APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2002, p. 372–378 Vol. 68, No. 1
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.1.372–378.2002

Identification and Characterization of a Putative Transcriptional Regulator Controlling the Expression of Fouling Inhibitors in *Pseudoalteromonas tunicata*

Suhelen Egan,[1,2] Sally James,[1,2] and Staffan Kjelleberg[1,2]*

School of Microbiology and Immunology[1] and Centre for Marine Biofouling and Bio-Innovation,[2] The University of New South Wales, Sydney 2052, New South Wales, Australia

Received 1 August 2001/Accepted 12 October 2001

The dark green pigmented marine bacterium *Pseudoalteromonas tunicata* colonizes living surfaces and produces a range of extracellular compounds that inhibit common fouling organisms, including marine invertebrate larvae, algae, bacteria, and fungi. We have observed a positive correlation between the antifouling activity of *P. tunicata* strain D2 and the expression of pigmentation. To address the hypothesis that pigmentation and antifouling may be jointly regulated in this organism and to begin to identify potential regulatory elements, we used transposon mutagenesis to generate a strain of *P. tunicata* deficient in antifouling activity. The data presented here describe the phenotypic and molecular characterization of a nonpigmented transposon mutant strain of *P. tunicata* (D2W2). Analyses of the antifouling capabilities of D2W2 demonstrate that this strain is deficient in the ability to inhibit each of the target fouling organisms. Genetic analysis of D2W2 identified a gene, designated *wmpR* (white mutant phenotype), with high sequence similarity to transcriptional regulators ToxR from *Vibrio cholerae* and CadC from *Escherichia coli*. Two-dimensional polyacrylamide gel electrophoresis analysis revealed that WmpR is essential for the expression of a significant subset of stationary-phase-induced proteins likely to be important for the synthesis of fouling inhibitors. The identification of a gene involved in the regulation of expression of antifouling phenotypes will contribute to the understanding of the interactions between bacteria and other surface-colonizing organisms in the marine environment.

An established biofouling community on surfaces in the marine environment consists of a complex mixture of microorganisms in addition to various sessile algae and animals. The formation of a biofouling community generally begins with the colonization of bacteria, followed by the adherence of diatoms and the settlement and attachment of algal spores and invertebrate larvae (2, 10, 32). Biological interactions between different surface-associated organisms play a major role in the development and maintenance of biofouling communities. Many sessile algae and animals have evolved defense mechanisms against fouling by producing metabolites that can influence the settlement, growth and survival of other organisms (3, 5, 17, 25). However, algae and animals lacking chemical defenses and nonchemical defenses such as surface sloughing are thought to rely on secondary metabolites produced by associated surface bacteria as their defense against fouling (14, 18, 29).

The marine surface-associated bacterium *Pseudoalteromonas tunicata* strain D2 produces a number of stationary-phase, extracellular inhibitors that affect the normal settlement and growth of a variety of common marine fouling organisms. These include larvae from the invertebrates *Ciona intestinalis* and *Balanus amphitrite*, spores from the algae *Ulva lactuca* and *Polysiphonia*, various bacteria, and fungi (13). Moreover, the active components appear to be target specific and include a polar, heat-stable antilarval molecule of less than 500 Da (14), a 190-kDa antibacterial protein (16), a heat-sensitive antialgal molecule of between 3 and 10 kDa (7), and a small, nonpolar antifungal molecule (S. Egan et al., unpublished data).

P. tunicata is dark green due to the production of a yellow and a purple pigment. A correlation between the expression of pigmentation and toxic activity has been observed; for example, growth on nutrient-rich medium results in pigment-less *P. tunicata* colonies that do not display activity against the settlement of invertebrate larvae or algal spores (C. Holmström, unpublished data). In addition, studies designed to address the frequency with which bacterial strains isolated from different marine surfaces show inhibitory activity against common fouling organisms found that a high proportion of dark-pigmented isolates inhibited the settlement of invertebrate larvae and the germination of marine algal spores (12). These bacteria include *P. tunicata* and closely related species (6, 8). From these observations the hypothesis was derived that the expression of pigmentation and the antifouling inhibitors may be jointly regulated in this organism.

Here we report the generation and characterization of a nonpigmented transposon mutant strain of *P. tunicata* D2. The data show that this strain is deficient in antifouling properties. In fact, all extracellular inhibitory compounds presented above are downregulated or absent in this mutant. DNA sequencing shows that this mutant has been disrupted in a gene that encodes a putative transcriptional regulator. Genetic and functional analysis of this gene is presented and evidence is provided to suggest that it functions as a regulator of antifouling activity and pigmentation in the marine bacterium *P. tunicata*.

* Corresponding author. Mailing address: School of Microbiology and Immunology, The University of New South Wales, Sydney 2052, New South Wales, Australia. Phone: (61) 2 93852102. Fax: (61) 2 93851591. E-mail: S.Kjelleberg@unsw.edu.au.

TABLE 1. List of bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli Sm10 λpir	Sm^s, mobRP4, π-replicase (pir)	11
P. tunicata D2	Wild-type strain, Sm^s/Km^s	13
P. tunicata D2Sm	Spontaneously resistant to streptomycin	15
P. tunicata D2W2	Transposon mutant strain, Km^r Sm^r, *wmpR*	This study
Plasmid pLOF/Km	Mini-Tn*10*(Km), Km^r Amp^r	11

[a] Sm^s, streptomycin sensitive; Sm^r, streptomycin resistant; Km^s, kanamycin sensitive; Km^r, kanamycin resistant; Amp^r, ampicillin resistant.

