Unravelling the role of the ToxR-like transcriptional regulator WmpR in the marine antifouling bacterium *Pseudoalteromonas tunicata*

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The dark-green-pigmented marine bacterium *Pseudoalteromonas tunicata* produces several target-specific compounds that act against a range of common fouling organisms, including bacteria, fungi, protozoa, invertebrate larvae and algal spores. The ToxR-like regulator WmpR has previously been shown to regulate expression of bioactive compounds, type IV pili and biofilm formation phenotypes which all appear at the onset of stationary phase. In this study a comparison of survival under starvation or stress between the wild-type *P. tunicata* strain and a *wmpR* mutant (D2W2) does not suggest a role for WmpR in regulating starvation- and stress-resistant phenotypes such as those that may be required in stationary phase. Both proteomic [2-dimensional PAGE (2D-PAGE)] and transcriptomic (RNA arbitrarily primed PCR) studies were used to discover members of the WmpR regulon. 2D-PAGE identified 11 proteins that were differentially expressed by WmpR. Peptide sequence data were obtained for six of these proteins and identified using the draft *P. tunicata* genome as being involved in protein synthesis, amino acid transamination and ubiquinone biosynthesis, as well as hypothetical proteins. The transcriptomic analysis identified three genes significantly up-regulated by WmpR, including a TonB-dependent outer-membrane protein, a non-ribosomal peptide synthetase and a hypothetical protein. Under iron-limitation the wild-type showed greater survival than D2W2, indicating the importance of WmpR under these conditions. Results from these studies show that WmpR controls the expression of genes encoding proteins involved in iron acquisition and uptake, amino acid metabolism and ubiquinone biosynthesis in addition to a number of proteins with as yet unknown functions.

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INTRODUCTION

The Gram-negative, motile bacterium *Pseudoalteromonas tunicata* is found worldwide in marine waters (Brown & Bowman, 2001; Egan *et al.*, 2000; Holmes *et al.*, 2001; Holmström *et al.*, 1998; Skovhus, 2004). This dark-green-pigmented, biofilm-forming bacterium has been primarily

studied for its ability to produce a range of novel bioactive compounds. These stationary-phase metabolites act to inhibit the colonization and growth of a number of common marine fouling organisms and thus are believed to be important as a natural fouling defence for the sessile marine organisms from which *P. tunicata* has been isolated (Egan *et al.*, 2000). The inhibitors are target-specific and include a novel 190 kDa antibacterial protein (James *et al.*, 1996), a polar, heat-stable antilaval molecule of less than 500 Da (Holmström *et al.*, 1992), a heat-sensitive antialgal molecule between 3 and 10 kDa (Egan *et al.*, 2001), a small, non-polar antifungal molecule (A. Franks, S. Egan, C. Holmstrom, H. Lappin-Scott & S. Kjelleberg, unpublished data), an anti-protozoal compound (C. Matz & S. Kjelleberg, unpublished data) and an unidentified compound that is active against diatoms. Moreover, *P. tunicata* is dark green in appearance

Abbreviations: 2D-PAGE, 2-dimensional polyacrylamide gel electrophoresis; CAS, chrome azurol sulfonate; DP, 2',2'-dipyridyl; NRPS, non-ribosomal peptide synthetase; OMP, outer-membrane protein; RAP-PCR, RNA arbitrarily primed PCR; RT, reverse transcriptase; sRT-PCR, semi-quantitative reverse transcriptase PCR.

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due to the production of two pigments – a yellow pigment, with structural similarity to a tambjamine (Franks *et al.*, 2005), and a purple pigment identified as violacean (C. Matz & S. Kjelleberg, unpublished data).

Recent studies have established that the expression of the pigments and bioactive compounds in *P. tunicata* is controlled by the protein WmpR, a homologue of the transcriptional regulator ToxR from *Vibrio* species and CadC in *Escherichia coli* (Egan *et al.*, 2002b). Regions of the ToxR protein are homologous within members of the family *Vibrionaceae* (Osorio & Klose, 2000), and in several pathogenic and non-pathogenic strains ToxR, in co-operation with its downstream enhancer ToxS, plays an integral part in the co-ordinate expression of genes in response to environmental stimuli. In the human pathogen *Vibrio cholerae*, ToxR controls expression of the virulence factors cholera toxin (CTX) and the toxin co-regulated pilus (TCP) in response to environmental signals such as bile salts, pH and temperature (Hung & Mekalanos, 2005; Krukoni & DiRita, 2003). ToxR in the fish pathogen *Vibrio anguillarum* regulates biofilm formation and resistance to bile, but not virulence genes (Wang *et al.*, 2002, 2003). The non-pathogenic piezophile *Photobacterium profundum* contains a ToxR homologue that regulates starvation response genes (Bidle & Bartlett, 2001). ToxR controls genes required for successful colonization of the light organ of certain fish and squid by the fish symbiont *Vibrio fischeri* (Reich & Schoolnik, 1994). CadC, a homologue of ToxR present in *E. coli*, responds to external pH by activating the CadA/B operon involved in acid tolerance (Watson *et al.*, 1992).

