Role of RsmA in the regulation of swarming motility and virulence factor expression in *Proteus mirabilis*

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Swarming by *Proteus mirabilis* involves differentiation of typical short vegetative rods into filamentous hyper-flagellated swarm cells that undergo cycles of rapid and co-ordinated population migration across surfaces and exhibit high levels of virulence gene expression. RsmA (repressor of secondary metabolites) and CsrA, its homologue in *Escherichia coli*, control many phenotypic traits, such as motility and pathogenesis in *Erwinia* species, glycogen biosynthesis, cell size and biofilm formation in *Escherichia coli* and swarming motility in *Serratia marcescens*. To investigate the role of RsmA in *Proteus mirabilis*, the *rsmA* gene from *Proteus mirabilis* (hereafter referred to as *rsmA*_{Pm}) was cloned. RsmA_{Pm} showed high sequence similarity to *Escherichia coli* CsrA and RsmA cloned from *Erwinia carotovora* subsp. *carotovora*, *Serratia marcescens*, *Haemophilus influenzae* and *Bacillus subtilis* and could complement an *Escherichia coli* *csrA* mutant in glycogen synthesis. A low-copy-number plasmid carrying *rsmA*_{Pm} expressed from its native promoter caused suppression of swarming motility and expression of virulence factors in *Proteus mirabilis*. mRNA stability assays suggested that RsmA_{Pm} inhibited virulence factor expression through promoting mRNA degradation. RsmA homologues cloned from *Serratia marcescens* and *Erwinia carotovora* subsp. *carotovora* could also inhibit swarming and virulence factor expression in *Proteus mirabilis*.

Received 11 July 2002

Accepted 12 August 2002

INTRODUCTION

Swarming motility is a type of population migration behaviour characteristic of some bacteria on solid media (Eberl *et al.*, 1999; Harshey, 1994; Harshey & Matsuyama, 1994). It is a cyclic process and is closely correlated with the expression of virulence factors in both *Proteus* and *Serratia* (Allison & Hughes, 1991; Allison *et al.*, 1992; Givskov *et al.*, 1997; Liaw *et al.*, 2000). In these bacteria, swarming migration involves the co-ordinated differentiation of short, motile vegetative cells bearing a few peritrichous flagella into multinucleate, aseptate swarm cells of up to 40 times the vegetative cell length and with a much greater surface density of flagella (Allison *et al.*, 1992; Givskov *et al.*, 1997). Swarming is influenced by multiple environmental signals, such as cell density and the presence of particular amino acids and possibly peptides (Allison *et al.*, 1993; Gaisser & Hughes, 1997; Rauprich *et al.*,

1996). Characterization of swarming-defective *Proteus* transposon mutants has indicated that many proteins are involved in the regulation of differentiation and subsequent swarming migration (Belas *et al.*, 1991, 1995; Gygi *et al.*, 1995a, b, 1997; Fraser & Hughes, 1999). These include FlhA, a protein involved in flagellum assembly and swarm-cell differentiation (Gygi *et al.*, 1995a), FlgN, a substrate-specific flagellar chaperone that prevents oligomerization of hook-associated proteins and thus facilitates flagellum assembly (Fraser *et al.*, 1999), FlhD₂C₂ (heterotetramers of FlhD and FlhC), a transcription activator that regulates the expression of the flagellar regulon (Fraser & Hughes, 1999), Umo proteins that upregulate the *flhDC* operon during differentiation (Fraser & Hughes, 1999), Lrp, a global transcriptional regulator that links physiological signals to swarming differentiation (Fraser & Hughes, 1999), CcmA, a protein that regulates cell shape and thus influences multicellular swarming (Hay *et al.*, 1999), and RsbA, a putative bacterial two-component sensor kinase involved in the regulation of swarming (Liaw *et al.*, 2001). Of these, FlhDC, Lrp and the Umo proteins probably function as part of a broader regulatory network that may include bacterial two-component systems and the chemotaxis phosphorelay (Fraser & Hughes, 1999).

The nucleotide and deduced amino acid sequences of *Proteus mirabilis* *rsmA* are available as supplementary material in JMM Online (<http://jmm.sgmjournals.org/>).

The GenBank/EMBL/DBJ accession number for the DNA sequence of *Proteus mirabilis* *rsmA* reported in this paper is AF403736.