MATERIALS AND METHODS

Transposon mutagenesis. In a study aimed at identifying mutants with different expressions of pigment and fouling inhibitory compounds, transposon mutagenesis was performed in *P. tunicata* (Egan et al., unpublished). Briefly, the methodology used to generate the transposon mutant bank was as follows. The suicide vector pLOF carrying the mini-Tn*10* transposon with a kanamycin resistance marker (11) was transferred to streptomycin-resistant *P. tunicata* cells (strain D2Sm) by conjugation with *Escherichia coli* Sm10 donor strain harboring the pLOF plasmid (Table 1). The cells were then plated onto VNSS agar plates (21) with kanamycin (85 μg ml^{-1}) and streptomycin (200 μg ml^{-1}) to select for recipient *P. tunicata* cells carrying the mini-Tn*10* transposon. In a screen of more than 6,000 mutants, the observation was made that, in general, mutants which lack pigmentation displayed a reduced ability to inhibit target fouling organisms (Egan et al., unpublished) One of these transposon mutants (D2W2) was analyzed further.

Phenotypic characterization of *P. tunicata* D2W2. The UV-visible light spectra of the pigments produced by both the wild type and the D2W2 mutant strain of *P. tunicata* were determined. Pigments were extracted by adding redistilled methanol (40 ml g^{-1} [wet cell weight]) to the cells and stirring the solution over gentle heat for 10 min. The solution was filtered (Whatman filter paper, 5 mm) to remove cell debris, and the UV-visible light spectra were determined by using a Beckman DU 640 spectrophotometer.

A comparison of the growth rates for *P. tunicata* D2W2 mutant strain and *P. tunicata* D2 wild-type strain was performed in 500-ml flasks containing 200 ml of VNSS medium for the wild type and VNSS medium with the antibiotics kanamycin (85 μg ml^{-1}) and streptomycin (200 μg ml^{-1}) for D2W2. One percent (vol/vol) of an overnight culture was inoculated into an appropriate flask and incubated with shaking at 23°C. Growth was monitored by absorbance readings (610 nm) over a 24-h period. This experiment was carried out in duplicates.

The effect of the transposon mutant of *P. tunicata* (D2W2) on the normal growth and behavior of common fouling organism was assessed by previously described protocols. Assays for activity against the growth of bacteria (target strains included *P. tunicata* and *Bacillus subtilis*) were performed in triplicates by the overlay method on agar plates as described by James et al. (16). Briefly, fresh colonies of the test bacteria were spot inoculated, air dried, and incubated for 7 days at 23°C. The test strains were thereafter overlaid with 3 ml of agar containing 0.4 ml of an overnight culture of a target bacterial strain. The antibacterial activity was determined after a further 24-h incubation by a zone of inhibition surrounding the target strains. Antifungal activity was assessed by an agar plate-based method whereby suspensions of target fungal (*Penicillium digitatum*) and yeast (*Saccharomyces cerevisiae*) strains were inoculated onto a VNSS agar plate and air dried. Thereafter, wild-type and transposon mutant strains of *P. tunicata* were stab inoculated from a fresh VNSS agar plate onto the plate containing the fungal or yeast suspension. Plates were incubated for 48 h or until the fungi had created an even lawn of growth. At that time, zones of inhibition were visible surrounding the bacterial inoculations. Assays to determine the antifungal activity were performed in triplicates.

The activity against the germination of algal spores was determined for both *Ulva lactuca* spores and *Polysiphonia* sp. spores by a previously described protocol (7). Briefly, overnight cultures of *P. tunicata* strains were used to inoculate 24-well culture or petri dishes (36 mm) containing VNSS medium with or without appropriate antibiotics. Dishes were incubated for 24 h to form biofilms and washed twice with sterile filtered seawater, and algal spores were added. The number of germinated spores was determined after 5 days and compared to controls containing sterile filtered seawater (i.e., no biofilm). The activity against the settlement of marine invertebrate larvae was determined by standard settlement assays against larvae of the tube worm *Hydroides elegans* and cyprid larvae of the barnacle *Balanus amphitrite* (5, 14). Biofilms of *P. tunicata* strains were prepared in petri dishes (36 mm) as described above. Biofilms were washed twice with sterile filtered seawater, and invertebrate larvae were added. The number of settling larvae was determined microscopically after 3 days of incubation at 25°C and compared to controls containing sterile filtered seawater. Algal spore germination and invertebrate larvae settlement bioassays were performed in triplicates.

DNA manipulation and sequencing. Genomic DNA was extracted from 2 ml of an overnight culture of *P. tunicata* D2W2 by using the XS buffer protocol (31). The DNA sequence of the gene disrupted by the transposon insertion was obtained by a protocol of Siebert et al. (28) and modifications by Tillett (30). Briefly, two specific primers (Tn10D, 5′-CCTCGAGCAAGACGTTTCCCG-3′; Tn10C, 5′-GCTGACTTGACGGGACGGCG-3′) were designed that were complementary to the 5′ and 3′ strands of the mini-Tn*10* transposon. Genomic DNA from the transposon mutant was digested with a variety of blunt-end producing restriction enzymes, and the resulting fragments were ligated to adapter oligonucleotides. The adapter-fragment chimeras were purified and used in a PCR with a primer complementary to the one of the adapter oligonucleotide and one of the transposon-specific primers (i.e., Tn10C or Tn10D). The resulting PCR allows amplification of a region from within the transposon to a site upstream or downstream of the transposon that is defined by the position of the blunt-end restriction enzyme site.

