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NOTE

Production of Avilamycin A is regulated by AviC1 and AviC2, two transcriptional activators

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Streptomycetes are typical soil-dwelling bacteria with intricate morphological and biochemical differentiation of colonies, resulting in onset of secondary metabolite production. Compounds produced during these stages of differentiation comprise nearly two-thirds of bioactive molecules synthesized by microorganisms, including antibiotics, antitumor agents and immunosuppressants.[1,2] The biosynthesis of antibiotics and other secondary metabolites is controlled by interactions of both global and pathway-specific regulators. However, in all cases, the influence of the environment is reflected by the activity of pathway-specific regulatory genes, which are located within a biosynthetic gene cluster and which control the expression of biosynthetic genes.[3] On account of high structural and functional similarity, such regulators were grouped into two families, namely *Streptomyces* antibiotics regulatory proteins (SARP), mostly found within the aromatic polyketides clusters, and large ATP-binding regulators of LuxR family (LAL), controlling the production of macrolides and glycopeptides.[4–6] Recent efforts in cloning and characterization of biosynthetic gene clusters for new groups of secondary metabolites revealed novel classes of transcriptional factors that differ from typical SARPs or LAL representatives.[7,8]

Avilamycins are secondary metabolites produced by *Streptomyces viridochromogenes* Tu57. They are active against Gram-positive bacteria. It is known that avilamycins bind to the 23S rRNA in the region proximal to the channel, where tRNA enters the A-site and blocks the protein synthesis.[9] Avilamycin resistance is mediated by 23S rRNA methylation and active avilamycin transport.[10]

The avilamycin resistance genes together with the structural genes were identified and cloned as an entire biosynthetic gene cluster containing 54 open reading frames.[11] The biosynthetic steps leading to the formation of the avilamycin molecule were established by the analysis of secondary metabolites produced by mutants.[11,12] Within the cluster also, two putative regulatory genes named *aviC1* and *aviC2* were identified. Both genes are located in close proximity to each other and are transcribed in the same direction (Figure 1). Genes are separated by a 271-bp non-coding region. We succeeded in identifying the presence of putative bacterial promoter sequences upstream of *aviC1* initiation codon by the use of BPROM bacterial promoter prediction server (Softberry Inc., Mount Kisco, NY, USA). It consists of a putative −10 box GGTTTTCAT (Score 34) and a −35 box ATGCGA (Score 12). Another putative promoter sequence is located upstream of the *aviC2* translation start site consisting of a −10 box GCCCATGAT (Score 31) and a −35 box at TTTCTA (Score 34) similar to consensus *Streptomycetes* promoters.[13]

AviC1 consists of 206 amino acid (aa) residues. The C-terminal region of the putative *aviC1* product is similar to response regulators, which contain a LuxR-type DNA-binding domain.[14] Examples are CitB from *Frankia* sp. EAN1pec (53% identical aa) and NarQ, a nitrate/nitrite response regulator from *Escherichia coli* (43% identical aa).[15] All these proteins possess common CheY-like N-terminal sensor domains that undergo phosphorylation by protein kinases and C-terminal LuxR-type Helix-Turn-Helix (HTH) DNA-binding domains.[14] However, the AviC1 N terminus does not resemble CheY-like signal receiver domains, supposing that AviC1 responds to some other signals than phosphorylation.

The product of *aviC2* consists of 192 aa residues. The C-terminal region of AviC2 exhibits similarity to proteins involved in the control of nystatin production in *S. noursei* (NysRIV, 56% identical aa)[7] and pimaricin production in *S. natalensis* (PimM, 53% identical aa).[16] These proteins contain N-terminal PAS domains and sense internal and external signals, such as redox potential or light.[17] The C-terminal part of these proteins represents a LuxR type of HTH DNA-binding motif, which is formed by two α-helixes (R145-E157 and R164-L174