Egan *et al.* (2002b) determined that in *P. tunicata*, WmpR regulates both the pigments and all of the bioactive compounds. WmpR also regulates the production of the type IV pili (Saludes, 2004) and is involved in biofilm formation and cell death due partly to its regulation of the antibacterial protein AlpP (Mai-Prochnow *et al.*, 2004). These previous studies have shown that WmpR positively regulates a number of phenotypes that appear when *P. tunicata* enters stationary phase. However, it is unclear whether WmpR is a specific regulator of the antifouling compounds and pigments or if it acts as a general stationary-phase regulator. To address this we subjected the *P. tunicata* wild-type and *wmpR* mutant strains to starvation and stress conditions such as those that may be encountered during stationary phase and compared their survival. The results did not indicate a role for WmpR in the regulation of stationary-phase physiology induced by carbon, phosphate or nitrogen starvation. To further assess the extent of the WmpR regulon in *P. tunicata* we performed both proteomic studies [2-dimensional PAGE (2D-PAGE)] and transcript analysis [RNA arbitrarily primed PCR (RAP-PCR)]. Results from these studies and a comparison of survival under iron limitation show that WmpR controls the expression of a number of genes, including those involved in the sequestering and uptake of iron, amino acid metabolism and a number of proteins with as yet unknown functions.

METHODS

Bacterial strains. *Pseudoalteromonas tunicata* wild-type and *wmpR* mutant strains (D2W2) were used in these studies. Generation of the D2W2 strain by transposon mutagenesis and characterization has been described previously by Egan *et al.* (2002b). The *wmpR* gene is monocistronic, and hence the mutation should not have polar effects on downstream genes (Egan *et al.*, 2002b). Strains were grown with shaking at room temperature in the complex marine medium VNSS (Marden *et al.*, 1985). For starvation and stress experiments, the defined Marine Minimal Medium (3M) was used (Östling *et al.*, 1991). Kanamycin was added at a concentration of 85 µg ml⁻¹ for the cultivation of D2W2, which has been shown previously not to affect the growth characteristics of this strain (Egan *et al.*, 2002b).

Starvation and stress of *P. tunicata*. We compared survival of the wild-type and D2W2 *P. tunicata* strains under carbon, nitrogen or phosphate starvation, which are general markers of stationary-phase physiology and have been used in previous starvation studies (Nelson *et al.*, 1997; Nyström, 1998; Nyström *et al.*, 1992). Two-hundred microlitres of an overnight culture was inoculated in 20 ml 3M+0.4% trehalose and grown to either mid-exponential phase (OD₆₀₀ of 0.35) or to early stationary phase (OD₆₀₀ of 1.2). Starvation conditions were obtained by pelleting cells at 12 000 g at 20 °C for 5 min, then washing twice with 3M lacking the addition of the nutrient which the cells were being starved of (starvation medium). The cell pellet was resuspended in 20 ml starvation medium in a 100 ml flask and incubated at 25 °C with shaking. Culturability (the number of colonies growing on an agar plate after 2 d: c.f.u. ml⁻¹) was measured at 1, 4, 6, 8, 12, 20 and 27 d on VNSS agar plates. To control for the effect of starvation, we included a treatment where the nutrient that the culture was starved of was added back after 24 h starvation. The experiment was performed in duplicate and repeated three times.

P. tunicata wild-type and D2W2 strains were stressed with UV or H₂O₂ as follows. Cultures were grown in 3M+0.4% trehalose to early stationary phase (OD₆₀₀ of 1.2) and 24 h into stationary phase. The number of c.f.u. ml⁻¹ was determined at time 0 by colony plate counts. For UV stress, 200 µl culture was pipetted onto a glass slide and exposed to UV-C (254 nm) using a UV cross-linker (Amersham Life Science) at 25, 50, 75 or 100 J m⁻². The number of c.f.u. ml⁻¹ was determined by colony plate counts. The experiment was performed three times, each in duplicate. For H₂O₂ stress, cultures were exposed to a final concentration of 1 mM H₂O₂ and incubated with shaking at room temperature. Aliquots were taken at 15, 30 and 60 min and the number of c.f.u. ml⁻¹ was determined by colony plate counts. The experiment was repeated three times, each in duplicate.

2D-PAGE. Total cellular protein sample preparation was performed according to the method of Egan *et al.* (2002b). *P. tunicata* wild-type and D2W2 strains were grown in VNSS to an OD₆₀₀ of 0.65 (the onset of stationary phase in this medium). Cultures (20 ml) were centrifuged for 10 min at 2000 g, washed with 0.2 M sucrose and centrifuged again. The cell pellets were resuspended in 500 µl molecular grade water and stored at -80 °C.

