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ORIGINAL RESEARCH PAPER

Inactivation of the extracytoplasmic function sigma factor Sig6 stimulates avermectin production in *Streptomyces avermitilis*

Libin Jiang · Yanping Liu · Pan Wang · Ying Wen · Yuan Song · Zhi Chen · Jilun Li

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Abstract The role of the extracytoplasmic function (ECF) σ factor Sig6 (SAV663) in avermectin production by *Streptomyces avermitilis* was investigated by gene-deletion, complementation and over-expression experiments. Inactivation of Sig6 had no major effect on growth, stress responses, or morphology. Avermectin yield was increased 2- to 2.7-fold ($\sim$680 μg/ml) relative to the wild-type strain by deletion of the *sig6* gene, and was restored to the wild-type level by introduction of a single copy of *sig6*. Introduction of extra multi-copy or integrative *sig6* vectors into the wild-type decreased avermectin yield by 56–63%. Taken together, these findings indicate that Sig6 plays a negative regulatory role in avermectin production in *S. avermitilis*. RT-PCR analysis demonstrated that this role of Sig6 is mediated by the pathway-specific activator gene *aveR*.

Keywords Avermectin · ECF sigma factor · Gene deletion · *Streptomyces avermitilis*

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L. Jiang · Y. Liu · P. Wang · Y. Wen · Y. Song · Z. Chen (✉) · J. Li
State Key Laboratories for Agro-biotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, People's Republic of China
e-mail: chenzhi@cau.edu.cn

L. Jiang
e-mail: jianglibin1984@163.com

Y. Liu
e-mail: liuypsg@163.com

P. Wang
e-mail: wangpan8296@yahoo.cn

Y. Wen
e-mail: wen@cau.edu.cn

Y. Song
e-mail: syuan@cau.edu.cn

J. Li
e-mail: lijilun@cau.edu.cn

Introduction

Bacterial σ factors are essential subunits of RNA polymerase that determine promoter specificity and thereby rates of transcription initiation (Helmann and Chamberlin 1988). In addition to the housekeeping σ factor, most bacterial genomes, especially in species with complex life styles, also encode alternative σ factors that redirect RNA polymerase to initiate transcription from alternative promoters after substituting for housekeeping σ factors. Extracytoplasmic function (ECF) σ factors, which constitute the largest and most diverse subfamily of alternative σ factors, play key roles in determining the level of transcription initiation for adaptation to extracellular signals (Staron et al. 2009). In the absence of a stimulus, most alternative σ factors are kept inactive by anti-σ factors through protein–protein interaction. When

subjected to an appropriate environmental signal such as redox change or cell envelope stress, an anti-σ factor is inactivated by either degradation or conformational change (Butcher et al. 2008). The ECF σ factor is then released and activated, and it can redirect gene expression to its target promoters after recruitment by the RNA polymerase core enzyme.

The current study concerns an ECF σ factor and avermectin production by *Streptomyces avermitilis*. This Gram-positive filamentous bacterium is found in soil and is well known for its ability to produce a series of 16-membered macrocyclic lactones, termed avermectins. Avermectins have potent anthelmintic and insecticidal properties, and are therefore applied widely in agriculture, veterinary medicine, and human medicine (Burg et al. 1979). The whole-genome sequencing project for *S. avermitilis* revealed the presence of 47 ECF σ factors (Omura et al. 2001; http://mistdb.com/bacterial_genomes/summary/143). This is far more than the six ECF σ factors per genome that is the approximate average for the bacterial kingdom (Staron et al. 2009), and is only slightly less than the 51 ECF σ factors in the model actinomycete *S. coelicolor* (Bentley et al. 2002). The unusually high number of ECF σ factors in this genus may account for its rapid and precise regulation of diverse stress response regulons for adaptation to complex environments. So far, only a few ECF σ factors, namely SigR, SigE, σBldN, and SigT, have been extensively studied in terms of modulation of stress responses, morphological development, and secondary metabolism in *S. coelicolor* (Bibb et al. 2000; Park and Roe 2008; Staron et al. 2009). Recent comparative transcriptome analyses revealed that expression of 50 genes (including several ECF σ factors: SAV213, SAV424, and SAV4785) was at least twofold greater in an avermectin high-producer, *S. avermitilis* ATCC31780, than in an avermectin low-producer, wild-type *S. avermitilis* ATCC31267, and that over-expression of SAV213 was correlated with increased avermectin production in ATCC31267 (Im et al. 2007; Duong et al. 2009). These findings indicate that avermectin biosynthesis is controlled by several levels of signal transduction, including ECF-dependent signaling.