PCR products were visualized on a 1% (wt/vol) agarose gel and were purified by using a Prep-a-Gene DNA purification kit (Bio-Rad) according to the manufacturer's instructions. Both strands of DNA from the PCR products were sequenced by using a primer walking strategy. Approximately 50 to 100 ng of template DNA was used in a thermocycling reaction with BigDye terminator cycle sequencing mix (Applied Biosystems) and analyzed on an ABI 377 DNA sequencing system at the Automated Sequencing Facility at the University of New South Wales.

The completed DNA sequence was compared to known sequences in the GenBank database by using the BLAST search algorithm (1), and open reading frames (ORFs) were defined using the ORF finder program, both available through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Further analysis was performed by using the appropriate programs in the GCG software package provided by the Australian National Genomic Information Service (http://www.angis.org.au/WebANGIS/) and molecular biology analysis tools available through the ExPASy website (http://expasy.proteome.org.au/index.html).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Total cellular protein samples were prepared from early-logarithmic- and early-stationary-growth phase cells of both the *P. tunicata* wild-type strain D2 and the transposon mutant strain D2W2. Two hundred microliters of an overnight culture was inoculated into 50 ml of VNSS medium with appropriate antibiotics for D2W2. Growth was monitored by optical density at 610 nm, and 5-ml samples were removed at an optical density of 0.3 for the early logarithmic sample and 0.6 for early stationary growth. These points represent cells, prior to and at the onset of pigment production and antifouling activities in a *P. tunicata* wild-type culture. Cells were collected by gentle centrifugation (2,000 × *g*, 10 min) and washed once in 0.2 mM sucrose solution, followed by a second centrifugation (2,000 × *g*, 10 min). The cell pellet was resuspended into 200 to 500 μl of sterile milli-Q water and stored at −80°C.

Sample preparation, isoelectric focusing, equilibration, and 2D-PAGE were performed according to the method of Fegatella et al. (9). For each sample, 50 μg of total cell protein was loaded onto 18-cm Immobiline DryStrips (pH 4 to 7, linear; Amersham Pharmacia), and isoelectric focusing was performed with a Multiphor II system (Pharmacia) according to the manufacturer's instructions. Separation in the second dimension was performed by sodium dodecyl sulfate-PAGE on 11.5% gels made with Duracryl (0.8% bisacrylamide; Genomic Solutions) and using a Protean II system (Bio-Rad). After the second dimension separation, gels were silver stained according to the method of Merril et al. (23) with the modification that the gels were soaked prior to staining in a 0.002% (wt/vol) sodium thiosulfate solution. After being stained, the gels were washed briefly in milli-Q water and scanned by using a Bio-Rad GS-700 Imaging Densitometer. Images were saved as tiff files, and the data were analyzed by using BioRad Melanie II software. Duplicates of each set of conditions were performed and compared. Only spots that appeared in both duplicates of one condition and were missing or downregulated sixfold in both of the duplicates of the other condition were considered to represent significant changes.