Procedures for 2D-PAGE were performed as described previously (Egan *et al.*, 2002b; Fegatella *et al.*, 1999) with some modifications. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Sigma), according to the manufacturer's instructions. Total cell protein (150 µg) was added to rehydration buffer up to 500 µl and loaded onto 18 cm immobilized dry strips (pH 4–7, linear; Amersham Pharmacia). Isoelectric focusing was performed overnight using a Multiphor II (Pharmacia), according to the manufacturer's instructions. The second dimension was performed in 11.5% SDS-PAGE gels made with Duracryl (0.8% bisacrylamide;

Genomic Solutions) and run on a Protean II system (Bio-Rad). Gels were silver-stained using a sensitive ammoniacal method, scanned with a Bio-Rad GS-700 Imaging Densitometer and analysed using the Z3 2D-PAGE Analysis System (Version 3.0.1; Compugen). Triplicates of each condition were performed and only protein spots that were consistently absent or up-regulated by at least six-fold in all sets were considered.

Protein digestion. Protein spots were excised and the gel pieces were destained using a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate until the colour disappeared from the gel pieces. The reaction was stopped by washing three times in deionized water and soaking in 200 mM ammonium bicarbonate for 20 min. The gel pieces were dehydrated in acetonitrile for 10 min then dried down in a vacuum centrifuge. Gel pieces were reduced for 1 h at 56 °C using 10 mM DTT and then alkylated for 45 min in the dark using 55 mM iodoacetamide. The gel pieces were washed with 100 mM ammonium bicarbonate for 10 min and dehydrated using acetonitrile. Then the gel pieces were rehydrated at 4 °C in buffer containing 10 ng trypsin μl^{-1} and 10 mM ammonium bicarbonate (pH 8) for approximately 15 min. Proteins were then digested overnight at 37 °C.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) amino acid sequencing. Nanoflow LC-MS/MS analysis was performed using a QTOF Ultima mass spectrometer (Waters/Micromass UK) employing automated data-dependent acquisition. The mass spectrometer was operated in positive ion mode with a source temperature of 80 °C and a counter-current gas flow rate of 150 l h^{-1} . The peptides were bomb-loaded onto a custom-made capillary reversed-phase column [75 μm i.d., 360 μm o.d.; Zorbax SB-C18 3.5 μm (Agilent)]. A nanoflow-HPLC system (UltiMate-Switchos2-Famos; LC Packings) was used for separation of the peptide mixture prior to MS detection. Peptides were eluted at 200 nl min^{-1} by an increasing concentration of acetonitrile (2 % min^{-1} gradient). An MS-time of flight (MS-TOF) survey spectrum was recorded for 1 s. The most intense ions present in the MS-TOF spectrum were selected and fragmented by collision-induced dissociation in the second quadrupole (4 s per MS/MS spectrum). The fragment ion spectra were manually interpreted to obtain enough amino acid sequences to search for similar sequences in protein databases using the short, nearly exact match search available through the NCBI website (www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1990).

RNA preparations. Early-stationary-phase cells (OD_{600} of 0.65) were harvested and RNA was extracted using Trizol (Invitrogen), a guanidinium thiocyanate phenol-based reagent, according to the manufacturer's instructions. Quality of RNA was determined by agarose gel electrophoresis prior to use, then quantified using a UV spectrophotometer and treated with DNase (Promega) for 1 h at 37 °C to remove traces of genomic DNA from the samples.

RAP-PCR. RAP-PCR was performed according to the method of Bidle & Bartlett (2001). Arbitrary 10-mer primers with G+C contents of 50% were obtained from Genosys Biotechnologies. First strand cDNA synthesis was performed using 500 ng heat-denatured RNA (65 °C, 10 min) in 20 μl reaction mixtures containing 200 μM dNTPs, 5 mM dithiothreitol, 100 U reverse transcriptase (RT; Invitrogen), 1 $\times$ RT reaction buffer and 0.4 μM arbitrary primer. The first strand reaction was performed under the following conditions: touchdown from 50 to 30 °C in 45 s increments, 1 h at 37 °C, then 95 °C for 5 min. Second strand synthesis was performed in 30 μl reactions containing 3 μl cDNA from the first strand reaction, 0.3 U *Taq* polymerase (Invitrogen), 20 μM each dNTP, 2 μM primer used in the first strand reaction, 2 μM different random primer, 2.5 μCi [α - ^{32}P]dCTP, 1 $\times$ *Taq* buffer and 1.5 mM MgCl_2 . Amplification of DNA was conducted using the following protocol:

94 °C for 30 s, 40 °C for 2 min and 72 °C for 1 min for 40 cycles, with a final step of 72 °C for 10 min. Products were stored at -20 °C for a maximum of 24 h. RAP-PCR samples were added to loading dye and heated for 2 min at 92 °C, then run on a 5 % denaturing acrylamide gel containing 33 % urea at 1500 V for approximately 5 h. The gel was then dried overnight, marked with phosphorescent tape for orientation and exposed to film for 6 to 24 h depending on the age of the [α - ^{32}P]dCTP.