RsmA is a homologue of CsrA (for carbon storage regulator) (Romeo *et al.*, 1993; Cui *et al.*, 1995), a critical component of the *Escherichia coli* Csr system, a global regulatory system that represses a variety of stationary-phase genes (Romeo *et al.*, 1993). CsrA inhibits glycogen biosynthesis and catabolism, gluconeogenesis and biofilm formation in *Escherichia coli* (Romeo *et al.*, 1993; Romeo, 1998). CsrA represses glycogen synthesis by causing rapid mRNA decay (Liu *et al.*, 1995). This leads to a decrease in intracellular levels of glycogen biosynthesis enzymes, which, in turn, decreases the rate of glycogen biosynthesis. A second component of the Csr regulatory system, CsrB, a non-coding RNA molecule, acts as an antagonist of CsrA, presumably by sequestering it (Liu *et al.*, 1997; Liu & Romeo, 1997). Searches in the GenBank databases have shown that homologues of *csrA* can be found in many Gram-negative bacteria and some Gram-positive bacteria (White *et al.*, 1996). RsmA, a homologue of CsrA, represses stationary-phase genes in *Pseudomonas fluorescens* (Blumer *et al.*, 1999) and negatively controls several genes involved in motility, secondary metabolism, pathogenesis and quorum-sensing in *Erwinia carotovora* subsp. *carotovora* (Cui *et al.*, 1995; Liu *et al.*, 1998; Mukherjee *et al.*, 1996).

Proteus mirabilis is an important pathogen of the urinary tract, especially in patients with indwelling urinary catheters (Warren *et al.*, 1982). It is believed that the ability of *Proteus mirabilis* to colonize the urinary tract is associated with its swarming motility. Moreover, the ability of *Proteus mirabilis* to express virulence factors, including urease, protease, haemolysin and flagellin, and to invade human uroepithelial cells is coupled to swarming differentiation (Allison & Hughes, 1991; Allison *et al.*, 1992; Liaw *et al.*, 2000). RsmA has been shown to repress swarming motility and the expression of virulence factors in many *Erwinia* species (Cui *et al.*, 1995; Liu *et al.*, 1998; Mukherjee *et al.*, 1996). However, in *Escherichia coli*, CsrA, a homologue of RsmA, positively regulates swarming motility and *flhDC* expression (Wei *et al.*, 2001). In this study, the *rsmA* allele of *Proteus mirabilis* has been identified and cloned and the effect of RsmA_{Pm} (RsmA encoded by *Proteus mirabilis*) on the swarming behaviour and the expression of virulence factors, including haemolysin, protease, urease and flagellin, in *Proteus mirabilis* has been investigated. The *trans*-acting effects of RsmA_{Ecc} (RsmA encoded by *Erwinia carotovora* subsp. *carotovora*) and RsmA_{Sm} (RsmA encoded by *Serratia marcescens*) (Ang *et al.*, 2001) on swarming-related traits of *Proteus mirabilis* were also examined.

METHODS

Enzymes and chemicals. DNA restriction and modification enzymes were purchased from Boehringer. *Taq* polymerase and PCR-related products were from Perkin Elmer or Takara Biomedicals. Other chemicals were purchased from Sigma.

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used are described in Table 1. Bacteria were cultured at 37 °C in Luria–Bertani (LB) medium. Swarming motility was examined on swarming-agar plates (LB solidified with 1.5 % agar)

by inoculating 5 µl of an overnight broth culture on to the centre of the agar plate. The plates were dried before inoculation and incubated at 37 °C. Hourly increases in the optical density of broth cultures at 600 nm were taken as a measurement of the growth rate.

Recombinant DNA techniques. Standard protocols were used for Southern hybridization, isolation of plasmid and chromosomal DNA, transformation, electroporation, PCR, restriction endonuclease digestion, agarose gel electrophoresis, recovery of DNA from agarose gels and the ligation of DNA fragments. DNA sequencing and analysis were performed using a Perkin-Elmer Autosequencer model 373A with a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The DNA sequence of PCR products was confirmed by sequencing both strands. DNA sequence similarity searches of GenBank were performed using programs of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984). Protein primary sequence comparisons were performed using BLASTP via the NCBI internet homepage (<http://www.ncbi.nlm.nih.gov/>). Promoter prediction was made through the Berkeley *Drosophila* Genome Project (<http://www.fruitfly.org/>). The oligonucleotide primers used in PCR and sequencing are summarized in Table 2.