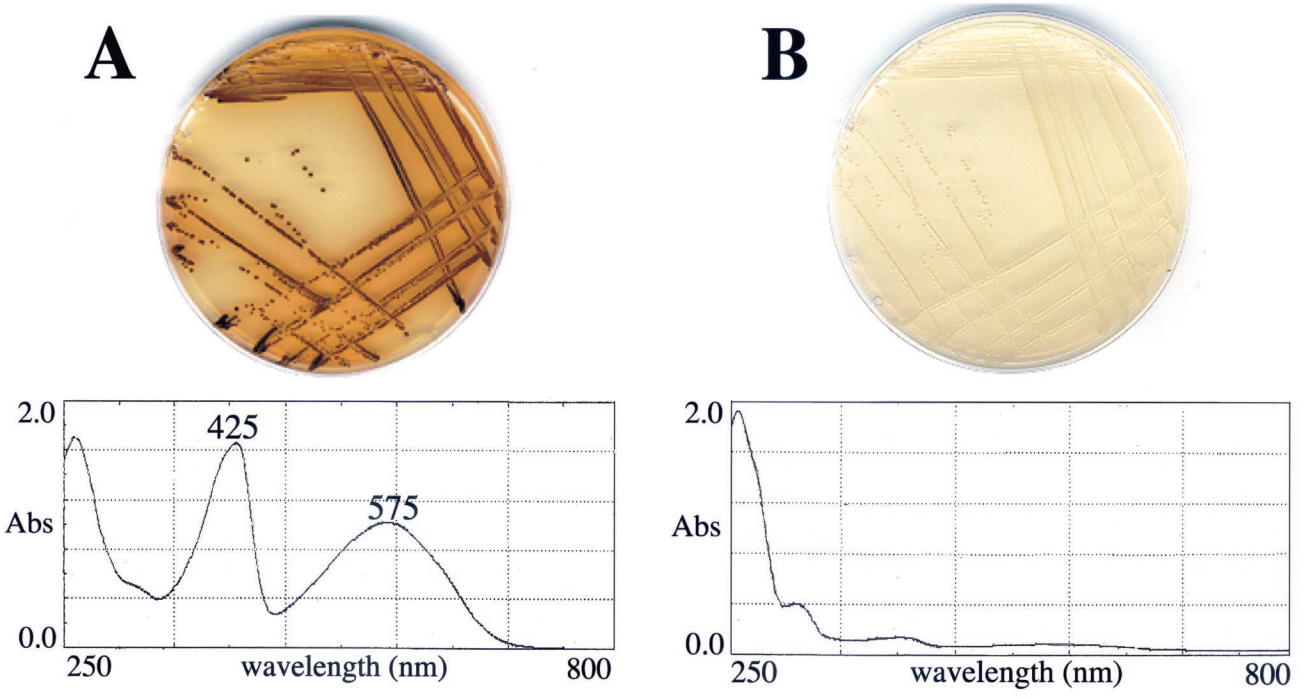

FIG. 1. Wild-type D2 (A) and transposon mutant D2W2 (B) strains of *P. tunicata* showing differences in pigmentation. D2W2 was generated by using the mini-Tn*10* mutagenesis system. UV-visible light spectra are shown below agar plates of each strain. The pigments are represented by peaks at 575 and 425 nm for the purple and yellow pigments, respectively.

RESULTS

A pigment-less transposon mutant is deficient in antifouling activity. To investigate the regulation of expression of antifouling properties in *P. tunicata*, mutants were generated by random transposon mutagenesis (Egan et al., unpublished). We identified one mutant that had lost all antifouling activities. This mutant also exhibited a white phenotype due to the loss of both the yellow and the purple pigments and was designated white mutant 2 (D2W2) (Fig. 1). To obtain a qualitative guide to the pigments produced by strain D2W2 compared to strain D2; UV-visible light spectrum scans were performed. Figure 1 shows that the dark green color of *P. tunicata* D2 is due to the combination of a purple pigment (575 nm) and a yellow pigment (425 nm). The transposon mutant strain D2W2 has lost the pigments corresponding to both peaks, confirming the loss of both the purple and the yellow pigments in this strain.

A detailed characterization of the antifouling activities of D2W2 showed that this strain had lost the ability to inhibit each of the target fouling organisms. Compared to the wild-type strain, D2W2 displayed a reduced ability to inhibit the settlement of larvae from the barnacle *Balanus amphitrite* and from the tube worm *Hydroides elegans*, and the germination of spores from the marine green algae *Ulva lactuca* and the marine red alga *Polysiphonia* sp. (Table 2). In addition, D2W2 was unable to inhibit the growth of a range of bacterial and fungal strains (Table 3).

To ensure that the differences seen in the antifouling bioassays were not due to differences in the growth of the transposon mutant D2W2 compared to the wild-type strain of *P. tunicata*, growth curves were determined. The results showed that the D2W2 displayed the same growth characteristics as the wild type when cultured on the complex marine medium VNSS (data not shown).

Characterization of the gene disrupted in *P. tunicata* D2W2. Phenotypic characterization of the transposon mutant D2W2 suggests that it is disrupted in a gene with global function. Therefore, the genomic DNA regions flanking the transposon in this strain were sequenced. A total of 3,674 bp of DNA sequence was obtained from the DNA region flanking the transposon in D2W2. Analysis of the region indicated that a 2,088-bp ORF encoding a potential transcriptional regulator had been disrupted. The ORF was designated *wmpR* (white mutant phenotype). Further sequence analysis of this region revealed a putative ribosome-binding site 5′-AAGAAG-3′ located 2 bp upstream of an ATG start codon. Promoter prediction analysis resulted in the identification of a putative transcriptional start point approximately 148 bp upstream of the

TABLE 2. Settlement of invertebrate larvae and germination of algal spores in the presence of biofilms of *P. tunicata* wild type and D2W2 transposon mutant

Target organism	% Settlement or germination (mean ± SD)[a]		
	Wild type (D2)	D2W2	No biofilm
Balanus amphitrite larvae	0	79 ± 6.5	95 ± 1.6
Hydroides elegans larvae	0	57 ± 7	60 ± 5.5
Ulva lactuca spores	0	91 ± 3	100
Polysiphonia sp. spores	0	87 ± 6	81.5 ± 2

[a] All values are means ± the standard deviations of three replicates.

TABLE 3. Antibacterial and antifungal activities of *P. tunicata* wild type and D2W2 transposon mutant

Target organism	Mean growth inhibition ± SD (mm)[a]	
	Wild type (D2)	D2W2
Pseudoalteromonas tunicata	6.5 ± 0.6	0 ± 0
Bacillus subtilis	3 ± 0.3	0 ± 0
Penicillium digitatum	5 ± 0.3	0 ± 0
Saccharomyces cerevisiae	3.5 ± 0.5	0 ± 0

[a] All values are means ± the standard deviations of three replicates and represent the radii (in millimeters) of the inhibition zones of the target organisms.

start codon; this region also contains potential −10 and −35 sequences. Following the translational stop of *wmpR* is a GC-rich inverted repeat that is followed by a series of thymidine residues. This region may act as a ρ-independent terminator of transcription (22), therefore suggesting that *wmpR* is monocistronic. The primary nucleotide sequence data for the *P. tunicata wmpR* gene has been deposited in the GenBank database under accession number AF394228.

