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Transcriptional regulation of the S-layer protein type I secretion system in *Caulobacter crescentus*

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Abstract

The Gram-negative *Caulobacter crescentus* exports RsaA, the crystalline S-layer subunit protein using a dedicated type I secretion system. The protein and two transporter genes (*rsaADE*) are located together, comparable to the *Escherichia coli* type I hemolysin *hlyCABD* operon, where read through of a stem loop following *hlyCA* results in reduced transcription of the *hlyBD*. Using two genetic approaches and a direct assessment of transcription from regions 5′ to the genes we learned that *rsaD* and *rsaE* were transcribed together as a separate transcript from *rsaA*. These results are contrary to previous assumptions about the *rsaADE* type I secretion gene control and add another theme to the area of type I secretion transcription regulation. It may be that to accommodate the high levels of RsaA secretion, the type I transporters must be transcribed independently from *rsaA*.

Keywords: Caulobacter; S-layer; Transcription; Type I secretion

1. Introduction

Gram negative type I secretion is a Sec-independent process which transports protein from the cytoplasm to the exterior of the cell without periplasm contact. It is composed of a three-component ATP-binding-cassette (ABC) based exporter containing an ABC transporter, membrane fusion protein (MFP) and outer membrane protein (OMP). In most type I systems the ABC transporter and MFP genes are adjacent to the secreted protein gene, whereas the OMP gene location can vary. In some cases, the genes for all three transport components are immediately adjacent to the substrate gene [1–3]. In others only the ABC-transporter and MFP genes are adjacent [4,5]. For example, the *Escherichia coli* TolC and the *Caulobacter crescentus* RsaFa and RsaFb OMP genes are distant from the other components [6].

Transcriptional regulation of the type I components has not been extensively studied. Until recently transcriptional regulation of type I systems was only described for the HlyA system of *E. coli*. where co-transcription of the transporter genes has been demonstrated [7]. Transcription of the four different genes in the *hlyCABD* operon was shown using gene probes for *hlyCA* as well as *hlyB* and *hlyD*. Two mRNA transcripts, estimated at 4 and 8 kb, corresponding to transcripts containing the *hlyCA* genes and the *hlyCABD* genes. It was concluded that *hlyB* and *hlyD* are transcribed using the *hlyCA* promoter by read through of a ρ-independent terminator between the *hlyA* and *hlyB* genes.

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As more type I secretion systems are characterized, the transcriptional regulation of type I systems is becoming more divergent. Two type I systems have been found to have separate promoters for the transported protein as well as the transporter components. The *Campylobacter fetus* (SapA) and *Serratia marcescens* (Lip) systems have separate promoters for the S-layer (or S-layer like) gene and the transporter genes [2,8]. The *sapDEF* genes are transcribed in the opposite direction from the *sapA* gene. The *lipBCD* genes are located immediately downstream of the *slaA* gene with a separate promoter for the *lipBCD* gene set. In *Serratia liquefaciens* the Lip type I transporter mediates transport of a lipase, metalloprotease and an S-layer protein and transcriptional control of the transporter is separate from that of the S-layer protein [9].

In *C. crescentus*, the S-layer monomer (encoded by *rsaA*) is also secreted by a type I secretion system and does so at high levels (approximately 10–12% of total cell protein). The promoter for *rsaA* was identified when the S-layer protein was initially characterized [10]. The genes encoding the ABC transporter (*rsaD*) and the membrane fusion protein (*rsaE*) were identified adjacent to the *rsaA* gene by Tn5 insertional inactivation [11]. Two OMPs were later found elsewhere [6]. The *rsaA* gene has a moderate strength promoter and has been used in gene expression studies as a reference gene [12] since *rsaA* is transcribed throughout the cell cycle [13]. A putative rho-independent terminator is found 40 bp 3′ of the *rsaA* translational stop, which is located 162 bp 5′ of the *rsaD* start codon. As no additional promoter sequences were immediately obvious in the DNA sequence of the *rsaADE* cluster upon its discovery, it was presumed that the *rsaA* promoter occasionally read through the *rho*-independent terminator, allowing for the transcription of *rsaD* and *rsaE*, in a manner comparable to the *E. coli* HlyA system [7].

