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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-