The deduced amino acid sequence of WmpR showed similarity with the amino terminus to a subgroup of the OmpR-like transcriptional activators. More specifically, WmpR was shown to be 38% identical and 66% similar (over 85 N-terminal amino acid residues) to *E. coli* CadC transcriptional regulator protein (GenBank accession number P23890); 38% identical and 64% similar (over 71 N-terminal amino acid residues) to the ToxR homologue from *Vibrio parahaemolyticus* (GenBank accession number Q05938), and 39% identical and 62% similar (over 71 N-terminal amino acid residues) to the *V. cholerae* transcriptional activator ToxR (GenBank accession number P15795). Interestingly, the large C-terminal domain showed no similarity to known proteins in the GenBank or Swissprot databases. Computer analysis indicates that the protein has a potential transmembrane region between amino acid residues 153 and 175, thus providing support that this protein is membrane integrated.

Global differences in protein expression between the wild-type (D2) and mutant (D2W2) strains of *P. tunicata*. Based on the phenotypic and genotypic study of *P. tunicata* D2W2, it would appear that the putative transcriptional regulator, WmpR, is responsible for the expression of genes and proteins required for the synthesis of pigments and fouling inhibitors. To investigate further the role of WmpR and to identify specific proteins that are influenced by this regulator, differences in the expression of proteins at a global level were examined by 2D-PAGE. Proteins expressed by both the wild type and D2W2 mutant strain were compared at two growth phases. The first samples were taken during early logarithmic growth, which represents a stage before the expression of pigments and inhibitors (14, 16; unpublished data). The second samples were taken during early-stationary-growth phase, when wild-type cells express the pigments and fouling inhibitors. Approximately 950 spots were detected on the gels and analyzed (Fig. 2). Specific differences in protein expression were found between the wild-type and mutant samples, and a summary of this analysis is given in Fig. 3. A total of 39 proteins were upregulated and 9 were downregulated when the early-logarithmic-phase wild-type sample was compared with the early-stationary-phase wild-type sample. Similarly, 24 protein spots were upregulated and 9 were downregulated in the mutant when the early logarithmic sample was compared with the early-stationary-phase sample. A comparison of the mutant with the wild type at the early stationary growth phase found 15 protein spots to be downregulated in the mutant. These proteins consisted of a subpopulation of those that were upregulated in wild-type cells as they enter the early stationary phase of growth. In contrast, no difference was found between the wild type and the mutant strain for the early-logarithmic-growth-phase samples, suggesting that WmpR is a regulator specific to stationary-phase physiology.

DISCUSSION

The marine epibiotic bacterium *P. tunicata* strain D2 is dark green in pigmentation and has been studied extensively for its ability to inhibit a wide range of ubiquitous fouling organisms. Studies have also indicated that there is a positive correlation between pigment production and the expression of antifouling traits in *P. tunicata* and related bacteria, leading to the hypothesis that these phenotypes are jointly regulated (12; Holmström, unpublished). In order to identify potential regulatory elements of antifouling activity, transposon mutagenesis of *P. tunicata* was performed. This study details the generation and characterization of the non-antifouling mutant of *P. tunicata* designated D2W2. This strain was demonstrated to have lost both the purple and the yellow pigments, as well as antifouling activity. This includes the inhibition of ubiquitous macrofoulers, including the settlement of larvae from sessile marine invertebrates such as barnacles (*Balanus amphitrite*) and tube worms (*Hydroides elegans*), as well as the germination of spores from common fouling algae including the green alga *Ulva lactuca* and the red alga *Polysiphonia* sp. In fact, the results presented in Table 2 for the settlement and germination of larvae and spores on surfaces coated with D2W2 are comparable to the values expected for a noninhibitory bacterial strain (8, 12). In addition, it was discovered that D2W2 had lost the ability to inhibit the growth of representative target bacterial, yeast, and fungal isolates, suggesting that this strain does not produce an active antibacterial protein or antifungal compound. It should be noted that the phenotypic differences observed in this study are not due to differences in the growth rate between D2 and D2W2 since both strains display similar growth characteristics. Overall, the phenotypic characterization of *P. tunicata* strain D2W2 supports previous observations that pigmentless *P. tunicata* colonies do not display antifouling activity (Holmström, unpublished).

Sequence analysis of the DNA region where the transposon had inserted in D2W2 revealed that a single ORF had been disrupted. Analysis of the DNA sequence surrounding the ORF suggests that it is monocistronic since a putative terminator of transcription is located downstream of the translational stop of the gene and no neighboring ORFs have been identified. This suggests that the mutation caused by the transposon insertion reflect the loss of function of the disrupted ORF rather than a polar or downstream effect. The deduced gene product of the ORF, designated WmpR, was found to be highly similar in the amino-terminal domain to a subgroup of

