represents approximately 2 kb. The inverted triangle indicates the location of the transposon in the *P. tunicata* SM5 MSHA mutant strain. Black shading, >45% identity; dark grey, 35–45% identity; pale grey, 25–35% identity; white, <25% identity.

functional pili. Transmission electron micrographs of *P. tunicata* wild-type and the SM5 mutant cells are shown in Fig. 2. It was observed that *P. tunicata* wild-type cells are surrounded by flexible pili on their surface, each with a diameter of 7–8 nm and a length of 90–150 nm. The mean number of surface pili per cell \pm SEM was calculated on 50 randomly selected cells. *P. tunicata* wild-type cells showed a mean number of surface pili per cell of 11.32 ± 0.7 . *P. tunicata* SM5 mutant cells were non-piliated, confirming that the putative MSHA biogenesis operon is required for pilus expression. In addition, we observed that wild-type cells grown using cellobiose or cellulose as the sole carbon source exhibited a significant increase in the mean number of pili per cell, 14.42 ± 0.2 and 17.22 ± 0.9 respectively, than those grown in VNSS ($P < 0.0001$).

Assay for agglutination of red blood cells

To further characterize the *P. tunicata* pilus, haemagglutination assays were performed. It was found that *P. tunicata* wild-type cells cause agglutination of horse red blood cells. Haemagglutination was observed when either exponential- or stationary-phase wild-type *P. tunicata* cells were used (Table 1). In contrast there was no haemagglutination observed in the presence of the SM5 mutant when cells were assayed during exponential phase; however, in stationary-phase cells, a low level of haemagglutination was observed. Addition of mannose to the assay mixture blocked haemagglutination by *P. tunicata* wild-type cells.

Pili promote the attachment of *P. tunicata* to abiotic surfaces

Polystyrene microtitre plates, widely used in bacterial attachment assays (O'Toole & Kolter, 1998; Taylor *et al.*, 2002), were used as an abiotic test surface. After 1 h, *P. tunicata* wild-type cells demonstrated considerably higher attachment compared to SM5 mutant cells as detected by the crystal violet staining of attached cells to wells of a polystyrene microtitre plate. The crystal-violet-stained *P. tunicata* wild-type cells displayed an OD_{590} of 0.97 ± 0.09 , which was 2.8-fold higher than the crystal-violet-stained SM5 mutant cells (0.34 ± 0.01).

The attachment of *P. tunicata* wild-type and SM5 mutant cells to cellulose was also tested. Cellulose is a major surface polymer of *C. intestinalis* (De Leo *et al.*, 1977) and *U. australis* (Chapman, 1979), and *P. tunicata* is frequently isolated from these surfaces. *P. tunicata* wild-type cells (grown with glucose as the carbon source) demonstrated

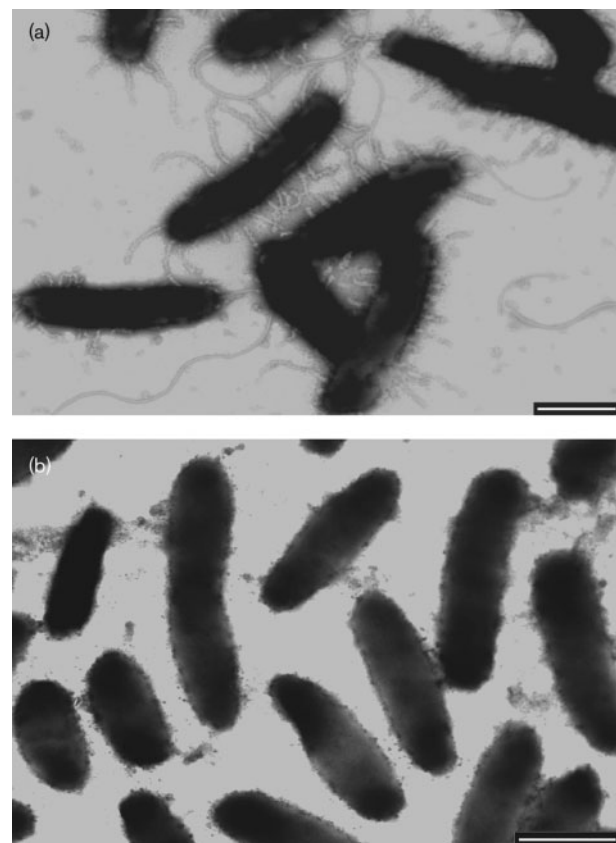


Fig. 2. Transmission electron micrographs showing (a) *P. tunicata* wild-type cells expressing pili on the surface, and (b) *P. tunicata* SM5 mutant cells displaying the non-piliated phenotype. Both strains were grown on VNSS agar plates for 24 h. All samples were negatively stained with 2% phosphotungstic acid. Bars, 1 μ m.