However, the high secretion levels of RsaA raised the question of whether such a low level transcription strategy would be capable of sustaining adequate levels of transporter components. One reason for additional investigation is that the *C. crescentus* S-layer secretion system has been adapted for recombinant protein secretion and remains as one of few alternatives to secretion of heterologous proteins by systems using sec-dependant pathways [14]. The expression system functions by fusing a heterologous segment to the *rsaA* C-terminal secretion signal. Thus if *rsaA*, *D* and *E* are transcribed as a single mRNA transcript, then the features (such as stability) of the heterologous mRNA would also directly affect transporter expression and could adversely impact secretion. To determine transcription of the transporter genes two genetic approaches were used to demonstrate that *rsaD* and *rsaE* are co-transcribed using a promoter distinct from the *rsaA* promoter. In addition, the promoter regions of *rsaD* and *E* were cloned and transcription assessed by a reporter assay.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

E. coli DH5α or DH10B (Invitrogen Corp., Carlsbad, CA) were used for all DNA manipulations, except when a non-methylated *Cla*I site was required, and then strain RB404 was used. *E. coli* was grown at 37 °C in Luria broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.3% agar for plates. *C. crescentus* CB15 strains were grown at 30 °C in PYE medium (0.2% peptone, 0.1% yeast extract, 0.01% $CaCl_2$, 0.02% $MgSO_4$) with 1.2% agar for plates. In *E. coli* ampicillin (Ap) was used at 50 μg/ml, kanamycin (Km) at 50 μg/ml, chloramphenicol (Cm) at 20 μg/ml, streptomycin (Sm) at 50 μg/ml and tetracycline (Tet) at 20 μg/ml. In *C. crescentus* Km was used at 25 μg/ml, Cm at 2 μg/ml, Sm at 10 μg/ml and Tet at 10 μg/ml.

2.2. Plasmid and DNA manipulations

Standard methods of DNA manipulation and isolation were used [15]. Electroporation of *C. crescentus* was performed as described [16]. PCR products were generated using Platinum *Pfx* DNA polymerase (Invitrogen) following the manufacturers protocols. Chromosomal DNA from *C. crescentus* strain NA1000 was used as template for the *rsaD* and *rsaE* PCR products. *C. crescentus* CB15 chromosomal DNA was used for *rsaA* deletion PCR products.

A fragment containing the *rsaD* gene was amplified by PCR using the primers 5′-CCGAATTCCATGTTCAAGCGCAGC-3′ and 5′-GCGGCCGCTCTGGACGCGCTGCAA-3′ incorporating *Eco*RI and *Not*I restriction sites. This gene fragment was inserted into the *Eco*RV site of the pBSKSI$^+$ plasmid. The pBSKSI$^+$: *rsaD* plasmid was cut with *Eco*RI and *Not*I releasing the *rsaD* fragment. This fragment was inserted into *Eco*RI-*Not*I cut pGEX4T3 plasmid. The pGEX4T3: *rsaD* construct is an in-frame insertion of the *rsaD* gene so that it contains a C-terminal GST tag.

Another fragment containing the *rsaE* gene was amplified by PCR using the primers 5′-CCGAATTCCATGAAGCCCCCCAAG-3′ and 5′-GCGGCCGCTCTCCTCGCGCATCGT-3′ and cloned into pGEX4T3 in a similar manner as the *rsaD* gene.

The plasmid pAL1 was constructed in order to create an in-frame deletion of the complete *rsaA* coding region. A PCR product encoding a 1.0 kb region upstream of the *rsaA* gene was amplified using the primers 5′-GGATCCGGCGTTCGAGCTGCTGCTGA-3′ and 5′-GAATTCTCACCTGGCGGGTGAGTGAG-3′ intro-

ducing *Bam*HI and *Eco*RI sites. Another PCR product was created using the primers 5′-GAAATTCCGCTC-GCCTAAGCGAACGTC-3′ and 5′-ACTAGTGGCC-GAGATCTTGCCGTCGA-3′ amplifying a 1.0 kb region containing the end of the *rsaA* gene and incorporating *Eco*RI and *Spe*I sites. Fragments were digested with *Eco*RI, *Bam*HI and *Spe*I, and ligated into *Bam*HI and *Spe*I cut pNPTS138 plasmid [17] using the pGEM-T Easy kit (Promega). This resulted in pAL1 which was transformed into *E. coli* by electroporation.