Isolating and cloning RAP-PCR fragments. Bands determined to be differentially expressed were excised from the gel by aligning the autoradiograph with the gel using the phosphorescent marks and using a clean scalpel blade to excise each band. DNA was eluted from the gel piece in 50 μl 10 mM Tris (pH 8) overnight at room temperature. Ten microlitres of this elution was used to reamplify the DNA using the same pair of primers used in the second strand reaction of the RAP-PCR. DNA was cloned using the pCR2.1-TOPO cloning kit (Invitrogen), according to the manufacturer's instructions.

DNA sequencing. Clones of the correct size were sequenced using an Applied Biosystems 3730 DNA sequencer at the Automated Sequencing Facility at the University of New South Wales, Australia. Sequence alignments were performed using Inherit software. Panhandle PCR was used, as described previously by Egan *et al.* (2002a), to obtain additional sequence information for the gene fragments.

DNA sequences were compared with sequences in the GenBank database made available through the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) BLASTx program (Altschul *et al.*, 1990).

Confirmation of RAP-PCR results by semi-quantitative RT-PCR (sRT-PCR). The procedure for sRT-PCR was performed as described previously (Goodchild *et al.*, 2004) with some modifications. Briefly, RNA was extracted as described above and the first strand reaction used 100 ng of random hexamer primers (Promega) instead of the arbitrary primer. The sRT-PCR was optimized to ensure that amplification was in exponential phase and that the two primer sets being used did not compete with each other. The level of 16S rRNA was used as a control to allow normalization and comparison of RNA levels between genes. Non-reverse-transcribed RNA was also used in each set of reactions as a control to confirm the absence of genomic DNA in the samples. Second strand synthesis was performed using a sequence-specific primer set (TonBOME, 5'-CGTTAGGAGCACGTACCG-3', and TonBOMR, 5'-CTAGGTCAA-GGTGGCGC-3'; NRPSF, 5'-CTGTCTCACCATTGTGGCG-3', and NRPSR, 5'-CGCTACAAAGAGGATGGC-3'; UNE, 5'-ACCATTGCAACAAACGC-3', and UNR, 5'-GGAAGACAACACTACATTGAGC-3') and a 16S rRNA primer set [63f (Marchesi *et al.*, 1998) and 1392r (Pace *et al.*, 1986)] with the following reaction conditions: 80 °C for 2 min, then 20 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min. PCR products were visualized on a 1 % agarose gel stained with SYBR Green 1 (Sigma) at 473 nm on a FLA-5000 (Fujifilm) and quantified using the IMAGEGUAGE program (Fujifilm). The ratio between the specific RNA and 16S rRNA was calculated to allow for initial RNA variations in the samples and control for PCR efficiency. The paired Student's *t*-test ($P \leq 0.05$) was performed on three RNA replicates from three separate experiments for each gene.

Growth under iron limitation. Growth under iron limitation was compared between the *P. tunicata* wild-type and D2W2 strains. Iron limitation was achieved by adding 0.1 mM of the commonly used iron chelator 2',2'-dipyridyl (DP) to 3M. An overnight culture grown in VNSS was diluted 1 in 100 into 3M in the presence or absence of 0.1 mM DP and incubated with shaking at room

temperature. Culturability (c.f.u. ml⁻¹) was measured over 160 h using colony plate counts. The experiment was carried out three times in duplicate.

Chrome azurol sulfonate (CAS) liquid assay. Overnight cultures of *P. tunicata* wild-type and D2W2 grown in VNSS were inoculated 1:100 into 20 ml 3M in 100 ml flasks. Aliquots (2 ml) were taken at 0, 24 (exponential phase) and 48 h (stationary phase), centrifuged at 12000 g for 5 min and the supernatant was used in the CAS assay. The liquid CAS assay was carried out according to the method of Schwyn & Neilands (1987). Culturability (c.f.u. ml⁻¹) was measured at each time point by colony plate counts.

RESULTS

Comparison of survival under starvation or stress conditions by *P. tunicata* wild-type and *wmpR* mutant (D2W2) strains

The effect of starving the wild-type and D2W2 strains of carbon, phosphate or nitrogen was compared. The results were similar for both carbon (Fig. 1) and phosphate starvation (results not shown), while nitrogen starvation had a lethal effect on both strains within 48 h (results not shown). Because the phenotypic and protein differences between the wild-type and D2W2 occur at stationary phase (Egan *et al.*, 2002b), the strains were grown to both mid-exponential phase and stationary phase and starved to determine if there was a survival difference between growth phases. When exponentially grown wild-type and D2W2 strains were resuspended in the starvation medium lacking either carbon or phosphate, the survival of the two strains was almost identical and showed a very steady decline in the number of culturable cells over time. When cells grown to the start of stationary phase were resuspended in starvation medium lacking either carbon or phosphate, the wild-type

P. tunicata strain showed a significant decrease in culturability compared to the D2W2 strain. However, after 20 d the c.f.u. ml⁻¹ counts for both strains were similar. As a control, after 24 h growth the nutrient (carbon, phosphate or nitrogen) was added and both the wild-type and D2W2 strains resumed growth (results not shown). The wild-type and D2W2 strains showed no difference in survival under UV or hydrogen peroxide stress (results not shown).