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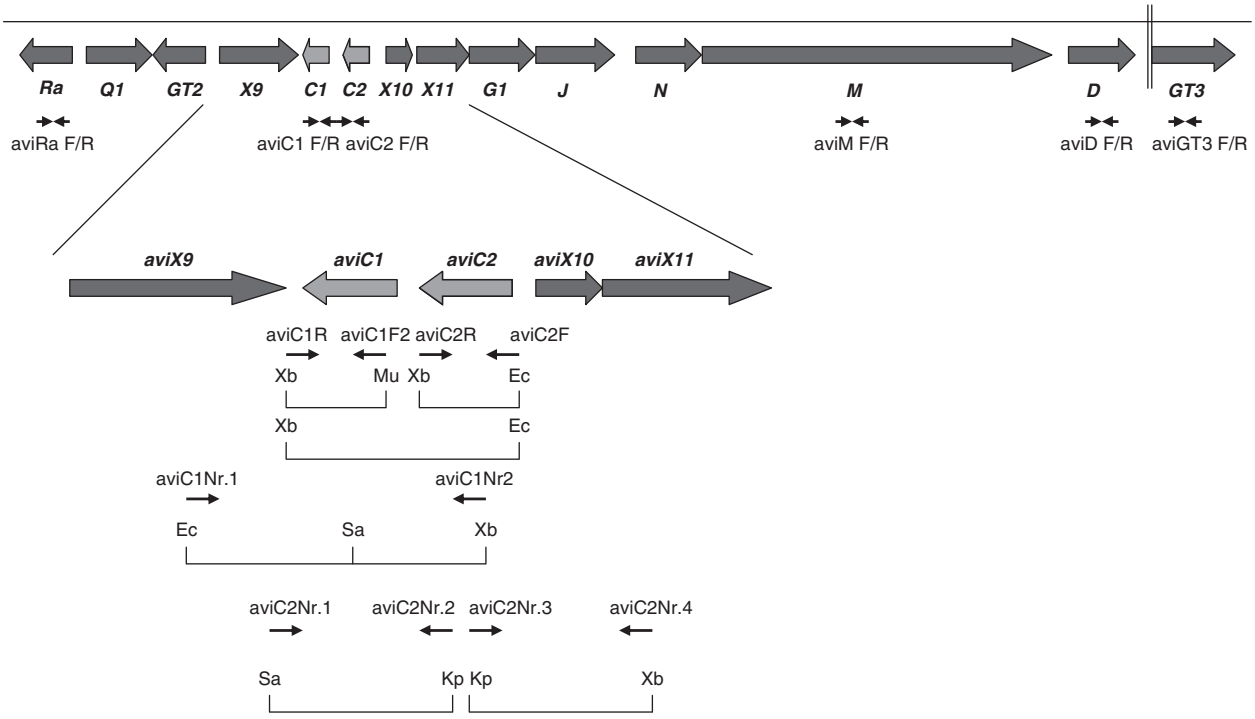

Figure 1 Genetic organization of a part of the avilamycin biosynthesis gene cluster in *S. viridochromogenes* Tu57. Open reading frames are shown as arrows. DNA fragments used for *aviC1* and *aviC2* disruption and expression experiments are shown below the genes. Primers used for gene cloning and reverse transcriptase PCR are indicated as small arrows. Sites for restriction endonucleases are abbreviated as follows: *Eco*RI (Ec), *Sac*I (Sa), *Xba*I (Xb), *Mun*I (Mu), *Kpn*I (K), *Pst*I (P).

in the case of AviC2). BLASTP search for homologs of the N-terminus of AviC2 did not give any significant matches.