The pUC19 RAT1 plasmid was made by removing the insert from pRAT1 [11] as an *Eco*RI–*Sst*I fragment and inserting the fragement into *Eco*RI and *Sst*I digested pUC19. Interruption of the *rsaA* gene in the pUC19 RAT1 construct was done with the omega antibiotic resistance cassette, ΩCm, from pHP45ΩCm [18]. The ΩCm cassette was removed as a *Bam*HI fragment and blunted with T4 polymerase. pUC19 RAT1 was digested with *Cla*I and blunted. The blunted ΩCm cassette was ligated into pUC19 RAT1 resulting in pUC19 RAT1 ΩCm, in order to make a broad host range construct containing the RAT1ΩCm fragment. The *Eco*RI–*Sst*I RAT1ΩCm fragment from pUC19 RAT1ΩCm was ligated into *Eco*RI–*Sst*I digested pBBR4, creating pBBR4 RAT1ΩCm.

To clone and assess potential promoter regions of *rsaD* and *rsaE* pXCA601 was selected. This is a broad host range plasmid containing a promoter-less but otherwise complete β-galactosidase gene, with Tet resistance as a selectable marker [19]. The *rsaD* promoter region selected began 85 bp 3′ of the *rsaA* stop codon, did not include the putative rho-independent terminator of *rsaA* and terminated with sequence corresponding to the first nine amino acids of RsaD, which were fused to the position corresponding to the fourth amino acid of β-galactosidase. Similarly, the selected *rsaE* promoter region began immediately after the *rsaD* stop codon, included the sequence corresponding to the first nine amino acids of RsaE and was joined to the fourth amino acid of β-galactosidase. To accomplish this the oligos JNRDP-1 5′TGCATGCATGCGCTACGCGCTGGC-CGGCCTTGC 3′ and JNRDP-2 – 5′GAGATCTCCC-GGCTTCGCGCCGCTGCGCTTGAACAT 3′ and the oligos JNREP-3 5′TGCATGCATCCGGGGCTG-CGCCGCCCCAGACGC 3′ and JNREP-45′GAGAT-CTCCCGGACGCTGGATCTTGGGGGGCTTCAT3′ were used to PCR amplify the potential promoter regions of *rsaD* and *rsaE*, respectively. *Nsi*1 and *Bgl*11 restriction sites are underlined. Two microliter of *C. crescentus* cell culture were directly used as the DNA template source for PCR amplification with Pfx polymerase. The PCR product fidelity was confirmed by sequencing. The PCR product was then digested with *Nsi*1 and *Bgl*11, ligated into the *Pst*1 and *Bam*H1 sites of pXCA601 and transformed into *E. coli*, and subsequently *C. crescentus*, by electroporation.

2.3. RsaADE gene transcription

A CB15 strain with a deleted *rsaA* gene was created using the pAL1 plasmid by conjugation with the *E. coli* LS980 (match maker) and MT607 helper strains (D. Alley). The helper strain utilizes the vector pRK600, a derivative of pRK2013, Cm^r, containing the Tn9 insertion, ColE1 ori, and tra functions from pRK2013 [20]. Homologous recombination was confirmed by PCR analysis (not shown) and the resulting CB15 was designated UJ2602.

The CB15A Tn5 mutant B15 was previously described [11]. The pBBR4: RAT1ΩCm plasmid was transformed into the B15 strain by electroporation and cells were selected for resistance to Km, Cm, and Sm.

2.4. Investigating promoter activity of rsaD and rsaE

A modified, standard β-galactosidase activity assay of the promoter constructs was performed [21]. Cultures of *C. crescentus* containing the promoter probe constructs were grown in PYE and normalized to the same $OD_{600\,nm}$ (0.3–0.4). One milliliter of cells was pelleted and suspended in an equal volume of Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 1 mM $MgSO_4$, 0.05 M β-mercaptoethanol pH 7.0). The $OD_{600\,nm}$ was again determined for use in calculations and cells were sonicated (10 one s pulses, using a microprobe). Eight hundred microliters of lysate and 200 μl of Z-buffer were mixed and equilibrated at 28 °C for 5 min. Two hundred microliters of ONPG substrate (4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside in 0.1 M sodium phosphate buffer pH 7.0) was added and incubated at 28 °C for 15 min followed by addition of 0.5 ml of 1 M Na_2C0_3 to stop the reaction. Cell debris was removed by centrifugation and absorbance measured at 420 and 550 nm. Enzyme activity in Miller units was calculated using standard methods.