Cloning of *Proteus mirabilis* P19 *rsmA*. The PCR primers *csrAR* and *csrAF* (Table 2), designed according to conserved regions in *Escherichia coli* *csrA* and *Erwinia carotovora* subsp. *carotovora* *rsmA*, were used to amplify a conserved DNA fragment from *Proteus mirabilis* P19 chromosomal DNA. A single DNA band with the expected size of 156 bp was detected. This 156-bp DNA fragment, which showed high sequence identity to *Escherichia coli* *csrA* and *Erwinia carotovora* subsp. *carotovora* *rsmA*, was used as a hybridization probe in the following cloning protocol (Fig. 1). Chromosomal DNA prepared from *Proteus mirabilis* P19 was first digested with *EcoRV*, followed by ligation to *EcoRV*-digested pZER02.1 (Table 1). The 5' half and upstream region of *rsmA*_{Pm} (2600 bp) was amplified by PCR using the primers M13R and *csrAR* (Table 2) and the 3' half and downstream region (500 bp) was amplified using the primers *csrAF* and M13F (Table 2). Southern hybridization using the labelled 156-bp DNA fragment as a probe and DNA sequencing confirmed that the amplified DNA fragments contained the *rsmA*-like sequence. The full-length *rsmA*_{Pm} was subsequently amplified using the primers *rsmAF* and *rsmAR* (Table 2), designed from the 5' and 3' flanking regions of *rsmA*_{Pm}. The amplified *rsmA*_{Pm} and its flanking sequences were then cloned into pCR2.1 to generate pSJ.

Complementation of an *Escherichia coli* *csrA* mutant with *rsmA*_{Pm}. The *csrA*-defective *Escherichia coli* mutant TR1-5BW3414 (Romeo *et al.*, 1993) was transformed with pACYC184, pRsmA (pACYC184 containing *rsmA*_{Pm}), pSJ (pCR2.1 containing *rsmA*_{Pm}) or pCSR10 (pUC19 containing *csrA*). Chloramphenicol- or ampicillin-resistant clones were selected and subjected to plasmid preparation to confirm the presence of the appropriate plasmid. These transformed strains together with the wild-type *Escherichia coli* strain, BW3414 (*csrA*⁺), and the TR1-5BW3414 mutant were inoculated on to plates containing Kornberg medium (1 % glucose, 0.85 % KH₂PO₄, 1.1 % K₂HPO₄, 0.6 % yeast extract, 1.5 % agar). After incubation overnight at 37 °C, the plates were treated with iodine solution (0.01 M I₂, 0.03 M KI) to detect the accumulation of glycogen produced by these strains.

Measurement of cell length, flagellin level and haemolysin, urease and protease activities. Measurements of cell length, flagellin level and haemolysin, urease and protease activities were performed as described previously (Liaw *et al.*, 2001). Briefly, 150 µl aliquots of stationary-phase LB cultures from pRsmA-containing and pACYC184-containing *Proteus mirabilis* were spread on to LB agar plates and incubated at 37 °C for various lengths of time. After incubation, cells from the entire surface were harvested by washing with 5 ml LB. These

Table 1. Bacterial strains and plasmids

Ap^r, Ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>Proteus mirabilis</i> P19	Clinical isolate	Liaw <i>et al.</i> (2001)
<i>Escherichia coli</i> Top10	For TA cloning	Invitrogen
<i>Escherichia coli</i> BW3414	Parental strain of TR1-5BW3414	Romeo <i>et al.</i> (1993)
<i>Escherichia coli</i> TR1-5BW3414	<i>csrA</i> : : <i>kanR</i>	Romeo <i>et al.</i> (1993)
Plasmids		
pZErO2.1	Cloning vector, Km ^r	Invitrogen
pCR2.1	TA cloning vector, Ap ^r Km ^r	Invitrogen
pACYC184	Tc ^r Cm ^r , replicon P15A	Biolabs
pSJ	Full-length <i>rsmA</i> _{Pm} in pCR2.1	This study
pRsmA	Full-length <i>rsmA</i> _{Pm} in pACYC184	This study
pSA1	Full-length <i>rsmA</i> _{Ecc} in pACYC184	Ang <i>et al.</i> (2001)
pSA4	Full-length <i>rsmA</i> _{Sm} in pACYC184	Ang <i>et al.</i> (2001)
pCSR10	Full-length <i>csrA</i> in pUC19	Romeo <i>et al.</i> (1993)

Table 2. Nucleotide sequences of primers used in PCR and sequencing

Primer	Sequence (5'→3')
Cloning <i>Proteus mirabilis</i> <i>rsmA</i> homologue	
csrAF	ATGCTGATTCTGACTCGTCGA
csrAR	GGATACGCTGGTAGATCTCTTCACGGT
Amplification of full-length <i>Proteus mirabilis</i> <i>rsmA</i>	
rsmAF	GCTAAGAATGTGAAAGGTAT
rsmAR	TCATTCAAGCATTTTATTTTC
Haemolysin gene mRNA-stability assay	
HpmF	TAAAATCGATGAACGCACAG
HpmR	GATTTTTCTGCATTGATAG
<i>Proteus mirabilis</i> <i>rsmA</i> homologue upstream sequencing	
rsmR	ATTTACGCCAATACGTACCT
fala	GGATAACGCTTTTTCAGGTA
<i>Proteus mirabilis</i> <i>rsmA</i> homologue cloning by reverse PCR	
M13F (–20)*	GTAACGACGGCCAG
M13R	CAGGAACAGCTATGAC