Based on the use of a whole genome-expression profile chip, our recent comparative transcriptome analysis of ATCC31267 versus avermectin high-producer *S. avermitilis* 76-02-e revealed that the gene SAV663 (*sig* 6) is down-regulated 4.6-fold and 12.5-fold at day 2 and 6, respectively, in 76-02-e (data not shown). SAV663 encodes a putative ECF σ factor. We now describe avermectin production in *sig6*-deletion and -overexpression mutants of ATCC31267. We also describe a potential strategy for obtaining strains with increased avermectin production.

Materials and methods

Strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *Escherichia coli* strains were cultured at 37°C in LB medium. *S. avermitilis* strains (including wild-type ATCC31267) were grown at 28°C on solid YMS medium for sporulation, or in modified liquid YEME medium (MacNeil and Klapko 1987) containing 25% sucrose for growth of mycelia, for DNA extraction, and protoplast preparation. Minimal medium (MM) and YMS medium were used for morphological studies (Kieser et al. 2000). RM14 medium (MacNeil and Klapko 1987) was used for regeneration of protoplasts. For avermectin production, seed medium contained 30 g soluble starch, 4 g yeast extract, 2 g soya peptone, and 10 mg $CoCl_2{\cdot}6H_2O$ per liter H_2O. Fermentation medium I contained 70 g soluble starch, 16 g yeast extract, 0.5 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 4 g KCl, 10 mg $CoCl_2{\cdot}6H_2O$, and 2 g $CaCO_3$ per liter H_2O. Liquid fermentation medium II (which was used to culture mycelia for biomass) contained 50 g soluble starch, 12 g yeast extract, 0.5 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 4 g KCl, and 10 mg $CoCl_2{\cdot}6H_2O$ per liter H_2O. Apramycin was added, if necessary, at 10 μg ml^{-1} for YMS, 5 μg ml^{-1} for YEME, 20 μg ml^{-1} for RM14, or 100 μg ml^{-1} for LB.

Construction of *sig6* gene-deletion mutant

The vector for *sig6* gene deletion was constructed as follows. With ATCC31267 genomic DNA as template, a 490-bp fragment upstream of *sig6* was amplified with primers 663-U1 (5′-CCCAAGCTTACCGAGGCAAGTACGAGG-3′; *Hin*dIII site underlined) and 663-U2 (5′-GGAGTTCGAGCCCAGGAGTCACCGAACCGAGCAACC-3′), and a 493-bp fragment downstream of

sig6 was amplified with primers 663-D1 (5′-GGTTG CTCGGTTCGGTGACTCCTGGGCTCGAACTCC-3′) and 663-D2 (5′-CGGAATTCGTCGATGCCGGTGAC AAC-3′; *Eco*RI site underlined). The two fragments were recovered using a PCR product recovery kit and used as templates at the molar ratio of 1:1. A 947-bp fragment was amplified with primers 663-U1 and 663-D2, digested by *Hin*dIII/*Eco*RI, and cloned into *Hin*dIII/*Eco*RI-digested pKC1139 to produce the *sig6* gene-deletion vector pKCD663.