2.5. Antibody production

Polyclonal antibodies were produced against the RsaD protein using a GST-RsaD fusion protein. The pGEX4T3: *rsaD* plasmid was expressed in *E. coli* (DH5α), and the protein was only produced in the form of inclusion bodies. Protein in inclusion bodies was isolated by growing cells to an OD_{600} of 1, centrifuging cells and suspending in 1 × PBS buffer, followed by addition of lysozyme (100 μg/ml) for 1 h at 25 °C and then additions of RNAseA (50 μg/ml) and DNAseI (1 μg/ml) for an additional hour at 25 °C. 10% SDS, SDS–PAGE sample buffer and samples were mixed at a 1:1:1 ratio. Samples were boiled for 5 min and then put on ice for 15 min and centrifuged at 16,000 × *g* for 10 min. The inclusion body pellet was recovered and dissolved in 4 M urea. The preparation was dialyzed

(30,000 MW cut-off) in dH_2O for two days with several dH_2O changes to remove urea. A New Zealand white rabbit was immunized with the recovered protein and Freund's incomplete adjuvant; the rabbit serum was collected and processed using standard protocols [15] and the immunoglobulin fraction was enriched by treatment of the serum with an equal amount of saturated ammonium sulfate and subsequent dialysis of the globulin precipitate against phosphate buffered saline.

RsaE antibodies were made using a GST-RsaE fusion protein. The pGEX4T3: *rsaE* plasmid was expressed in *E. coli* JM109 and soluble protein was produced and purified. JM109 cells with the pGEX4T3: *rsaE* plasmid were grown to $OD_{600} \sim 0.8$ and then incubated with 0.1 mM IPTG at 30 °C for 3 h. Cells were then pelleted and resuspended in cold buffer (PBS/0.5% Tween-20/1 M NaCl/10 mM DTT/1 mM PMSF). Resuspended cells were sonicated and then centrifuged and the supernatant was saved. The supernatant was added to glutathione–Sepharose beads (Sigma) and mixed for 1 h. After rocking, beads were centrifuged and the supernatant was aspirated. Beads were washed three times using PBS. GST-tagged RsaE protein was eluted using 50 mM Tris–HCl pH 8/10 mM reduced Glutathione (Sigma). Eluted protein fractions were pooled and dialyzed against dH_2O. Dialyzed protein was then injected into New Zealand white rabbit and antisera recovered as above.

2.6. Protein techniques

S-layer protein from *C. crescentus* cells was extracted by low pH extraction as previously described [22]. To compare the amounts of surface layer protein extracted from different mutants, normalized levels of cells (determined by OD_{600}) growing at log phase were used and equal amounts of extracted protein samples were loaded onto Sodium dodecyl sulfate (SDS)–poly-acrylamide gel electrophoresis (PAGE) gels.

Whole cell protein preparations were done as above, cultures were centrifuged and cell pellets washed twice with 10 mM Tris–HCl pH 8. Pellets were resuspended in 10 mM Tris–HCl pH 8; lysozyme (100 μg/ml) was added and incubated at 25 °C for 15 min, followed by RNAseA (50 μg/ml) and DNAseI (1 μg/ml) and incubation at 37 °C for 30 min. Equal amounts of whole cell preparations were loaded onto protein gels.

SDS–PAGE was done using 7.5% or 12% (as indicated) separating gels. Coomassie stained SDS–PAGE gels and western immunoblotting were performed as previously described [15]. Western blots were probed with primary rabbit polyclonal antibodies, and antibody binding was visualized by either horseradish peroxidase colorimetric [10] or chemiluminescence developing methods. Chemiluminescent blotting was done using the Amersham Biosciences ECL western blotting kit in accordance with the manufacturer's protocol and Kodak X-OMAT LS film was used for visualization. Primary anti-RsaA antibodies (anti-188–784) were made against portions of the RsaA protein as described [6] and used at 1/15,000 for colorimetric and 1/30,000 for chemiluminescent western blotting. The primary antibodies, anti-RsaD and anti-RsaE were diluted at 1/1000 and 1/7500 for colorimetric and 1/3000 and 1/10,000 for chemiluminescent western blotting, respectively.