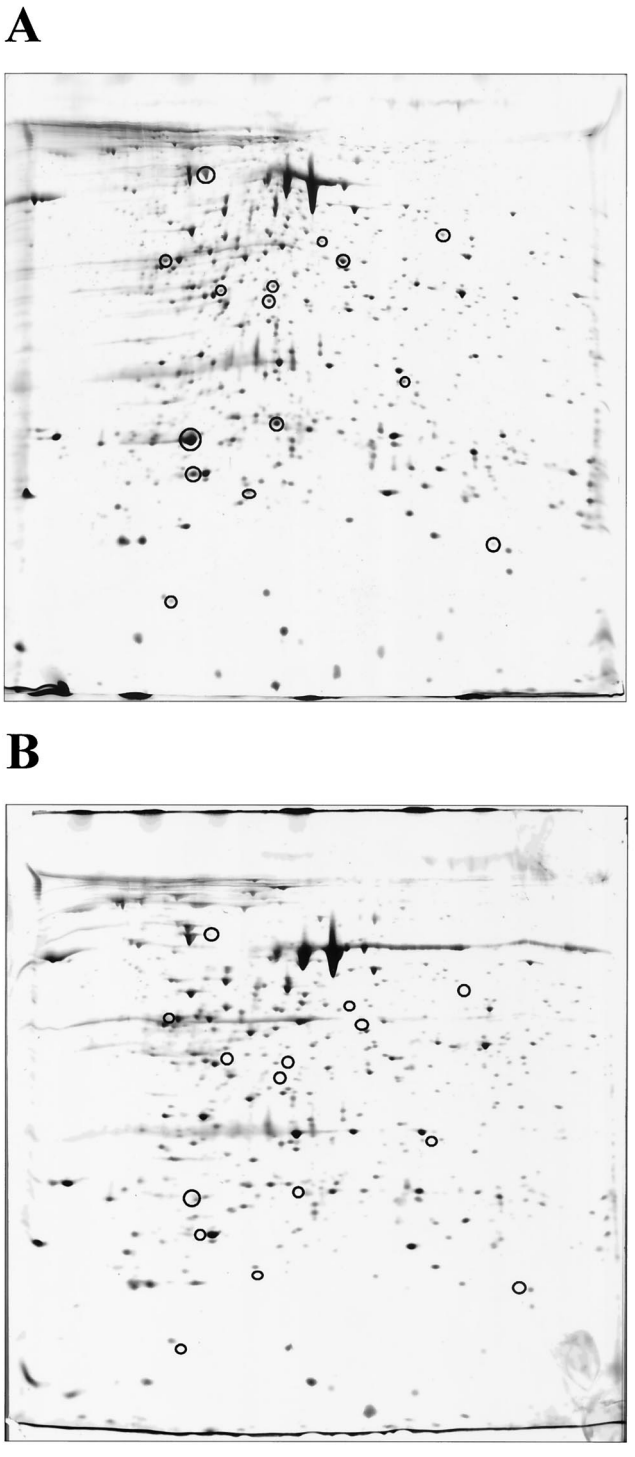

FIG. 2. 2D-PAGE gels of the total cell protein from *P. tunicata* wild type (D2) and transposon mutant (D2W2) cells in the early stationary phase of growth. A total of 50 μg of protein was separated, and the gels were silver stained. Protein spots that are circled indicate proteins found in D2 during early stationary growth (A) but not in D2W2 during early stationary growth (B).

the OmpR-like transcriptional activators, including ToxR from *V. cholerae* and CadC from *E. coli*. This class of regulators differs from other members of the OmpR group since they are membrane bound and are able to both sense and respond to changes within the external environment (24, 33). In addition, these regulators have their DNA-binding domain located at the amino terminus rather than at the carboxy terminus of the protein (24, 33). While the exact physical location of WmpR has not been experimentally determined, computer-assisted analysis of the deduced WmpR primary structure predicts a 23-amino-acid sequence (residues 153 through 175) that resembles the membrane-spanning regions of other proteins. This putative transmembrane segment also aligns with the transmembrane segments from ToxR and CadC (24, 33).

ToxR coordinately regulates the expression of a number of virulence genes, such as cholera toxin and the toxin coregulated pilus, in *V. cholerae*. The ToxR protein is a transmembrane DNA-binding protein which also functions as a sensor and whose activity is enhanced by the presence of ToxS. The genes encoding for ToxR and ToxS are expressed as a single operon separate from the structural genes they regulate. Homologues to ToxR have been characterized in a number of other species within the *Vibrionaceae*, including *V. parahaemolyticus* (20), *V. fischeri* (27), *V. vulnificus* (19), and *Photobacterium profundum* (34). The common theme among this group is the need to respond to changes in environmental conditions. These include changes from a free-living state in seawater to a form closely associated with their host, as is the case of *V. cholerae* and *V. fischeri*, or may be in response to high pressure as for the deep-sea bacterium *P. profundum*.

In *E. coli* a ToxR homologue, CadC, is also a membrane-bound transcriptional activator that is capable of sensing and responding to changes in environmental conditions. The CadC protein activates the transcription of *cadBA* under conditions of low external pH and in the presence of lysine. The *cadBA* operon encodes proteins involved in the decarboxylation of lysine to cadaverine (CadA) and for lysine and/or cadaverine transport (CadB) (4, 33). The production and excretion of cadaverine leads to an increase in the external pH, and it has been suggested that this may provide a selective advantage for the bacterium under acidic growth conditions (26). Unlike *toxR* and the *toxR* homologues found in members of the *Vibrionaceae*, *cadC* is located in close proximity to the structural genes for which it regulates, and it does not appear to be associated with a *toxS* homologue.

Sequence analysis of the DNA surrounding the *wmpR* gene of *P. tunicata* has not identified a *toxS* homologue. In addition,

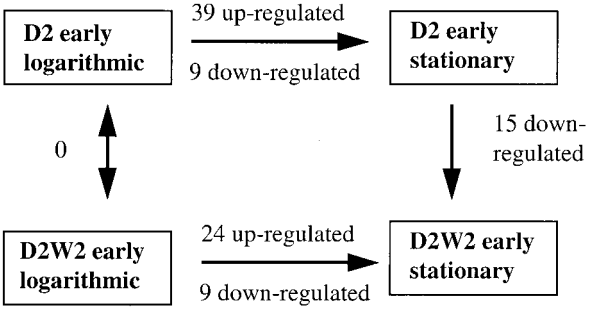

FIG. 3. Differences in the number of proteins expressed by the wild type (D2) and the transposon mutant strain (D2W2) of *P. tunicata* in the early logarithmic and early stationary phases of growth as detected by 2D-PAGE.

the predicted periplasmic space domain of both CadC and WmpR are larger then those of ToxR, which reflects the much larger CadC and WmpR proteins in comparison to other ToxR-like proteins. These observations suggest that WmpR may function at a molecular level in a way similar to CadC rather than ToxR. Therefore, based on the above phenotypic and genetic analysis of D2W2, we propose that WmpR may act as a global regulator for pigment production and antifouling activity in *P. tunicata*.