Table 1. Haemagglutination of horse erythrocytes by bacteria at different growth stages

Values represent the reciprocal of the dilution in which haemagglutination can be observed. The results of the assay performed in the presence of 1% α -methyl-D-mannoside were zero for all strains tested in both growth phases.

Bacterial strain	Stationary phase	Exponential phase
<i>Vibrio cholerae</i> M1615 (+ control)	>50	>50
<i>P. tunicata</i> wild-type	20	20
<i>P. tunicata mshJ</i> (SM5) mutant	<2	0

attachment to microcrystalline cellulose which resulted in a 22.2% reduction in the final OD₄₀₀ of the assay mixture (Fig. 3). SM5 mutant cells, however, attached less effectively to cellulose showing only 4% reduction. Moreover, attachment of wild-type *P. tunicata* was enhanced when cells were pre-grown on cellulose and cellobiose (29.4 and 40.7% reduction, respectively). SM5 mutant cells pregrown in cellobiose and cellulose showed 26.7 and 14.5% reduction of the final OD₄₀₀ of the assay mixture, respectively. It was also demonstrated that mannose significantly inhibits attachment of *P. tunicata* wild-type cells to cellulose, displaying only 3.7% reduction of the final OD₄₀₀ of the attachment assay mixture.

We also compared the attachment of the two strains to cellulose using fluorescently tagged cells and CLSM. Transmission fluorescence images revealed that substantial numbers of *P. tunicata* wild-type cells attached to microcrystalline cellulose (Fig. 4b). In contrast, the SM5 mutant

cells attached less to cellulose both during attachment in pure culture and mixed with wild-type *P. tunicata* cells (Fig. 4c and a, respectively).

Pili promote attachment of *P. tunicata* to the surface of the green alga *U. australis*

Axenic discs of *U. australis* were prepared and resulted in over 90% reduction of the natural microbial community as previously demonstrated (Rao *et al.*, 2006). Axenic *U. australis* thallus discs were incubated with *P. tunicata* wild-type:GFP and *P. tunicata* SM5:GFP cells, and attachment assays were carried out in PBS, pH 7.4. The total cell counts per mm² of algal surface for *P. tunicata* wild-type and the SM5 mutant were 2377 ± 607 and 996 ± 362 cells, respectively. *P. tunicata* wild-type cells demonstrated 2.4-fold enhanced attachment compared to the SM5 mutant on the surface of *U. australis* (Fig. 5).

DISCUSSION

Surface-associated bacteria on marine sessile organisms may provide benefits to their hosts. For example, *P. tunicata* produces a range of inhibitory compounds and may offer protection against colonization of biofouling and host-pathogenic organisms (Egan *et al.*, 2000, 2001b; Holmström *et al.*, 1992; James *et al.*, 1996). Such interactions are initiated by the attachment and initial colonization of bacteria to the host surface, and may be facilitated by the expression of surface adhesins. In *V. cholerae* El Tor, the attachment to phytoplankton and zooplankton surfaces is mediated by the MSHA pilus (Chiavelli *et al.*, 2001). Recently, it was reported that an MSHA pilus promotes interaction between *V. cholerae* El Tor and haemolymph of the mussel *Mytilus galloprovincialis* (Zampini *et al.*, 2003). In this study, we investigated the role of an MSHA-like pilus in the attachment of *P. tunicata* to the surface of *U. australis*, a living surface from which *P. tunicata* has been isolated in the marine environment.

This study describes a gene locus proposed to be involved in the export and assembly of an MSHA pilus in *P. tunicata*. We have sequenced the DNA flanking a Tn10-disrupted ORF with high homology to *mshJ* of *V. cholerae* (Marsh & Taylor, 1999), and together with analysis of the flanking region in the recently obtained draft genome of *P. tunicata*, revealed 17 contiguous ORFs (*mshI1I2JKLMNEGFACDOPQ*) with