3. Results and discussion

3.1. Absence of an rsaE promoter is predicted by genetic methods

The *rsaE* gene is located 135 bp after the *rsaD* gene. When initial studies were carried out [11] there were no convenient tools to identify a possible promoter and the regulation of this region was assumed to be analogous to HlyA. Production of polyclonal antibodies against RsaD and RsaE proteins allowed for indirect determination of *rsaE* promoter activity. Using the previously created B15Tn5 *rsaD* knockout strain [11] whole-cell protein samples of the B15 Tn5 *rsaD* and wild type NA1000 strains were run on SDS–PAGE gels, and western blots were probed with anti-RsaD antibodies (Fig. 2(a)). As expected, the NA1000 strain contained RsaD whereas no RsaD was present in B15Tn5 *rsaD*. RsaD antiserum was generated using protein from inclusion bodies resulting in high background activity to other Caulobacter proteins. Therefore we generally used anti-RsaE for western analysis because more definitive results could be obtained. Western blots using anti-RsaE antibodies showed the expected presence of RsaE for NA1000 while B15Tn5*rsaD* did not (Fig. 2(b)). If a promoter was located in the intergenic space between *rsaD* and *rsaE,* then presumably it would be active despite the Tn5 insertion. Since no *rsaE* product could be detected in the *rsaD* Tn5 mutant, it is likely the *rsaD* and *rsaE* genes are transcribed using the same promoter.

3.2. Prediction of an rsaD promoter

Possible promoters were identified in the 242 bp region between the stop codon of *rsaA* and the start codon of *rsaD* using in-silico and manual scanning methods. Scanning software (BPROM) predicted a promoter ~140 bp 5′ of *rsaD* (Fig. 1(a)) with a putative −35 site (*rsaD* −35(1)) located 147–141 bp upstream from the *rsaD* start codon. The putative −10 site is located 123–115 bp 5′ of the *rsaD* start site. The ribosomal binding site was predicted to be in the region 11–7 bp 5′ of the start codon.

a

TTGACAGCTCGGCTGGCGCGACCTTCGTCAGCGGCGCTGACGCGGTGATCAAGCTGAC
CGGTCTGGTCACGCTGACCACCTCGGCCTTCGCCACCGAAGTCCTGACGCTCGCCTAA
GCGAACGTCTGATCCTCGCCTAGGCGAGGATCGCTAGACTAAGAGACCCCGTCTTCCG
AAAGGGAGGCGGGGTCTTTCTTATGGGCGCTACGCGCTGGCCGGCCTTGCCT
AGTTCCGGTGGCTATGATTTAGCGGGACTGGGGGGCTTGCTCACTTTCCGCCACAAT
TTCGTGGTCGAGACGGCGCCTTAGTTGTTACTGTACATGGCCGCGTCGGTTCGCGCGG
CGTCCTGAAGGCTCACAATGTTCAAGCGCAGCGGCGCGAAGCCGACGATCTTCGACCA
GGCCGTGCTGGTCGCCCGCCCGGCGGTGATCACCGCCATGGTCTTCAGCTTCTTCATC
AACATTCTGGCCCTGGTCAGCCCGCTGTACATG

b

Consensus –35 - TTGACG
rsaD –35(1) - TgG cCG
rsaD –35(2) - TTG cCt

Consensus –10 – GCTANA(A/T)C
rsaD –10 – GCTATg A t

Fig. 1. In-silico predicted *rsaD* promoter orientation and binding sites: (a) prediction of the *rsaD* promoter using the Softberry BPROM program. The sequence shown begins with the end of *rsaA* (indicated by dark shadowing) and concludes with the beginning of *rsaD* (medium shadow). The two sequences for the predicted *rho*-independent terminator of *rsaA* are also indicated by medium shadow and the predicted ribosome binding region of *rsaD* by underscore. Identified −10 and −35 sites are indicated as bold text ~115 bp upstream of *rsaD*. Two possible −35 sites are shown since both show similarity to consensus *C. crescentus* promoter sequences. The promoter site is located far enough away from the ρ-independent terminator to allow for polymerase binding and (b) the predicted *rsaD* promoter sequences compared to consensus promoter sequences. Both −35 sites are similar to the consensus site, but the rsaD −35 (2) appears a better fit since the consensus TTG bases are identical.