In order to further investigate the regulatory function of WmpR, we used 2D-PAGE to look at changes in protein expression. Total cellular proteins were compared between D2 and D2W2 at two different growth phases, before (early logarithmic) and at the onset (early stationary) of the expression of pigment and inhibitors in the wild type (Fig. 2). Analysis of the 2D-PAGE patterns for the wild type and mutant revealed no differences in proteins expressed by the two strains during the early logarithmic phase of growth. However, a comparison during the early stationary growth phase found 15 proteins to be missing or largely downregulated (>6-fold) in the mutant. The missing proteins consisted of a subpopulation (40%) of those found to be upregulated in the wild type when the cells entered the early stationary phase of growth (Fig. 3). The results of the 2D-PAGE analysis show a difference in protein expression, which coincides with pigment and/or inhibitor production in *P. tunicata*. The D2W2 strain expresses fewer proteins than did the wild type, a finding which correlates well with the loss of pigment or inhibitor production in this strain. Furthermore, the upregulation of 15 proteins in the wild type and not in the D2W2 mutant indicates that WmpR is an activator of protein expression rather than a repressor.

The nature of the signal(s) and other means of information transfer needed for WmpR-mediated expression of the pigments and inhibitors is unclear. The large periplasmic C-terminal domain of the protein suggests that it is responding to external environmental conditions such as the presence or absence of specific nutrients, toxins, or signal molecules. Observations of pigment or inhibitor expression under different culture conditions indicate that the response is mediated by various environmental signals. For example, in nutrient-rich media a reduction in the expression of pigment occurs and, when grown at close to the maximum growth temperature, only the yellow pigment is expressed (data not shown). In a natural setting WmpR may be required by *P. tunicata* to sense and respond to environmental conditions and signals generated by the host organism (e.g., tunicates and green algae), which in turn lead to the expression of antifouling phenotypes that are beneficial for the host. Thus, the ability of *P. tunicata* to sense environmental conditions and to respond by increasing the expression of fouling inhibitors may be an important means by which it effectively competes with other surface-associated organisms in a biofouling community on living surfaces.

In conclusion, this study has identified the first regulator involved in controlling the expression of both pigment and fouling inhibitors in the marine antifouling bacterium *P. tunicata*. 2D-PAGE analyses revealed a stationary-phase dependency of WmpR regulation in addition to a set of proteins under the control of WmpR. In fact, WmpR appears to control a significant subset of the stationary phase-induced proteins in *P. tunicata*. It is suggested that further studies of the CadC/ToxR homologue in *P. tunicata* will aid in the understanding of how this organism is able to sense and adapt to environmental conditions and coordinate the expression of pigments and the specific extracellular inhibitory compounds. Furthermore, we believe that this information will be of great benefit to the application and development of marine bacteria and their metabolites as effective antifouling and biocontrol agents.

ACKNOWLEDGMENTS

This work was supported by the Australian Research Council and by the Centre for Marine Biofouling and Bio-Innovation.