*Also used for *rsmA* downstream sequencing.

cells were then subjected to several assays. For cell-elongation measurements, bacteria were fixed in 4 % paraformaldehyde and examined by light microscopy at a magnification of 1000× under oil-immersion using an Olympus BH2 microscope equipped with a graticule. The lengths of 100 cells in each sample were determined and the mean calculated. Cell membrane-associated haemolysin activity was assayed as described previously (Koronakis *et al.*, 1987). Protease activity was determined by the method of Gibson & Macfarlane (1988). The urease activity of whole-cell suspensions was determined by the phenol red colorimetric assay (Jones & Mobley, 1988). Flagellin determination was performed as described previously (Gygi *et al.*, 1995a).

mRNA stability assay. *Proteus mirabilis* P19 cells transformed with the pACYC184 vector or pRsmA were grown at 37 °C in LB to an OD₆₀₀ of 0.5. At 0, 2, 4, 6 and 8 min after addition of rifampicin to a final concentration of 400 µg ml^{–1} to block further transcription, aliquots (10 ml) were collected in tubes containing 1 ml freshly prepared stop solution (5 % phenol in ethanol). Total RNA was extracted by the hot-phenol method (Magni *et al.*, 1995) and Northern blot analysis was performed to detect the haemolysin mRNA as described previously (Liu *et al.*, 1998). RNA samples were loaded on formaldehyde (6 %)/agarose (1.2 %) gels and electrophoresed in MOPS buffer (0.02 M MOPS, 0.005 M sodium acetate, 0.001 M EDTA). After transfer to a nitrocellulose membrane which was washed in 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate), the RNAs were cross-linked to the membrane by UV irradiation. The haemolysin gene (*hpmA*) probe (1 kb) was amplified from genomic DNA with a PCR DIG Probe Synthesis kit (Boehringer Mannheim) using primers HpmF and HpmR (Table 2), designed from the *hpmA* sequence (Uphoff & Welch, 1990). Pre-hybridization (4 h at 58 °C) and hybridization (18 h at 58 °C) were performed in DIG Easy Hyb* buffer (Boehringer Mannheim). After hybridization, membranes were washed twice for 5 min at 50 °C in 2× SSC, 0.1 % SDS, then twice for 15 min at 50 °C in 0.1× SSC, 0.1 % SDS, and examined by using the DIG DNA Luminescence Detection kit (Boehringer Mannheim). A 0.24–9.5 kb RNA ladder (Gibco-BRL) was used as a size marker.

RESULTS

Cloning of the *rsmA* gene from *Proteus mirabilis*

In order to know whether *rsmA* exists in *Proteus mirabilis* and might play a role in the regulation of *Proteus mirabilis* swarming and virulence factor expression, *rsmA*_{Pm} was first cloned. The PCR primers csrAR and csrAF (Table 2), designed according to conserved regions in *Escherichia coli* *csrA* and *Erwinia carotovora* subsp. *carotovora* *rsmA*, amplified a conserved DNA fragment of *Proteus mirabilis* P19 chromosomal DNA to give a single DNA band with the expected size of 156 bp. This had high DNA sequence

Fig. 1. Scheme for cloning of *Proteus mirabilis* *rsmA*. For details, refer to Methods.

identity to sequences of *Escherichia coli* *csrA* and *Erwinia carotovora* subsp. *carotovora* *rsmA* (unpublished observations). Despite repeated attempts, it was not possible to clone the complete *rsmA*_{Pm} gene via a colony hybridization protocol (a genomic library had been constructed in the high-copy-number plasmid pZErO2.1 and it is possible that overexpression of *rsmA*_{Pm} was lethal). Therefore, PCR was used to clone the complete *rsmA*_{Pm} gene as described. The nucleotide sequence and the deduced amino acid sequence of the open reading frame (ORF) of the *rsmA*_{Pm} gene-containing fragment are available as supplementary material in JMM Online (<http://jmm.sgmjournals.org/>). The fragment contains 1021 bp in total and an ORF (nt 555–743) that could encode a 6.8-kDa polypeptide of 62 amino acid residues. A putative Shine–Dalgarno sequence (5′-AGGAG-3′) is located 6 bp upstream of the ATG start codon. Putative –10 and –35 regions are also shown. Fig. 2 shows the alignment of deduced amino acid sequences among *Escherichia coli* *CsrA*, *Proteus mirabilis* *RsmA*_{Pm}, *Erwinia carotovora* subsp. *carotovora* *RsmA*_{Ecc} and other *RsmA* homologues in *Serratia marcescens*, *Haemophilus influenzae* and *Bacillus subtilis*.