Analysis of 2D-PAGE

Using 2D-PAGE we compared the protein expression of *P. tunicata* wild-type to that of D2W2 in stationary phase. A previous study by Egan *et al.* (2002b) had shown that there is no difference in protein expression between the two strains at exponential phase; however, on entry into stationary phase 15 proteins were up-regulated in the wild-type. The current study detected approximately 600 spots on each gel and found 10 proteins unique or up-regulated by at least sixfold in the wild-type and identified one protein which is up-regulated in D2W2 (Fig. 2). The difference in the number of up-regulated proteins in the current study versus the previous study by Egan *et al.* (2002b) can be attributed to the different silver-staining methods used in the two studies. The silver-staining method used in this study, while being less sensitive, was more compatible with mass spectrometry analysis. Of the 11 proteins identified as differentially expressed in the wild-type compared to D2W2, six were not detected in the D2W2 gels and five were found to be significantly up-regulated by at least sixfold in the wild-type compared to the D2W2 gels, using Z3 software. Spot 11 was found to be up-regulated by at least sixfold in the D2W2 gels, suggesting WmpR down-regulates this protein on entry into stationary phase.

Identification of proteins in the WmpR regulon

Amino acid sequence data were obtained for six out of the 11 differentially regulated proteins. The identity of five of the 11 proteins was not obtained due to low abundance of the proteins or an insufficient amount of peptides following trypsin digestion. We identified six proteins by matching the amino acid sequences with proteins in the recently available draft annotated *P. tunicata* genome (Table 1). Spot 2 matched to coenzyme Q4 (Coq4) homologues involved in ubiquinone biosynthesis from several prokaryotes and eukaryotes, including the cyanobacterium *Nostoc punctiforme* (31 % identical, 52 % similar over 181 aa; accession no. ZP_00112374), the fungus *Aspergillus fumigatus* (30 % identical, 49 % similar over 145 aa; EAL92927) and mouse *Mus musculus* (30 % identical, 49 % similar over 170 aa; NP_848808). Spot 10 is a putative aspartate aminotransferase (AspAT) that matches to putative AspATs from *Desulfovibrio desulfuricans* (35 % identical, 54 % similar over 360 aa; YP_387599) and *Aquifex aeolicus* (28 % identical, 46 % similar over 379 aa; NP_214350). Spot 11 matched closely to elongation factor Tu (EF-Tu) from *Pseudoalteromonas haloplanktis* (89 % identical and 94 % similar over 393 aa; YP_338773) and *V. cholerae* 01 (88 %

Fig. 1. Effect of carbon starvation on wild-type and *wmpR* mutant (D2W2) *P. tunicata* strains. Wild-type (squares) and D2W2 (circles) were grown to exponential (open symbols) or stationary phase (closed symbols), resuspended in starvation medium lacking carbon and starved for 27 d. Error bars represent SD.

Fig. 2. Comparison of 2D-PAGE protein profiles for wild-type (a) and *wmpR* mutant (D2W2) (b) strains of *P. tunicata* at the onset of stationary phase. Circles identify proteins differentially expressed between the wild-type and D2W2.

identical and 95 % similar over 393 aa; AAF93494), in addition to EF-Tus from many other organisms.

Spots 3 and 4 had the same molecular mass but differed slightly in isoelectric point. These spots were identified as the same hypothetical protein. Post-translationally modified proteins are frequently identified using 2D-PAGE; these appear as horizontal or vertical rows of spots in the gel (Gorg *et al.*, 2004). Spot 1 was also identified as a hypothetical protein.

Identification of genes in the WmpR regulon by RAP-PCR

Seventeen differentially regulated bands were obtained using 17 different primer combinations in this RAP-PCR analysis. Of these bands, 10 were unique to the wild-type strain and

seven to the D2W2 strain. Due to an inability to amplify eight of the bands (seven of which were unique to the wild-type strain and one unique to D2W2), this study considered only nine of the bands. The problem of reamplification of differentially expressed bands has been reported in other studies of this kind (Bidle, 2003; Bidle & Bartlett, 2001). One example of a standard autoradiograph obtained is shown in Fig. 3(a). RAP-PCR gene fragments were determined to be differentially expressed when there was no matching fragment in the adjacent lane. The deduced partial amino acid sequence from clone 10-1 matched with high similarity to TonB-dependent outer-membrane proteins (OMPs) from several bacteria (for example, 36 % identity, 55 % similarity over 430 aa to a TonB-dependent OMP from *Xylella fastidiosa*; NP_779483). The deduced amino acid sequence of clone 7-3 contained conserved domains found in non-ribosomal peptide biosynthesis proteins, including a