We thank Carola Holmström for help with invertebrate larvae bioassays and Torsten Thomas for helpful discussions and review of the manuscript.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Clare, A. S., D. Rittschof, D. J. Gerhart, and J. S. Maki.** 1992. Molecular approaches to nontoxic antifouling. Invertebr. Reprod. Dev. **22:**67–76.
3. **Davis, A. R., and A. E. Wright.** 1990. Inhibition of larval settlement by natural products from the ascidian, *Eudistoma olivaceum* (Van Name). J. Chem. Ecol. **16:**1349–1357.
4. **Dell, C. L., M. N. Needly, and E. R. Olson.** 1994. Altered pH and lysine signalling mutants of *cadC*, a gene encoding a membrane-bound transcriptional activator of the *Escherichia coli cadBA* operon. Mol. Microbiol. **14:**7–16.
5. **De Nys, R., P. D. Steinberg, P. Willemsen, S. A. Dworjanyn, C. L. Gabelish, and R. J. King.** 1994. Broad spectrum effects of secondary metabolites from the red algal *Delisea pulchra* in antifouling assays. Biofouling **8:**259–271.
6. **Egan, S., C. Holmström, and S. Kjelleberg.** 2001. *Pseudoalteromonas ulvae* sp. nov., a bacterium with antifouling activities isolated from the surface of a marine alga. Int. J. Syst. Evol. Microbiol. **51:**1499–1504.
7. **Egan, S., S. James, C. Holmström, and S. Kjelleberg.** 2001. Inhibition of algal spore germination by the marine bacterium *Pseudoalteromonas tunicata*. FEMS Microbiol. Ecol. **35:**67–73.
8. **Egan, S., T. Thomas, C. Holmström, and S. Kjelleberg.** 2000. Phylogenetic relationship and antifouling activity of bacterial epiphytes from the marine alga *Ulva lactuca*. Environ. Microbiol. **2:**343–347.
9. **Fegatella, F., M. Ostrowski, and R. Cavicchioli.** 1999. An assessment of protein profiles from the marine oligotrophic ultramicrobacterium, *Sphingomonas* sp. strain RB2256. Electrophoresis **20:**2094–2098.
10. **Henschel, J. R., and P. A. Cook.** 1990. The development of a marine fouling community in relation to the primary film of microorganisms. Biofouling **2:**1–11.
11. **Herrero, M., V. de Lorenzo, and K. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
12. **Holmström, C., S. James, S. Egan, and S. Kjelleberg.** 1996. Inhibition of common fouling organisms by marine bacterial isolates with special reference to the role of pigmented bacteria. Biofouling **10:**251–259.
13. **Holmström, C., S. James, B. Neilan, D. White, and S. Kjelleberg.** 1998. *Pseudoalteromonas tunicata* sp. nov., a bacterium that produces antifouling agents. Int. J. Syst. Bacteriol. **48:**1205–1212.
14. **Holmström, C., D. Rittschof, and S. Kjelleberg.** 1992. Inhibition of attachment of larval barnacles, *Balanus amphitrite* by *Ciona intestinalis* a surface colonizing marine bacterium. Appl. Environ. Microbiol. **58:**2111–2115.
15. **James, S.** 1998. Ph.D. thesis. University of New South Wales, Sydney, New South Wales, Australia.
16. **James, S., C. Holmström, and S. Kjelleberg.** 1996. Purification and characterization of a novel anti-bacterial protein from the marine bacterium D2. Appl. Environ. Microbiol. **62:**2783–2788.
17. **Jensen, P., K. Jenkins, D. Porter, and W. Fenical.** 1998. Evidence that a new antibiotic flavone glycoside chemically defends the sea grass *Thalassia testudium* against zoosporic fungi. Appl. Environ. Microbiol. **64:**1490–1496.
18. **Kon-ya, K., N. Shimidzu, N. Otaki, A. Yokoyama, K. Adachi, and W. Miki.** 1995. Inhibitory effect of bacterial ubiquinones on the settling of barnacle, *Balanus amphitrite*. Experientia **51:**153–155.
19. **Lee, S. E., S. H. Shin, S. Y. Kim, Y. R. Kim, D. H. Shin, S. S. Chung, Z. H. Lee, J. Y. Lee, K. C. Jeong, S. H. Choi, and J. H. Rhee.** 2000. *Vibrio vulnificus* has the transmembrane transcriptional activator ToxRS stimulating the expression of hemolysin gene *vvhA*. J. Bacteriol. **182:**3405–3415.
20. **Lin, Z., K. Kumagal, K. Baba, J. Mekalanos, and M. Nishibuchi.** 1993. *Vibrio parahaemolyticus* has a homolog of the *Vibrio cholerae toxRS* operon that mediates environmentally induced regulation of the thermostable directed hemolysin gene. J. Bacteriol. **175:**3844–3855.

21. **Marden, P., A. Tunlid, K. Malmcrona-Friberg, G. Odham, and S. Kjelleberg.** 1985. Physiological and morphological changes during short-term starvation of marine bacterial isolates. Arch. Microbiol. **142:**326–332.
22. **Mathews, C. K., and K. E. van Holde.** 1990. Biochemistry. The Benjamin/Cummings Publishing Company, Redwood City, Calif.
23. **Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Elbert.** 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science **211:**1437–1438.
24. **Miller, V., R. Taylor, and J. Mekalanos.** 1987. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell **48:**271–279.
25. **Mizobuchi, S., K. Abachi, and W. Miki.** 1996. Antifouling polyhydroxysterols isolated from a Palauan octocoral of *Sinularia* sp. Fish. Sci. **62:**98–100.
26. **Neely, M. N., and E. R. Olson.** 1996. Kinetics of expression of the *Escherichia coli cad* operon as a function of pH and lysine. J. Bacteriol. **178:**5522–5528.
27. **Reich, K., and G. Schoolnik.** 1994. The light organ symbiont *Vibrio fischeri* possesses a homolog of the *Vibrio cholerae* transmembrane transcriptional activator ToxR. J. Bacteriol. **176:**3085–3088.
28. **Siebert, P., A. Chenchik, D. Kellogg, K. Lukyanov, and S. Lukyanov.** 1995. An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res. **23:**1087–1088.
29. **Thomas, R. W. S. P., and D. Allsopp.** 1983. The effects of certain periphytic marine bacteria upon the settlement and growth of *Enteromorpha*, a fouling alga. Biodeterioration **5:**348–357.
30. **Tillett, D.** 2000. Ph.D. thesis. University of New South Wales, Sydney, New South Wales, Australia.
31. **Tillett, D., and B. A. Neilan.** 2000. Rapid nucleic acid isolation from cultured and environmental cyanobacteria: novel techniques based on xanthogenate. J. Phycol. **36:**251–258.
32. **Wahl, M.** 1989. Marine epibiosis. I. Fouling and antifouling: some basic aspects. Mar. Ecol. Prog. Ser. **58:**175–186.
33. **Watson, N., D. Dunyak, E. Rosey, J. Slonczewski, and E. Olson.** 1992. Identification of elements involved in transcripitional regulation of the *Escherichia coli cad* operon by external pH. J. Bacteriol. **174:**530–540.
34. **Welch, T., and D. Bartlett.** 1998. Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium *Photobacterium* species strain SS9. Mol. Microbiol. **27:**977–985.