<i>E. coli</i>	MLILTRRVGE	TLMIGDEVTV	TVLGVKGNQV	RIGVNAPKEV
<i>S. m.</i>	MLILTRRVGE	TLMIGDEVTV	TVLGCKGNQV	RIGVNAPKEV
<i>E. c.c.</i>	MLILTRRVGE	TLIIGDEVTV	TVLGVKGNQV	RIGVNAPKEV
<i>P. m.</i>	MLILTRRVGE	TLMIGDDTV	TVLGVKGNQV	RIGVNAPKEV
<i>H. i.</i>	MLILTRKVG	SVLIGDDISI	TVLSVRGNQV	KLGVAPKEV
<i>B. s.</i>	MLVLSRKINE	AIQIGADIEV	KVIAVEGDQV	KLIGDAPKHI
<i>E. coli</i>	SVHREEIYQR	IQAEKSQQSS	Y	
<i>S. m.</i>	SVHREEIYQR	IQAEKSADDL	LIPKQRLVAX	
<i>E. c.c.</i>	SVHREEIYQR	IQAEKSQPTS	Y	
<i>P. m.</i>	SVHREEIYQR	IQAEKTQPTD	NY	
<i>H. i.</i>	SVHREEIYQR	IKQTKDEPYL	GSS	
<i>B. s.</i>	DIHRKEIYLT	IQEENNRAAA	LSSDVISALS	SQKK

Fig. 2. Alignment of the deduced amino acid sequences of *RsmA* homologues in *Escherichia coli* K-12 (*E. coli*; Romeo *et al.*, 1993), *Serratia marcescens* (*S. m.*; Ang *et al.*, 2001), *Erwinia carotovora* subsp. *carotovora* (*E. c. c.*; Cui *et al.*, 1995), *Proteus mirabilis* (*P. m.*), *Haemophilus influenzae* (*H. i.*; Fleischmann *et al.*, 1995) and *Bacillus subtilis* (*B. s.*; Mirel & Chamberlin, 1989). Vertical lines indicate residues identical to those in *Escherichia coli* K-12.

Amino acid sequence comparison revealed that *RsmA*_{Pm} showed 96 % identity to *Escherichia coli* *CsrA* and *RsmA*_{Sm}, 94 % identity to *RsmA*_{Ecc} and respectively 70 and 45 % identity to *RsmA* from *H. influenzae* and *B. subtilis*. As with *Escherichia coli* *CsrA* (Romeo, 1998), the predicted product of *rsmA*_{Pm} also contains a putative RNA-binding domain (see supplementary material) that is similar to the KH (K protein homology) motif found only in proteins associated with RNA (Siomi *et al.*, 1994).

Complementation of an *Escherichia coli* *csrA* mutant with *rsmA*_{Pm}

To investigate whether *RsmA*_{Pm} had similar functions to *Escherichia coli* *CsrA*, an *Escherichia coli* *csrA*-defective mutant, TR1-5BW3414 (Romeo *et al.*, 1993), was transformed respectively with the vectors pACYC184, pRsmA (*rsmA*_{Pm} in low-copy-number vector pACYC184), pSJ (*rsmA*_{Pm} in high-copy-number vector pCR2.1) or pCSR10 (*csrA* in high-copy-number vector pUC19). The ability of *RsmA*_{Pm} and *CsrA* to suppress the glycogen-overproducing activity of the TR1-5BW3414 mutant was tested. As shown in Fig. 3, the TR1-5BW3414 mutant and its pACYC184 transformant stained dark-brown due to excess glycogen accumulation. The TR1-5BW3414 mutant transformed with pRsmA stained yellowish brown, similar to the staining

Fig. 3. Complementation of an *Escherichia coli* *csrA* mutant with *rsmA_{Pm}* detected by iodine staining. Cultures were streaked on to Kornberg medium and incubated overnight at 37 °C before treatment with iodine solution. Plate segments were streaked with: 1–3, mutant strain TR1-5BW3414 transformed with pACYC184 (1), pSJ (2) and pCSR10 (3); 4, wild-type strain BW3414; 5, mutant strain transformed with pRsmA; 6, mutant strain TR1-5BW3414.

pattern of the wild-type strain BW3414, indicating that the glycogen-overproducing phenotype of the TR1-5BW3414 mutant was suppressed by RsmA_{Pm}. The TR1-5BW3414 mutants transformed with pSJ or pCSR10 showed a yellow staining pattern, suggesting that RsmA_{Pm} and CsrA had similar abilities to suppress glycogen production by the mutant. These results suggested that *rsmA_{Pm}* could complement the glycogen-excess phenotype of the *csrA* mutant and that RsmA_{Pm} had similar functions to CsrA.