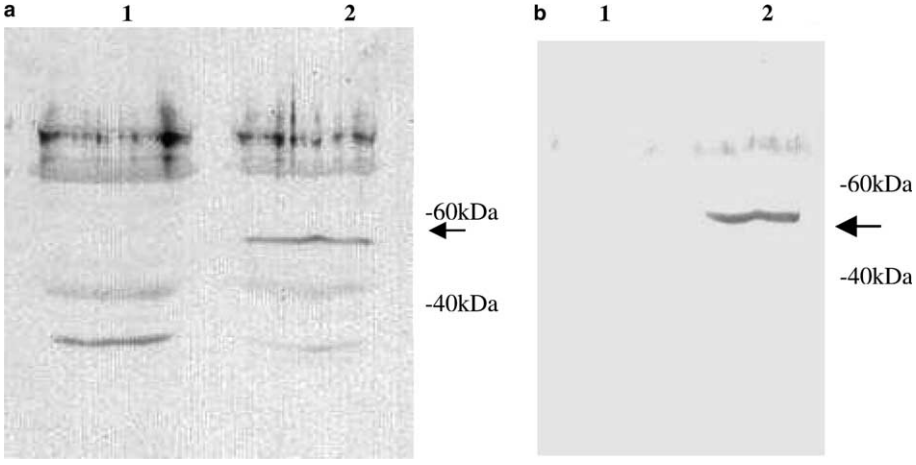

Fig. 2. Characterization of CB15A B15: (a) colorimetric western blot of whole culture preparations using: anti-RsaD. Lane 1, CB15A B15 (*rsaD*⁻), Lane 2, NA1000 wild type, demonstrating RsaD expression (arrow) and (b) colorimetric western blot of whole culture preparations using anti-RsaE. Lane 1, CB15A B15 (*rsaD*⁻) demonstrating loss of RsaE, Lane 2, NA1000 wild type.

An alternative −35 site (*rsaD* −35(2)) was found closer to the −10 site after analysis of the intergenic space using the previously predicted consensus *C. crescentus* promoter sequence [23]. The *rsaD* −35(1) site lies 19 bp away from the −10 site, whereas the *rsaD* −35(2) site is only 10 bp from the −10 site. Of the two predicted −35 sites, the latter −35(2) site makes the spacing of the predicted (−35, −10) binding sites similar to those identified for promoters in *C. crescentus*. As well, the *rsaD* −35(2) site has a better fit with the consensus −35 site as the TTG bases are highly conserved in *Caulobacter* with the second T being conserved for all identified promoter sites (Fig. 1(b)). Although BPROM claims 80% accuracy in promoter identification, this is based on the *E. coli* sigma 70 promoters and may not relate directly to *C. crescentus*. We therefore suggest that the architecture of the promoter region consists of the *rsaD* −35(2) site and the predicted −10 site. These results support the later molecular based findings described below.

In addition, the 135 bp region separating *rsaD* and *rsaE* was also analyzed to determine if there was a potential promoter. BPROM identified no promoters in the region, and no sites show significant similarity to predicted *C. crescentus* promoter sequences, confirming the western results.

3.3. Identification of the rsaDE promoter by genetic methods

A chromosomal deletion removing the region starting 242 bp 5′ of the translational start site of *rsaA* to 8 bp 5′ of the *rsaA* translational stop codon was constructed in CB15 (strain UJ2602). Loss of *rsaA* was confirmed by PCR (not shown) and western immunoblotting using the anti-RsaA antibodies demonstrated that no RsaA was produced (Fig. 3(a)). In this mutant, the entire *rsaA* promoter and gene have been removed while retaining the putative *rsaA* transcription terminator. Also, the promoter immediately upstream of *rsaA* is transcribed in the opposite direction [13], so there was little possibility of read-through causing transcription of *rsaD* and *rsaE*. Western blots show that both the wild-type NA1000 and UJ2602 strain produce RsaD (data not shown) and RsaE (Fig. 3(b)). To confirm that these transporter proteins were active in UJ2602, a plasmid borne copy of *rsaA* was expressed in UJ2602 and secretion of S-layer was evident in the complemented strain (Fig. 3(c)). The level of RsaA secretion was comparable to wild-type, suggesting that production of RsaD and RsaE was not affected by the loss of the chromosomal copy of *rsaA* and its promoter.