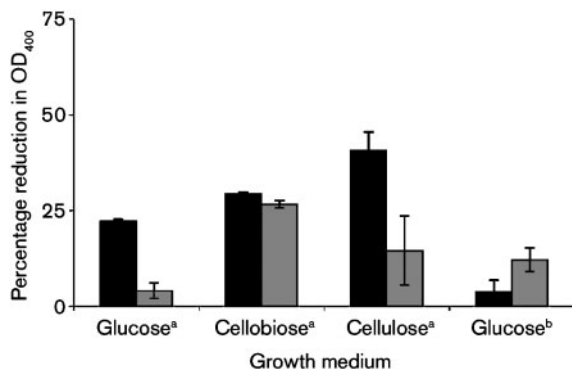


Fig. 3. Attachment of *P. tunicata* wild-type (black bars) and SM5 mutant (grey bars) to microcrystalline cellulose (Avicel). The microcrystalline cellulose was mixed with bacterial cells ($\sim 10^7$ c.f.u. ml⁻¹) for 1 h and the final OD₄₀₀ of the cellulose and bacterial cell suspension was measured after 1 h incubation. The attachment of bacteria to cellulose was scored by the reduction in the final OD₄₀₀ of the suspension. ^a, Bacteria were grown in this medium prior to the attachment assay which was performed in the absence of mannose; ^b, bacteria were grown in this medium prior to the attachment assay which was performed in the presence of mannose. Error bars represent the SEM of triplicate cultures.

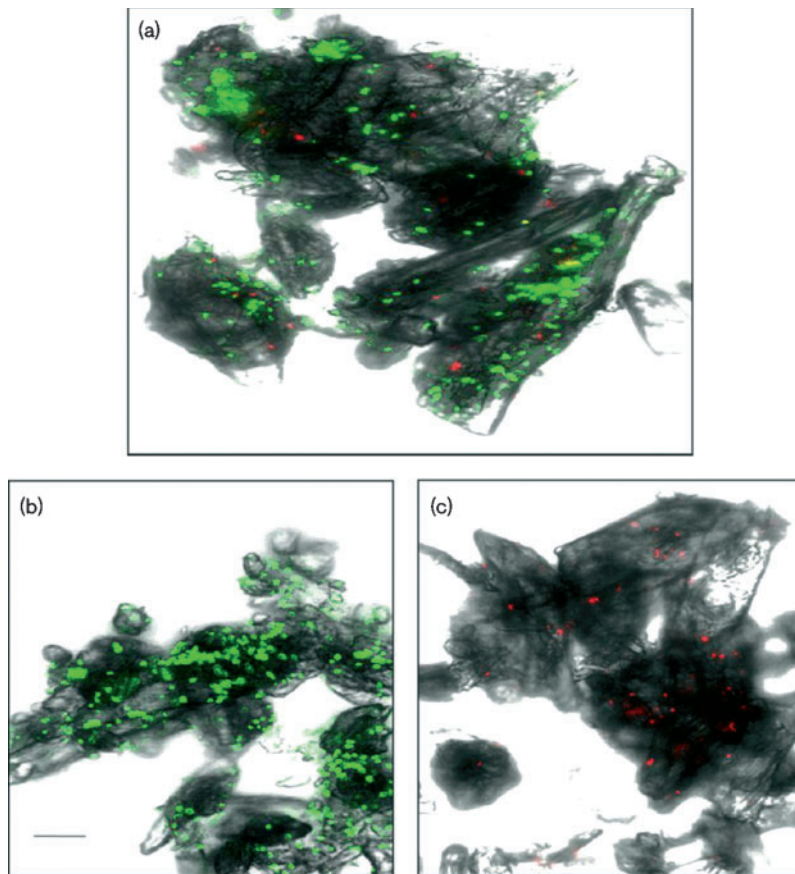


Fig. 4. Transmission fluorescent micrographs using CLSM showing attachment of bacterial strains to microcrystalline cellulose (Avicel) after the adherence assay. (a) *P. tunicata* wild-type: GFP and SM5 mutant: RFP added to the plant as a 1:1 mixture; (b) *P. tunicata* wild-type: GFP only and (c) SM5 mutant: RFP only. Bar, 20 µm.

similar genetic organization and high homology to MSHA pili biogenesis and secretory proteins of various Gram-negative bacteria, including *Pseudoalteromonas haloplanktis*, *Shewanella baltica*, *V. cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. The best characterized MSHA pilus gene locus is that of *V. cholerae* El Tor (Marsh & Taylor, 1999). The 17 *P. tunicata* ORFs identified in this study share major similarities with the MSHA pili secretory and structural genes of *V. cholerae*, including (1) homology of the predicted amino acid sequences, (2) similarity of the predicted location of the gene products, (3) similarity in their organization, orientation and arrangement, and (4) the presence of polycistronic genes, where overlapping stop and start codons of ORFs have been identified.