To investigate the function of *aviC1* and *aviC2* genes, respective mutants were generated. For this purpose, plasmids pSPaviC1X and pSPaviC2X, were obtained. For inactivation of *aviC1*, the entire gene and flanking DNA regions were amplified by PCR using cosmid F4 as template, and primers aviC1Nr.1 (TCGCCACCGAATTCGGGCTG GACC) and aviC1Nr.2 (ACGTCATCTAGATGATGGAGCGCTTC) (Figure 1). The 1.5 kb PCR fragment was cloned into the *Eco*RI and *Xba*I sites of pSP1. The resulting plasmid pSPaviC1 was restricted by *Sac*I and treated with T4 DNA polymerase. After ligation, pSPaviC1X was obtained. Sequencing confirmed an insertion of 4 bp in the *aviC1*-coding region. To inactivate *aviC2*, two PCR fragments containing parts of *aviC2* were amplified using primers aviC2Nr.1 (TATGAGCG TGAGCTCCTTGCATGC), aviC2Nr.2 (GGTGAGGGTACCACCTG CATCG) and aviC2Nr.3 (ACTCCTTGGTACCCAACACTCGTTC), aviC2Nr.4 (CAGGGTCTAGATGGCGTGGACGG), respectively (Figure 1). PCR products were T/A cloned into pGEM vector (Promega, Mannheim, Germany) to yield pGEMaviC2l and pGEMaviC2r. Fragment aviC2r was restricted using *Kpn*I and *Xba*I, and cloned into the respective sites of pGEMaviC2l. The resulting plasmid was digested using *Sac*I and *Xba*I, and the fragment containing *aviC2* with a deletion of 153 bp was subcloned into pSP1 to give pSPaviC2X.

For gene inactivation, *S. viridochromogenes* GW4 was used as a host. This strain lacks *aviGW4*, a methyltransferase gene involved in methylation of the orsellinic acid moiety of avilamycin A. Desmethyl-avilamycin derivatives are, namely, gavibamycin A1 (desmethyl-avilamycin A) and A3 (desmethyl-avilamycin C).[18] pSPaviC1X and pSPaviC2X were introduced into *S. viridochromogenes* GW4 by protoplast transformation and selection for erythromycin resistant strains. Integration of the plasmids into the chromosome was shown by PCR analysis using primers aviC1F and aviC1R, and aviC2Nr.1 and aviC2Nr.4.

For the generation of *S. viridochromogenes* GW4-C1 and *S. viridochromogenes* GW4-C2 single-crossover mutants were screened for loss of resistance as a consequence of a double-crossover event. Insertion and deletions of the respective genes were confirmed by PCR and Southern hybridization. Analysis of secondary metabolites produced by *S. viridochromogenes* GW4-C1 and *S. viridochromogenes* GW4-C2 verified that both mutants did not produce either gavibamycin A derivatives or its intermediates, as analyzed by HPLC–MS.[12] For complementation and 'overexpression' experiments, *aviC1* and *aviC2* genes were PCR amplified using primers aviC1F (AACGCGT CAATTGAGCCACTGTCAC), aviC1R (CTGTACGTCTAGACGCGG TACACC) and aviC2F (ACGCCACGAATTCGCAGGCGTCGAGGA) aviC2R (GATGCACCTCTAGAAATCCGGCATCC), respectively (Figure 1). PCR products were cloned behind the P_{ErmE} promoter into the *Mun*I and *Xba*I sites of pSET-1cerm. The resulting plasmids were named pSETerm-aviC1 and pSETerm-aviC2. For simultaneous expression of *aviC1* and *aviC2*, plasmid pSETerm-C1C2 was constructed. A 1.7-kb fragment containing both genes was amplified using primers, aviC1F and aviC2R, and using cosmid F4 as a template. Introduction of pSETerm-aviC1 and pSETerm-aviC2 into *S. viridochromogenes* GW4-C1 and *S. viridochromogenes* GW4-C2, respectively, restored production of gavibamycin derivatives, indicating that the mutations did not have any polar effect on other genes of the cluster. On the basis of the results obtained, we suppose that both *aviC1* and *aviC2* gene products are positively controlling avilamycin production.