Table 1. Proteins up- or down-regulated by WmpR and identified by proteomics

Spot no.	Regulation by WmpR*	Observed pI/MM†	Predicted pI/MM†	No. of peptides matched	Putative protein function
2	Up (ND)	4·9/22	4·8/25	4	Coenzyme Q4 homologue
10	Up (ND)	5·2/40	5·1/41·6	8	Aspartate aminotransferase
11	Down	5·3/43·5	5·1/43·2	6	Elongation factor Tu
1	Up	4·7/16	4·98/24	8	Hypothetical protein
3	Up	4·9/27	4·93/25·2	6	Hypothetical protein
4	Up	4·8/27	4·93/25·2	3	Hypothetical protein

*ND, Not detected in D2W2 gels.

†pI, Isoelectric point; MM, molecular mass.

Fig. 3. (a) Example of a RAP-PCR autoradiograph from this study. Wt, Wild-type; D2W2, *wmpR* mutant. Arrows indicate differentially expressed gene fragments. Approximate gene fragment sizes are given on the left. (b) Confirmation of differential gene expression of RAP-PCR fragments. sRT-PCR was performed with stationary-phase *P. tunicata* wild-type (Wt) and D2W2 RNA using gene-specific primers. Relative amounts of each target gene were calculated after normalization using 16S rRNA (for further details, see Results). Clone designations are given beneath.

condensation domain and an AMP-binding domain. More specifically, the partial amino acid sequence was found to be 47 % identical and 64 % similar (over 492 aa) to arthrofactin synthetase C from *Pseudomonas* sp. MIS38 (BAC67536); 45 % identical and 63 % similar (over 520 aa) to syringomycin synthetase from *Pseudomonas syringae* (T14593) and 45 % identical and 60 % similar (over 500 aa) to a non-ribosomal peptide synthetase (NRPS) from *Erwinia carotovora* (YP_049593). Further sequencing of the gene fragment from clone 5-4 allowed an ORF to be

identified which matched to a hypothetical protein from the deep-sea bacterium *Photobacterium profundum* (27 % identical, 49 % similar over 191 aa; YP_129958) (Vezzi *et al.*, 2005). The protein from clone 5-4 also matched closely to another protein in the *P. tunicata* genome, found to be up-regulated by WmpR using 2D-PAGE (spot 1).

Six other bands (all up-regulated in D2W2) were identified as either 16S or 23S rRNA. rRNAs are common artifacts associated with RAP-PCR, due to the higher relative abundance of rRNAs compared to mRNAs (Nagel *et al.*, 2001).

Confirmation of RAP-PCR results by sRT-PCR

An sRT-PCR method was used to confirm that the transcript levels of the genes identified in RAP-PCR were differentially expressed in the wild-type compared to the D2W2 strain (Fig. 3b), standard practice when using the RAP-PCR technique (Bidle, 2003; Bidle & Bartlett, 2001; Shepard & Gilmore, 1999). The relative gene expression ratios (wild-type:D2W2) were determined to be 3, 5.2 and 6.4 for the NRPS gene (clone 7-3), the TonB-dependent OMP gene (clone 10-1) and the unidentified gene (clone 5-4), respectively. Statistical analysis ($P \leq 0.05$) confirmed that all three genes were significantly up-regulated in the wild-type strain compared to the D2W2 strain. Therefore WmpR positively regulates these genes.

Growth under iron limitation

Under iron limitation (in the presence of 0.1 mM DP, a commonly used iron chelator) *P. tunicata* D2W2 had a lower culturability compared to the wild-type grown under the same conditions and to D2W2 grown in the presence of iron (absence of 0.1 mM DP) (Fig. 4). D2W2 grown with iron limitation lost all culturability by 160 h, while the wild-type grown under the same conditions displayed a 2-log

Fig. 4. Effects of iron limitation on *P. tunicata* wild-type and *wmpR* mutant (D2W2). The wild-type (squares) and D2W2 (circles) were grown in the presence (closed symbols) or absence (open symbols) of iron. Culturability was measured as c.f.u. ml⁻¹. Error bars represent SD.

reduction in culturability compared to the wild-type grown in the presence of iron.

The liquid CAS assay

The supernatants of the wild-type and D2W2 exponential- and stationary-phase cultures were analysed for the presence of extracellular iron-binding compounds (Fig. 5). A decrease in A_{630} indicates the presence of iron-binding compounds that remove Fe^{3+} from the CAS/ Fe^{3+} /HDTMA complex. The growth of the two strains was comparable over time; however, the A_{630} of the CAS complex, and hence the presence of iron-binding compounds in the supernatants, differed significantly after 48 h growth (stationary phase) (Student's *t*-test, $P \leq 0.05$). The wild-type produced approximately fivefold more iron-binding compounds than the D2W2 strain.