Effect of RsmA_{Pm} on the swimming and swarming activities of *Proteus mirabilis* P19

The above results suggested that the cloned *rsmA_{Pm}* gene was a true *csrA* homologue. Because CsrA and its homologue RsmA regulate motility in *Escherichia coli* and *Erwinia* species, the effect of RsmA_{Pm} on the swimming and swarming activities of *Proteus mirabilis* was examined. Because

expression of *rsmA_{Pm}* from a high-copy-number vector, such as pZER02.1 or pCR2.1, in *Proteus mirabilis*, resulted in inhibition of growth (unpublished observations), the *rsmA_{Pm}* gene and its flanking sequences were cloned into the low-copy-number vector pACYC184 (Table 1) to generate pRsmA. The pRsmA plasmid was transformed into *Proteus mirabilis* P19 to establish an RsmA_{Pm}-expressing strain. As shown in Fig. 4, while *Proteus mirabilis* P19 transformed with the pACYC184 vector swarmed normally, the RsmA_{Pm}-expressing *Proteus mirabilis* strain, which expresses RsmA_{Pm} from its native promoter, lost its ability to swarm. In contrast, the swimming motility of *Proteus mirabilis* was not affected by the presence of RsmA_{Pm} (unpublished observations). These results indicated that RsmA_{Pm} could specifically inhibit the swarming activity of *Proteus mirabilis* P19.

Effect of RsmA_{Pm} on swarming differentiation and virulence factor expression in *Proteus mirabilis*

Swarming motility is associated with the expression of virulence factors in *Proteus mirabilis* (Allison & Hughes, 1991; Allison *et al.*, 1992; Liaw *et al.*, 2000). Because RsmA_{Pm} inhibited the swarming activity of *Proteus mirabilis* P19, it was of interest to determine whether RsmA_{Pm} could also inhibit swarming differentiation and virulence factor expression in *Proteus mirabilis*. The RsmA_{Pm}-expressing *Proteus mirabilis* P19 strain and the vector-transformed *Proteus mirabilis* P19 strain were spread on to LB swarming plates and the cell length (as an indicator of swarming differentiation) and expression of virulence factors were determined 2 h after seeding and hourly thereafter. *Proteus mirabilis* transformed with the pACYC184 vector expressed haemolysin, protease, urease and flagellin in the same differentiation-dependent manner as the parental *Proteus mirabilis* P19 strain (Fig. 5) (Liaw *et al.*, 2001). They differentiated to the longest cells 4 h post-seeding and expressed the highest levels of these virulence factors 4 or 5 h post-seeding. In contrast, the RsmA_{Pm}-expressing *Proteus mirabilis* strain did not differentiate into long cells and expressed virulence factors at only a basal level during the whole differentiation cycle (Fig. 5). These results indicated that RsmA_{Pm} could inhibit swarming differentiation and virulence factor expression in *Proteus mirabilis*.

Fig. 4. Swarming of pRsmA-transformed (a) and pACYC184-transformed (b) *Proteus mirabilis* P19 on LB swarming plates. Aliquots (5 µl) of bacterial culture were inoculated centrally on to LB swarming plates. The plates were incubated at 37 °C and observed after 10 h incubation.

Fig. 5. Cell length and expression of virulence factors after plating of pACYC184-transformed (open bars) and pRsmA-transformed (filled bars) *Proteus mirabilis* P19 on LB agar plates and incubation at 37 °C. The increase in cell length was taken as a sign of cell (swarming) differentiation. For protease activity measurements, the value obtained with the pACYC184-transformed *Proteus mirabilis* P19 at 5 h post-seeding was set at 100 % and all other values were expressed relative to this value. For all other measurements, the values obtained with the pACYC184-transformed *Proteus mirabilis* P19 at 4 h post-seeding were set at 100 %. The data represent means of three independent experiments with standard deviations.

Effect of RsmA_{Pm} on mRNA stability

Because both RsmA_{Ecc} and CsrA regulate gene expression by affecting the stability of mRNA (Liu *et al.*, 1995, 1998), it was possible that RsmA_{Pm} inhibited virulence-gene expression through a similar mechanism. Therefore, the stability of haemolysin mRNAs isolated from the vector-transformed *Proteus mirabilis* P19 and pRsmA-transformed *Proteus mirabilis* P19 was compared. As shown in Fig. 6, the haemolysin mRNA in the RsmA_{Pm}-transformed cells was completely degraded 8 min after rifampicin treatment, while that in the vector-transformed cells was not. These results indicated that RsmA_{Pm} promoted mRNA degradation in *Proteus mirabilis* P19. Therefore, RsmA_{Pm}, like CsrA and RsmA_{Ecc}, could inhibit virulence factor expression by affecting mRNA stability.