To confirm that a putative *rsaDE* promoter was actually driving transcription of *rsaD* and *rsaE*, a second molecular method was undertaken. Strain B15Tn5*rsaD* showed no secretion of RsaA since it lacks a functional copy of *rsaD* [11] (see Fig. 2). A strategy using *trans*-complementation of a plasmid borne copy of *rsaADE* was adopted to determine transcriptional control. The plasmid-borne RAT1 genome fragment [11], which includes *rsaADE* as well as flanking regions (total length ~11 kb) was modified so that its copy of *rsaA* was knocked out using the ΩCm antibiotic resistance cassette which contains transcription terminators at both ends. This presumably eliminates transcription read-through from the *rsaA* promoter on the plasmid vector. Western blots of low pH extracted protein showed that the B15Tn5*rsaD* mutant containing the modified RAT1 fragment secreted RsaA (Fig. 4(a)). This indicated the

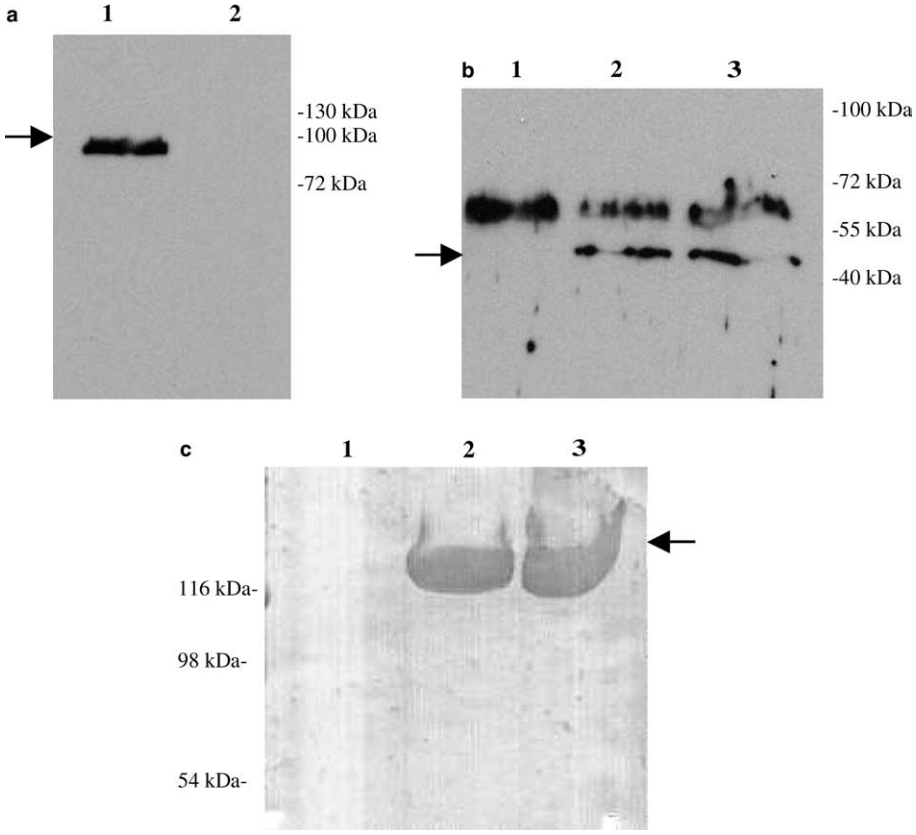

Fig. 3. Characterization of CB15 UJ2602: (a) chemiluminescent western blot of whole culture preparations using anti-188/784 RsaA. Lane 1, NA1000, demonstrating wild type RsaA expression. Lane 2, UJ2602, demonstrating the absence of RsaA. RsaA position marked by arrow; (b) chemiluminescent western blot of whole culture preparations using anti-RsaE. Lane 1, CB15A Tn5 mutant B15 shows absence of RsaE. Lane 2, UJ2602 demonstrating presence of RsaE. Lane 3, NA1000 wild type, demonstrating RsaE expression. RsaE position is marked with arrow. Binding activity at ~60–65 kDa is a non-specific binding activity characteristic of this antisera (it is also seen, but to a lesser extent in Fig. 2) and (c) colorimetric western of low pH extracted protein using anti-188/784 RsaA. Lane 1, UJ2602 demonstrating the loss of RsaA. Lane 2 UJ2602: pWB9: *rsaA*, indicating restoration of RsaA secretion. Lane 3, NA1000 demonstrates wild type RsaA secretion levels. RsaA position marked by arrow.

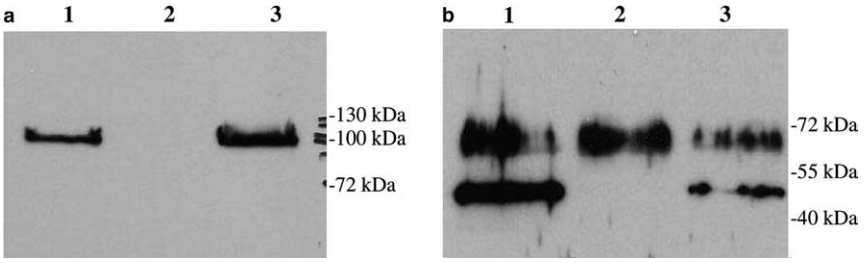

Fig. 4. Characterization of CB15A B15: RAT1ΩCm: (a) chemiluminescent western blot of whole culture preparations using anti-188/784 RsaA. Lane 1, NA1000 wild type. Lane 2, CB15A B15 (*rsaD*$^-$) indicating a loss of RsaA production. Lane 3, CB15A B15: RAT1ΩCm, indicating restoration of RsaA secretion; (b) chemiluminescent western blot of whole cell preparation using anti-RsaE. Lane 1, CB15A B15: RAT1ΩCm, indicating restoration of RsaE expression. Lane 2, CB15A B15 strain (*rsaD*$^-$)) demonstrates the loss of RsaE. Lane 3, NA1000, wild type levels of RsaE.