DISCUSSION

This study demonstrates that WmpR is an important regulator in *P. tunicata*, regulating a TonB-dependent outer-membrane receptor, an NRPS, a coenzyme Q4 homologue, aspartate aminotransferase, elongation factor Tu and several unknown proteins. Previous work showed that WmpR directly or indirectly regulates genes involved in producing the yellow and purple pigments, the antifouling compounds, type IV pili and biofilm formation phenotypes in *P. tunicata* (Egan *et al.*, 2002b; Mai-Prochnow *et al.*, 2004; Saludes, 2004). The phenotypic differences observed between the *P. tunicata* wild-type and *wmpR* mutant (D2W2) strains occur at the onset of stationary phase (Egan *et al.*, 2002b). Therefore, we investigated whether WmpR is a general stationary-phase regulator in this organism or a specific regulator of bioactive compounds. The

results of the starvation and stress studies did not indicate a role for WmpR in resistance to conditions that may be encountered during stationary phase (Fig. 1 and data not shown), unlike ToxR in other bacterial species, including *V. anguillarum* (Wang *et al.*, 2002, 2003) and *Photobacterium profundum* (Bidle & Bartlett, 2001). Interestingly, when stationary-phase wild-type cells were starved of either carbon or phosphate, survival was significantly poorer than that of starved stationary-phase D2W2 cells. This result could be due to the presence of the autotoxic antibacterial protein in stationary- rather than exponential-phase *P. tunicata* wild-type cells, causing cell death similar to that seen in biofilms established for 48 h or longer (Mai-Prochnow *et al.*, 2004).

Since WmpR did not appear to be a starvation or stress regulator, but nevertheless regulates key stationary-phase phenotypes in this organism, we aimed to determine the role for WmpR in *P. tunicata*. We used two methods to discover members of the WmpR regulon: RAP-PCR to determine regulation by WmpR at a transcriptional level and 2D-PAGE to identify members of the regulon at a translational level. To the best of our knowledge, this is the first study to use these techniques simultaneously in an attempt to identify all members of a bacterial regulon. Proteomic analysis allowed us to identify four of the proteins up-regulated by WmpR, using the draft *P. tunicata* genome, as coenzyme Q4 (Coq4), aspartate aminotransferase (AspAT) and two hypothetical proteins. Coq4 is essential for the synthesis of ubiquinone in yeast; however, its function remains unknown (Belogrudov *et al.*, 2001). Ubiquinone is an electron carrier in aerobic respiration and also functions as an antioxidant in *E. coli* (Søballe & Poole, 2000). AspAT (EC 2.6.1.1) is a transaminase that catalyses the reversible reaction of aspartate and 2-oxoglutarate to oxaloacetate and glutamate. Interestingly, ToxR, in addition to regulating virulence factors, also regulates protein synthesis, energy metabolism and amino acid biosynthesis genes in *V. cholerae* *in vivo* (Bina *et al.*, 2003). We also found that WmpR down-regulates EF-Tu, which participates in the elongation cycle of translation, binding GTP and bringing an aminoacylated tRNA to the ribosome complex. During stationary phase, ribosomal and translational proteins, including EF-Tu, are down-regulated compared to exponential phase (Xu *et al.*, 2003) as the requirement for synthesis of new proteins is reduced when the cell stops growing. However, there remains the possibility that the increase in EF-Tu in the D2W2 strain is due to addition of the translational inhibitor kanamycin to maintain the transposon insertion. Such inhibition may lead to an accumulation of translational proteins.

Using RAP-PCR we identified two genes up-regulated by WmpR that matched closely to a TonB-dependent OMP and an NRPS. TonB-dependent OMPs take up molecules that are too large to pass through porins, using energy provided by the membrane-spanning protein TonB. Examples include uptake of iron-loaded siderophores by FepA and vitamin B₁₂ by BtuB in *E. coli* (reviewed by Faraldo-Gomez

Fig. 5. Production of iron-binding compounds and growth of *P. tunicata* wild-type (squares) and D2W2 (circles) strains. An increase in iron-binding activity of the wild-type and the D2W2 strains was measured by a decrease in A_{630} of the CAS complex (open symbols). Growth was measured as c.f.u. ml⁻¹ (closed symbols).

& Sansom, 2003). Siderophores are low-molecular-mass compounds that have a high affinity for ferric iron (for a review, see Andrews *et al.*, 2003). Iron is an essential element for most organisms; however, the predominant form of iron available (Fe^{3+}) is extremely insoluble and therefore difficult to obtain. In addition, greater than 99% of dissolved iron in sea water is complexed by organic ligands (Gledhill & Van den Berg, 1994; Rue & Bruland, 1995). The identification of a TonB-dependent OMP up-regulated by WmpR indicates that WmpR may be important in regulating iron uptake in *P. tunicata*. ToxR has also been found to regulate iron uptake in *V. cholerae* *in vivo* (Bina *et al.*, 2003).