RsmA_{Ecc} and RsmA_{Sm} trans-suppress swarming and virulence factor expression in *Proteus mirabilis* P19

To investigate whether swarming and virulence factor ex-

pression in *Proteus mirabilis* could also be inhibited by other RsmA homologues, the pSA1 plasmid (Table 1), which contained the *rsmA_{Ecc}* gene (*rsmA* from *Erwinia carotovora* subsp. *carotovora*) under the control of its native promoter, was transformed into *Proteus mirabilis*. As shown in Figs 7 and 8, while the vector-transformed *Proteus mirabilis* could differentiate, swarm and express virulence factors normally, the pSA1-transformed *Proteus mirabilis* strain lost its abilities to differentiate, swarm and express normal levels of virulence factors. Similarly, RsmA_{Sm} (RsmA encoded by *Serratia marcescens*) also inhibited the ability of *Proteus mirabilis* to swarm and express virulence factors (unpublished observations). Together, these data confirmed that swarming and virulence factor expression in *Proteus mirabilis* could be suppressed by RsmA_{Pm} and its homologues.

DISCUSSION

In many host–pathogen interactions, disease development requires co-ordinated expression of sets of genes in response to various signals and environmental cues (Cotter & Miller,

1998). Regulation of these genes is subject to both transcriptional and post-transcriptional control. RsmA–RsmB constitutes an important regulatory system responsible for post-transcriptional regulation (Liu *et al.*, 1998; Cui *et al.*, 1999). RsmA, an RNA-binding protein, promotes the decay of many mRNA species (Liu *et al.*, 1998). RsmB, an untranslated regulatory RNA, on the other hand, neutralizes the effect of RsmA by forming a ribonucleoprotein complex with the latter (Liu *et al.*, 1998). The RsmA regulatory system has been conserved in many enterobacterial species (Romeo *et al.*, 1993; Cui *et al.*, 1995; White *et al.*, 1996) and has been shown to control diverse phenotypes including motility and expression of virulence factors (Cui *et al.*, 1995; Mukherjee *et al.*, 1996). In this study, we cloned the *rsmA_{Pm}* gene from *Proteus mirabilis* and investigated the effect of RsmA_{Pm} on swarming and virulence factor expression in *Proteus mirabilis*. *rsmA_{Pm}* was found to complement the defectiveness of an *Escherichia coli* *csrA* (*rsmA* homologue) mutant (Romeo *et al.*, 1993) and could act as *rsmA_{Ecc}* (Ang *et al.*, 2001) and *rsmA_{Sm}* (Ang *et al.*, 2001) to suppress swarming and virulence factor expression in *Proteus mirabilis*.

Several lines of evidence suggest that the *rsmA_{Pm}* gene we cloned is a true *rsmA* homologue. Firstly, the DNA sequence and the predicted product of *rsmA_{Pm}* showed high similarity to *rsmA* cloned from other bacteria (Fig. 2). Secondly, like other RsmAs, the predicted *rsmA_{Pm}* product bore a putative

RNA-binding motif (Siomi *et al.*, 1994). Thirdly, *rsmA_{Pm}* could complement the glycogen-excess phenotype of an *Escherichia coli* *csrA* mutant (Fig. 3). Furthermore, complementation of the *csrA* mutant with *rsmA_{Pm}* also restored the cell length to wild-type and abolished its formation of a biofilm (unpublished observations). Fourthly, RsmA_{Pm} could act as RsmA_{Ecc} and RsmA_{Sm} to suppress swarming and virulence factor expression in *Proteus mirabilis*.

The role of RsmA/CsrA in regulating bacterial virulence is of considerable interest and has been established in *Erwinia* species that are plant pathogens. A transposon-insertion mutation in *rsmA* in *Erwinia* caused hypervirulence, overproduction of lytic enzymes and elevation of their corresponding transcripts (Cui *et al.*, 1995; Chatterjee *et al.*, 1995). In *Pseudomonas fluorescens*, RsmA is also a negative regulator of virulence factor expression (Blumer *et al.*, 1999). Motility is an important survival mechanism and a distinct advantage for host-adapted species. Overexpression of *rsmA* has been demonstrated to suppress motility and flagella production in *Erwinia carotovora* subsp. *carotovora* (Mukherjee *et al.*, 1996). Our finding that RsmA_{Pm} could suppress swarming and virulence factor expression in *Proteus mirabilis* is consistent with the above observations. Our results also suggest that RsmA_{Pm} may play an important role in regulating virulence in *Proteus mirabilis*.