plasmid borne *rsaD* and *rsaE* genes were transcribed to allow RsaA secretion. Western blots confirmed that both RsaD (not shown) and RsaE (Fig. 4(b)) were present in the complemented strain.

3.4. Direct assessment of promoter activity

As an additional confirmation of promoter activity the regions adjacent to predicted expressed proteins from *rsaD* and *rsaE* were cloned and assessed using a β-galactosidase reporter vector. For the region 5′ of *rsaD* 983 (±107) relative (Miller) units of activity were recorded, compared to 57 (±24) units for the *rsaE* 5′ region and 9 (±0.6) units for the null plasmid control. Thus significant transcription activity for *rsaD* was obtained while activity for *rsaE*, though above background, was judged as negligible.

Taken together, the results indicate that the *rsaD* and *rsaE* genes must be transcribed using a promoter just downstream of *rsaA*, presumably after the putative *rsaA* rho-independent terminator.

Thus, the *rsaA* promoter was not involved in the transcription of the downstream transporter components *rsaD* and *rsaE* and so may explain why previous unsuccessful attempts to express the *rsaA*, *D* and *E* genes in *E. coli* were not due to improper antitermination of the rho-independent terminator as suggested [24], but are more likely due to *E. coli* not recognizing the newly found *rsaDE* promoter.

We propose that in *C. crescentus*, the *rsaD* and *rsaE* genes are transcribed separately from the *rsaA* gene in order to produce a larger amount of transporter units to enable high levels of protein secretion. If transcription of the *rsaD* and *rsaE* genes relied on leakiness of the rho independent terminator, it is possible that not enough copies of the transport apparatus would be produced. The amount of protein secreted by the *E. coli* HlyA system (where presumably transcriptional leakiness in the HlyA system allows adequate levels of secretion) is 0.5–2% of the total cell protein where as the S-layer of *C. crescentus* accounts for 10–12% of the total cell protein. Determining transcription of the RsaA transport components may aid in further increasing levels of S-layer secretion.

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