To establish the hierarchy of both regulatory proteins, cross-complementation experiments were performed. However neither *aviC1* in *S. viridochromogenes* GW-C2 nor *aviC2* in *S. viridochromogenes* GW-C1 were able to restore antibiotic production. The absence

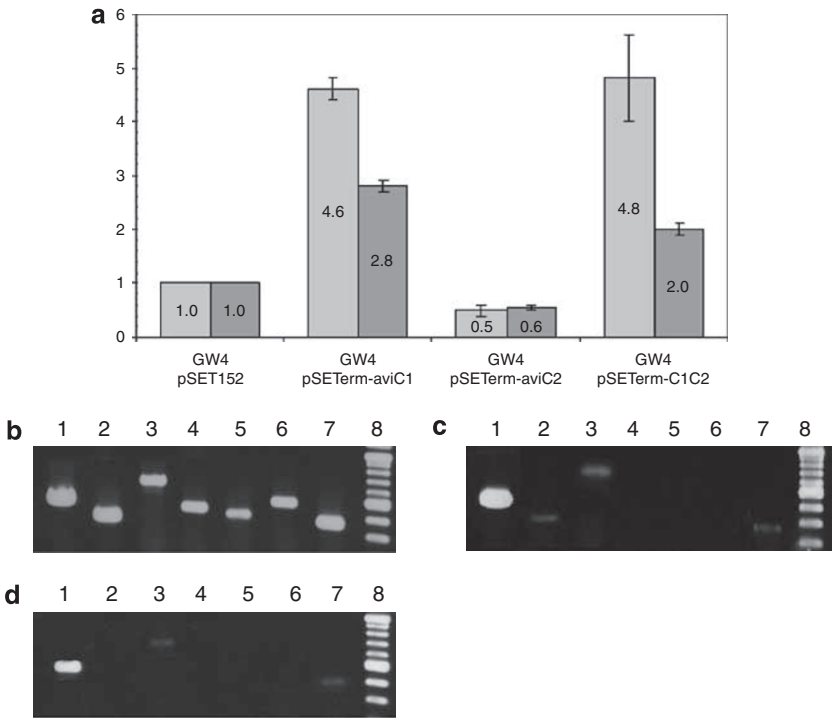

Figure 2 Production of gavibamycin derivatives by *S. viridochromogenes* GW4 strains harboring extra copies of *aviC1* and *aviC2* (**a**), and agarose gel electrophoresis of fragments obtained by reverse transcriptase PCR using total RNA of *S. viridochromogenes* GW4 (**b**), *S. viridochromogenes* GW4-C1 (**c**) and *S. viridochromogenes* GW4-C2 (**d**) as templates. (**a**: Level of gavibamycin A1 is shown in light gray, level of gavibamycin A3 is shown in dark gray; **b**, **c**, **d**: Results obtained by PCR using primers specific for 16S rRNA amplification (lane1), for *aviRa* amplification (lane 2), for *aviD* amplification (lane 3), for *aviM* amplification (lane 4), for *aviGT3* amplification (lane 5), for *aviC2* amplification (lane 6), for *aviC1* amplification (lane 7) and marker (8–100 bp) (lane8)).

of cross-complementation between *aviC1* and *aviC2* suggests that both genes are acting at the same level of regulation.

As *aviC1* and *aviC2* encode positive regulatory proteins, both proteins were also expressed in *S. viridochromogenes* GW4. A strain containing *aviC1* was producing significantly increased levels of gavibamycin A1 (4.6 times higher compared with *S. viridochromogenes* GW4) and gavibamycin A3 (2.8 times higher), major products of *S. viridochromogenes* GW4 (Figure 2a). Interestingly, production of gavibamycins was reduced to twofold compared with the wild type when *aviC2* was expressed in *S. viridochromogenes* GW4 (Figure 2a). Overexpression of AviC2 causes repression of avilamycin biosynthesis either by unspecific binding or by binding to low affinity sites within the *avi* promoters. This phenomenon was described for GerE, a transcriptional factor of the LuxR family controlling spore formation in *Bacillus.*[19] GerE can act both as activator and repressor of the same gene depending on its level of expression. AviC2 might control avilamycin production by a precise expression of *avi* genes depending on the physiological state of the producer strain. When both genes *aviC1* and *aviC2* were introduced into *S. viridochromogenes* GW4, the productivity of the strain was increased again (4.8-fold increase in gavibamycin A1 production and twofold increase in gavibamycin A3 production) (Figure 2a).