NRPSs are large multienzyme complexes that are made up of a series of modules joined together. Each module is responsible for the addition of a specific amino acid which together form small non-ribosomal peptides (including antibiotics, surfactins and siderophores) (Challis & Naismith, 2004; Moffitt & Neilan, 2000). The NRPS up-regulated by WmpR could be making a siderophore or one of the anti-fouling compounds. Several hundred siderophores have been described, including two new siderophores from *Pseudoalteromonas* sp. KP20-4 (Kanoh *et al.*, 2003). In addition to the TonB-dependent OMP and the NRPS genes, a third gene was identified by RAP-PCR as up-regulated in wild-type *P. tunicata* compared to D2W2. The protein encoded by this gene was found to match closely to one of the hypothetical proteins identified as up-regulated by WmpR in the 2D-PAGE study and to a novel protein of unknown function from *P. profundum*. It would be of interest to determine the function of this hypothetical protein, as it appears to be important enough for *P. tunicata* to have two very similar copies, both of which are up-regulated by WmpR.

Genes identified in the RAP-PCR experiment suggested a role for WmpR in the acquisition and uptake of iron. To provide physiological support for such a proposed role for WmpR we compared the survival of *P. tunicata* wild-type and D2W2 under iron-limiting conditions. D2W2 showed poor survival under these conditions, with eventual death by 160 h (Fig. 4). In contrast, the viability (c.f.u. ml^{-1}) of the wild-type grown under iron-limiting conditions showed a 2-log value reduction only over 160 h compared to wild-type grown in the presence of iron. Using the liquid CAS assay (Schwyn & Neilands, 1987) the wild-type showed a significantly higher production of siderophores than D2W2 at stationary phase ($P \leq 0.05$) (Fig. 5). Taken together, these results indicate that WmpR is important for survival under low-iron conditions, such as those found in sea water, and that the TonB-dependent OMP and NRPS up-regulated by WmpR may be involved in the production and uptake of siderophores.

A hypothetical model summarizing our current knowledge of the WmpR regulon is shown in Fig. 6. In this model, WmpR responds to environmental conditions (such as low levels of iron) and coordinates expression of genes involved in iron scavenging and uptake and non-ribosomal peptide synthesis, as well as genes involved in the expression of the pigment, antifouling, type IV pili and biofilm phenotypes. This model highlights the importance of WmpR as a regulator of key phenotypes in *P. tunicata*. At present it is unclear which environmental signals WmpR is responding to. In *Pseudomonas aeruginosa*, the siderophore pyoverdine is co-regulated with at least two other virulence factors (exotoxin A and an endoprotease) (Lamont *et al.*, 2002). Ferri-pyoverdine acts as a signalling molecule, regulating its own production as well as production of the virulence factors (Lamont *et al.*, 2002). In the same way, the siderophore

Fig. 6. Hypothetical model of the WmpR regulon (based on current knowledge). WmpR recognizes a signal (e.g. iron) and coordinates the expression of several stationary-phase genes identified in this study, including iron acquisition and translation, in addition to the genes behind the phenotypic differences we have observed (pigmentation, antifouling compounds, type IV pili and biofilm formation). TonB-DOMR, TonB-dependent outer membrane receptor.

regulated by WmpR, which has bound iron, could be used as a signal for WmpR in a positive feedback loop.

Neither method identified genes involved in the previously observed phenotypes regulated by WmpR. In the RAP-PCR experiment, this may be because coverage of the mRNA is limited by the primer sequences and the number of primer combinations used (Bidle & Bartlett, 2001). When Bidle & Bartlett (2001) used RAP-PCR to determine the regulon of ToxR in *P. profundum*, they did not identify the *ompL* or *ompH* transcripts, which are known to be regulated by ToxR. The 2D-PAGE study detected several differentially regulated proteins; however, not all of these proteins could be identified due to insufficient amounts of protein for sequencing. Furthermore, the amounts of the proteins involved in the previously observed phenotypes may be below the limit of detection of the silver-staining technique. Using the two methods simultaneously did not identify overlapping genes/proteins regulated by WmpR, which is probably a result of the intrinsic differences between the two methods: 2D-PAGE displays mainly abundant proteins, while RAP-PCR selects random, potentially single copy, mRNA (Lopez-Hellin *et al.*, 2005).

In this study we have shown that the ToxR-like regulator WmpR is not likely to regulate stationary-phase physiology induced by carbon, phosphate or nitrogen. However, using transcriptomic and proteomic approaches we have determined that the function of the WmpR regulon extends beyond regulation of bioactive compounds to an important role in iron regulation, amino acid metabolism and ubiquinone biosynthesis.

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