Plasmid pKCD663 was introduced into *E. coli* ET12567 to propagate non-methylated DNAs before their transformation into protoplasts of *S. avermitilis* strains. Protoplast preparation, transformation, and regeneration were performed as described previously (MacNeil and Klapko 1987). Because *S. avermitilis* does not sporulate on RM14 medium, transformants regenerated on RM14 plates were transferred to solid YMS medium for sporulation. Double-crossover recombinant strains were selected as described previously (Li et al. 2008). The putative gene-deleted strain was termed 663d, and gene deletion was confirmed by PCR using primer 663-V1 (5′-CGCCAGCACACT CAGACC-3′), primer 663-D2, primer 663-V2 (5′-CT CAGGTCGACCACGTGC-3′), and primer 663-U1. Primers 663-V1 and 663-V2 flank the gene-deletion region. When primer pairs 663V1/663D2, 663V2/663U1 and 663V1/663V2 were used for PCR analysis of 663d total DNA, the predicted products were 1020-, 1013-, and 1086-bp DNA fragments respectively. When total DNA of wild-type ATCC31267 was used as template, the predicted products were 1677-, 1670-, and 1743-bp DNA fragments. These results confirmed that the *sig6* gene was deleted by double-crossover recombination in 663d (Fig. 1).

Complementation of the *sig6*-deletion mutant and introduction of extra copy *sig6* into *S. avermitilis*

An 1,193-bp DNA fragment carrying the promoter and coding regions of *sig6* was amplified with primers 663-O1 (5′-CGGGATCCAGGGTCTGCAGGGCGA TC-3′; *Bam*HI site underlined) and 663-O2 (5′-CG GAATTCAGACTGGGCGGGACTCT-3′; *Eco*RI site underlined) using ATCC31267 genomic DNA as template, and then cloned into the *Eco*RI/*Bam*HI-digested multi-copy vector pKC1139 and integrative

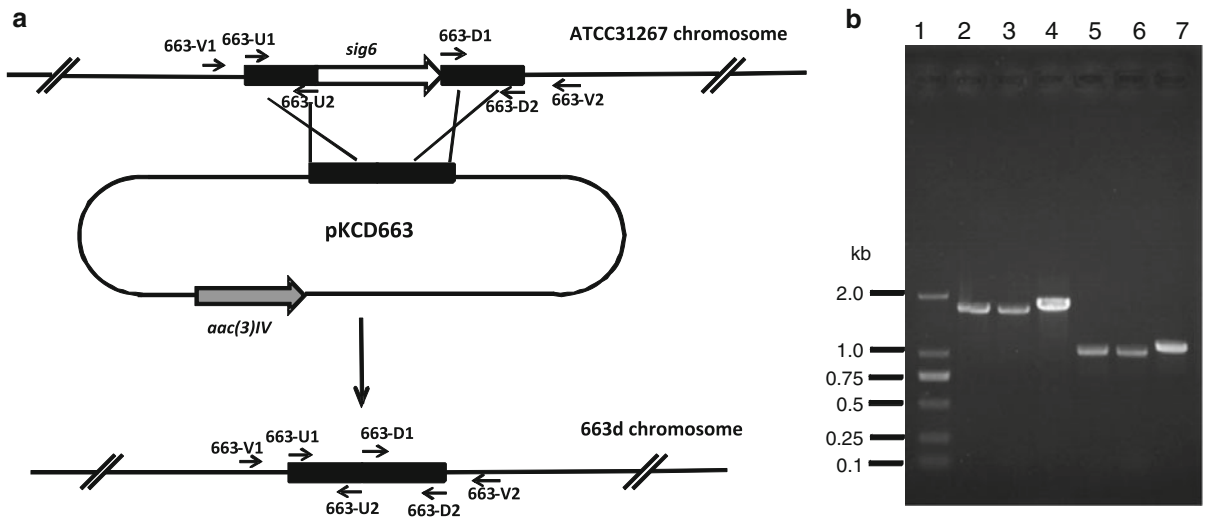

Fig. 1 Construction of the *sig6* gene-deletion mutant. **a** Strategy for deletion of the *sig6* gene. *Long white arrow* gene and its direction. *Short black arrows* positions of primers used for cloning of exchange regions and confirmation of gene deletions, as described in "Construction of *sig6* gene-deletion mutant" section. Double-crossover recombination led to *sig6* deletion. **b** PCR analysis to confirm deletion of *sig6* in mutant 663d. Agarose gel electrophoresis of PCR products from 663d and wild-type ATCC31267 using primer pairs 663V1/663D2 (*lanes 2 and 5*), 663V2/663D1 (*lanes 3 and 6*), and 663V1/V2 (*lanes 4 and 7*). *Lane 1*, DL2000 marker; *lanes 2–4*, PCR products from ATCC31267; *lanes 5–7*, PCR products from 663d