To prove that AviC1 and AviC2 act as transcriptional regulators of structural avilamycin biosynthesis genes, reverse transcriptase (RT)-PCR was performed. Total RNA of *S. viridochromogenes* GW4, *S. viridochromogenes* GW4-C1 and *S. viridochromogenes* GW4-C2 were isolated using the hot-phenol extraction method.[20] RT-PCR was performed using the ImProm II Reverse Transcription System (Promega). Total RNA was extracted from the mycelia after 72 h of culture growth that coincides with the active phase of antibiotic production by the wild-type strain. We performed the transcription analysis of *aviM*, encoding a polyketide synthase type I (primers avMF (CTGCACCTACGACTATGGC) and avMR (CAGCAGCTTGATGAC CAGC)), *aviD* encoding a dTDP glucose synthetase (primers avDF (ATTACGCATACCTCGGCCAA) and avDR (CTTCCAGTACCGGTC GATC)), *aviGT3* encoding a glycosyltransferase (primers avGT3F (TCTGCTACGTCGACAACGAC) and avGT3R (GTGAAGAGGTAG TAGTAGCG)), *aviC1* (primers avC1F (ATGGTCGGATCCCTCTGCA) and avC1R (CATGCAAGGACCACACGCT)), *aviC2* (primers avC2F (TCAATTGACGCGTTCCTGCA) and avC2R1 (ATGATCGTGAA GTCCGGTCA)) and resistance gene *aviRa* encoding a rRNA methyl-transferase (primers avRaF (GCGGATCGACAGTTCCGAT) and avRaR (AGATAGGAGGGCTTGCCGA)) (Figure 1). Primers specific to the 16S rRNA of *S. coelicolor* (rrnAF (CACATGCAAGTCGAAC GATG) and rrnAR (GCTGCTGGCACGTAGTTAG)) were used as positive control. Samples were analyzed by 2% agarose gel electro-phoresis, scanned and band density was measured using ImageJ 1.34s software (NIH, Bethesda, MD, USA).

In *S. viridochromogenes* GW4, both regulatory genes as well as biosynthetic and resistance genes were actively transcribed (Figure 2b). The transcription of *aviM* seemed to be much weaker than the activity of other structural genes, which might be explained either by the instability of the long *aviM* transcripts or by a low level of the *aviM* promoter activity. The latter has been described for PKSI-encoding genes from the nystatin biosynthesis gene cluster of *S. norsei.*[7]

In the case of *S. viridochromogenes* GW4-C1, no PCR fragment was obtained for *aviM, aviGT3* and *aviC2*, whereas very tiny fragments

were obtained for *aviD, aviRa and aviC1* (Figure 2c). In the case of *S. viridochromogenes* GW4-C2, no PCR fragment was obtained for *aviM, aviRa, aviGT3* and *aviC2*, whereas very tiny fragments were obtained for *aviD* and *aviC1* (Figure 2d).

On the basis of the obtained results, we suggest that both *aviC1* and *aviC2* gene products are positively controlling avilamycin production at the level of transcription of structural genes. Surprisingly, in both mutants, transcripts of *aviC1* were detectable at a very low level, but transcripts of *aviC2* were not. This might indicate that AviC1 can control *aviC2* expression and that AviC2 acts as autoregulator influencing its own gene transcription.

In conclusion, we have studied two novel regulatory genes controlling avilamycin production in *S. viridochromogenes* Tu57 that differ from the well-characterized SARP and LAL families of antibiotic biosynthesis regulators.

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