RsmA regulates gene expression by controlling mRNA stability and, thus, is a global regulator of gene expression in many bacteria (Romeo, 1998). RsmA_{Pm} also regulated gene expression through regulating mRNA stability and could promote mRNA degradation (Fig. 6). It is possible that the activity of RsmA_{Pm} must be rigorously controlled in the cells (Cui *et al.*, 1999; Mukherjee *et al.*, 1998). Overexpression of *rsmA* from high-copy-number plasmids or artificial strong promoters is generally detrimental to cell physiology and, in certain hosts, is even lethal (Cui *et al.*, 1999). In our study, overexpression of *rsmA_{Pm}* from the high-copy-number plasmids pZErO2.1 and pCR2.1 was inhibitory to the growth of *Proteus mirabilis* and *Escherichia coli*, whereas *rsmA_{Pm}* expressed from the low-copy-number pACYC184 was not (unpublished observations). These observations suggest that the level of RsmA_{Pm} must be delicately regulated in *Proteus mirabilis* and are supported by findings implying that the *rsmA_{Pm}* gene is essential for *Proteus mirabilis*. Repeated attempts to isolate *rsmA_{Pm}*-

Fig. 6. Stability of the haemolysin mRNA in pACYC184-transformed (a) and pRsmA-transformed (b) *Proteus mirabilis* P19. Rifampicin (400 µg ml⁻¹) was added to the cultures at an OD₆₀₀ of 0.5 and RNA was extracted 0, 2, 4, 6 and 8 min after the addition of rifampicin. Northern hybridization was performed at 58 °C with the DIG-labelled haemolysin probe. Each lane in row (a) was loaded with 10 µg total RNA and each lane in row (b) was loaded with 20 µg total RNA.

Fig. 7. Swarming of pSA1-transformed (a) and pACYC184-transformed (b) *Proteus mirabilis* P19 on LB swarming plates. Aliquots (5 µl) of bacterial culture were inoculated centrally onto LB swarming plates. The plates were incubated at 37 °C and observed after 10 h incubation.

Fig. 8. Cell length and expression of virulence factors after plating of pACYC184-transformed (open bars) and pSA1-transformed (filled bars) *Proteus mirabilis* P19 on LB agar plates and incubation at 37 °C. For further details see legend to Fig. 5.

knockout *Proteus mirabilis* mutants by using the pKO3 gene-replacement vector (Link *et al.*, 1997) and the screening of ~10 000 clones have proved unsuccessful. *rsmA/csrA* is not essential in *Escherichia coli* or *Serratia marcescens* (Romeo *et al.*, 1993; Ang *et al.*, 2001). However, a *csrA*-knockout mutation is deleterious in *Salmonella enterica* serovar Typhimurium (Altier *et al.*, 2000) and possibly also in *Yersinia* and *Legionella* species (T. Romeo, personal communication). Further experiments from construction of *rsmA_{Pm}* temperature-sensitive mutants should be performed to determine the essentiality of *rsmA_{Pm}* in *Proteus mirabilis*.

Regulation of swarming and virulence factor expression is a complex process and involves many factors and regulatory pathways (Fraser & Hughes, 1999). Previously, we demonstrated that RsbA, a sensor kinase homologue of the bacterial two-component system, was a negative regulator of swarming and virulence factor expression in *Proteus mirabilis* (Liaw *et al.*, 2001). *rsbA*-defective *Proteus mirabilis* mutants exhibit a super-swarming phenotype and overproduce virulence factors. When *rsmA_{Pm}* was transformed into these mutants, swarming was drastically suppressed (unpublished observations). These results suggest that the RsbA regulatory pathway and the *RsmA_{Pm}* regulatory pathway may cross-talk to each other. In *Pseudomonas fluorescens* and *Erwinia caroto-*

vora subsp. *carotovora*, the two-component system GacA/GacS has been shown to control the production of extracellular enzymes by regulating the expression of the *rsmA/rsmB* system (Mukherjee *et al.*, 1998; Cui *et al.*, 2001). It is possible that RsbA may also regulate swarming and virulence factor expression through modulating the expression of the *rsmA/rsmB* system. Experiments aimed at elucidating the relationship between RsbA and *RsmA_{Pm}* are in progress.

In addition to *rsmA_{Pm}*, we also found that *rsmA* homologues exist in many bacteria including *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Morganella morganii*, *Klebsiella pneumoniae*, *Aeromonas hydrophila*, *Citrobacter diversus*, *Helicobacter pylori* and *Streptococcus sanguis* (unpublished observations). Whether these *rsmA* homologues also regulate virulence in these bacteria is of interest and warrants further investigation.

ACKNOWLEDGEMENTS

We thank Dr Tony Romeo for providing us with *Escherichia coli* strains TR1-5BW3414 and BW3414 and for helpful discussions. This work was supported by grants from the National Science Council and the National Taiwan University Hospital, Taipei, Taiwan.

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