TEM studies revealed the presence of flexible pili on the cell surface of *P. tunicata*, with ultrastructural characteristics similar to pili of other Gram-negative bacteria. In contrast, the *P. tunicata* SM5 mutant, disrupted in the MSHA gene locus, showed no expression of pili on the cell surface. Biogenesis of pili requires numerous gene products, including a structural prepilin subunit, ancillary proteins with prepilin-like leader sequences, inner- and outer-membrane proteins and nucleotide-binding proteins (Alm & Mattick, 1997). It has been reported that a mutation in any of these genes is sufficient to prevent the assembly of functional pili (Strom & Lory, 1993). For example, mutation of any one of the secretory genes *pilO*, *pilP* or *pilQ* in

Pseudomonas aeruginosa resulted in loss of pili, confirming the importance of these mutated genes in pilus biogenesis (Martin *et al.*, 1995). These secretory genes belong to an operon for secretion and export which includes two other genes (*pilM* and *pilN*) required in the biogenesis of fimbriae in *Pseudomonas aeruginosa* (Martin *et al.*, 1995). In *V. cholerae*, it has been reported that the expression of MSHA to form functional pili on the bacterial surface is completely dependent on the transcription and expression of two operons: secretory and structural (Martin *et al.*, 1995). Deletion in any of the putative promoter regions upstream of *mshI*, a secretory gene, or *mshB*, a structural gene, abolished MSHA pilus assembly, secretion and expression (Martin *et al.*, 1995). Additionally, mutation in *mshE*, a secretory gene in the MSHA biogenesis locus, showed abolished haemagglutination (Hase *et al.*, 1994). The fact that the *P. tunicata* SM5 mutant displayed a non-piliated phenotype and was unable to mediate haemagglutination suggests that the MSHA gene locus is required for pilus biogenesis in *P. tunicata*.

Gram-negative bacteria bind to surfaces via the tip adhesins of the pili (Strom & Lory, 1993). These adhesins may have different specific receptors. For example, *V. cholerae* El Tor strains express a haemagglutinin pilus with a preference for mannose receptors (Jonson *et al.*, 1991). We have demonstrated that the *P. tunicata* pilus causes haemagglutination of horse red blood cells. As for *V. cholerae* El Tor, the

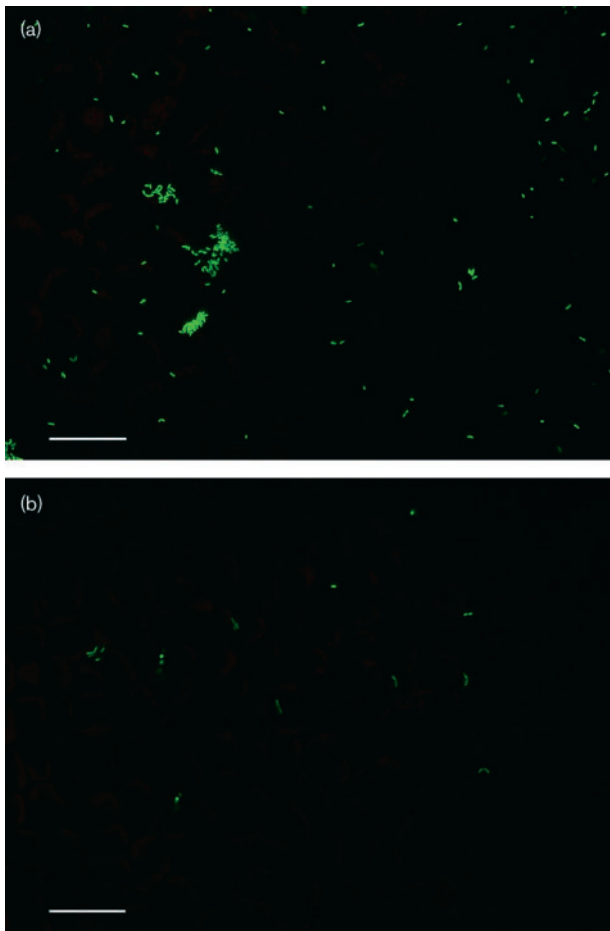


Fig. 5. CLSM images of (a) *P. tunicata* wild-type GFP and (b) *P. tunicata* (SM5) *mshJ* mutant GFP attached to the surface of *U. australis*. Bars, 50 μm .

clumping of red blood cells was abolished in the presence of mannose.

The MSHA pili expressed by *V. cholerae* cells are known to be involved in the attachment to and colonization of surfaces. The MSHA pilus is an important attachment factor in aquatic environments and has been shown to mediate attachment to solid substrates (Chiavelli *et al.*, 2001; Watnick *et al.*, 1999). It was reported that the *V. cholerae* El Tor MSHA mutant strain is unable to form biofilms and shows decreased adherence to abiotic and biotic surfaces (Watnick *et al.*, 1999). In this study, we first tested attachment of *P. tunicata* wild-type and SM5 mutant cells to polystyrene and to cellulose, the surface polymer of *U. australis* and *C. intestinalis* from which *P. tunicata* has been isolated. The SM5 mutant cells showed less attachment to both polystyrene and cellulose surfaces, in comparison with the piliated wild-type strain. This suggests a key role for the *P. tunicata* MSHA pilus in the attachment to both surfaces.

To determine if the MSHA pilus is involved in mediating the attachment of *P. tunicata* cells to living surfaces we

compared the ability of the piliated and non-piliated strains to attach directly to the surface of the green alga *U. australis*. The attachment assays revealed significantly higher numbers of attached *P. tunicata* wild-type cells compared to SM5 mutant cells at the surface of *U. australis*. However, a small number of SM5 cells did attach to the algal surface, suggesting that other mechanisms may be involved in the adhesion of *P. tunicata* to the surface of *U. australis* (for example non-specific physicochemical interactions between the bacterial cell and plant surface). Nevertheless, our findings indicate that the MSHA pilus is a major determinant for attachment of *P. tunicata* to *U. australis*.

This study also showed that specific growth conditions affect the expression of the *P. tunicata* MSHA pilus. *P. tunicata* cells grown in cellulose or cellobiose were found to be hyperpiliated when examined under TEM. These specific substrates also promote attachment of *P. tunicata* to cellulose. We observed that wild-type cells pregrown in cellulose or cellobiose displayed enhanced attachment to cellulose compared to cells grown on other carbon sources (a smaller increase in adhesion to cellulose of the SM5 mutant grown in cellulose or cellobiose was also observed, although the reason for this is unclear). These studies indicate that cellulose or cellobiose may serve as environmental signals which induce expression of the MSHA pilus and thus promote attachment of *P. tunicata* to cellulose-containing surfaces. Our findings concur with previous reports that chemosensory mechanisms can control pilus expression. In *Pseudomonas aeruginosa*, expression of *pilA* is controlled by a two-component sensor-regulator gene pair, *pilS* and *pilR* (Hobbs *et al.*, 1993). The PilS protein is a sensor protein located upstream of the regulator protein PilR, thought to be responsible for sensing unknown environmental signals (Boyd, 2000). In *V. cholerae*, the response of *tcp* (toxin-coregulated pili) genes to environmental stimuli is mediated by the ToxR regulon (Strom & Lory, 1993). The ToxR protein is a major sensor and regulator protein that transmits signals from the periplasmic side of the inner membrane and regulates transcription of virulence factors, including the toxin-coregulated pili. Interestingly, a putative transcriptional regulator, WmpR with homology to ToxR has been identified in *P. tunicata* (Egan *et al.*, 2002b). This gene is involved in the regulation of the expression of antifouling compounds in *P. tunicata* (Egan *et al.*, 2002b). The *P. tunicata* *wmpR* mutant is devoid of pili on its surface when examined using TEM (data not shown). It is possible that the regulatory activity of *wmpR* may explain the surface-sensing mechanisms demonstrated by *P. tunicata* in response to environmental stimuli. In the marine ecosystem, the *P. tunicata* *wmpR* gene product may sense an environmental signal (e.g. cellulose or surface polymers of *C. intestinalis* and *U. australis*) and respond by increasing the expression of MSHA pili on the cell surface, hence promoting the attachment of *P. tunicata* to the surfaces of these marine organisms.

In summary, we have identified a gene locus (*mshI112JKLMNEGFACDOPQ*) with similar genetic

organization and high homology to MSHA pilus biogenesis operons of marine vibrios, and have shown that the *msH* gene locus is required for the expression of a MSHA pilus on the *P. tunicata* cell surface. Our findings demonstrate that production of the MSHA pilus mediates the attachment of *P. tunicata* to surfaces and that this pilus is a key determinant of attachment of *P. tunicata* to both abiotic and living surfaces. We also provide evidence that expression of MSHA pilus is enhanced in the presence of cellulose or cellobiose and propose that substrate-sensing mechanisms and pilus expression in *P. tunicata* have a profound effect on the ecological distribution of this bacterium in marine surface ecosystems.

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