pSET152 to produce *sig6* expression vectors pKCE663 and pSEE663, respectively. In both plasmids, the sequence of the *sig6* gene was verified by nucleotide sequencing. For complementation analysis of the *sig6*-deletion mutant, vector pSEE663 was integrated into the *attB* site of the *S. avermitilis* chromosome after transformation. For *sig6* over-expression, pKCE663 and pSEE663, together with pSET152 and pKC1139 as controls, were introduced into ATCC31267. After transformation, the pKC1139-based vector contained multiple copies of *sig6* per cell, while the pSET152 derivative was integrated into the chromosome, thereby introducing one extra copy of *sig6*.

RNA extraction and semi-quantitative RT-PCR analysis

Mycelia of *S. avermitilis* grown in the fermentation medium were collected on days 2 and 6, frozen in liquid N_2, and ground to a fine powder. RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions, and was treated with DNase I (TaKaRa, Shiga, Japan) to remove chromosomal DNA contamination. Reverse transcription (RT)-PCR was performed using the TaKaRa RNA PCR kit (AMV, ver. 3.0), according to the manufacturer's protocol. Primer pairs, which are listed in Supplementary Table 2, were used to analyze transcript levels of *aveR*, *aveA1*, *olmR1*, and *olmR2* in the various strains of *S. avermitilis*. The *hrdB* gene, which encodes the major σ factor in *Streptomyces*, was used as the internal control. Samples (5 μl) of PCR amplification products were subjected to 2.0% agarose gel electrophoresis and stained with ethidium bromide. Negative controls to rule out the possibility of DNA contamination were conducted using PCR amplification without prior reverse transcription.

Stress survival tests

To assess susceptibility of the *sig6*-deletion mutant to various stress factors, 5 μl of a serially suspension of fresh spores (initially $= 4 \times 10^7$/ml) were dotted onto YMS plates containing NaCl (4%), SDS (0.02%), H_2O_2 (1 mM), EDTA (0.3 mM), Triton X-100 (0.04%), or toluene (3%). Wild-type ATCC31267 was used as the control. Survival in comparison to cells grown on non-treated YMS plates was determined after 7 days at 28°C.

Fermentation and HPLC analysis of avermectin production

Spores from various *S. avermitilis* strains cultured on YMS plates for 7 days were added to 250-ml flasks containing 50 ml seed medium; the cultures were incubated for 24 h at 28°C on a rotary shaker (180 rpm). 2.5 ml of the culture was then transferred into three 250 ml flasks each containing 50 ml fermentation medium and the cultures were grown for 10 days at 28°C on a rotary shaker (220 rpm). Fermentation broth (1 ml) was extracted with 4 ml methanol for 30 min, and centrifuged at $4{,}000 \times g$ for 10 min. The supernatant was directly applied to an HPLC system with a C18 column (10 μm; internal diameter 4.6 × 250 mm) developed with methanol/water (85:15 v/v) at 1 ml/min. Avermectins were detected at 246 nm with authentic samples of avermectin B1 used as internal standards.

Results and discussion

Characterization of *sig6*-deletion mutant strain

SAV663 (*sig6*) encodes a putative RNA polymerase ECF σ factor. Expression of this factor at 6 days is down-regulated >12-fold in the avermectin high-producer *S. avermitilis* strain 76-02-e compared to wild-type ATCC31267. To evaluate the involvement of *sig6* in avermectin production, we constructed a *sig6*-deletion mutant (termed 663d) by homologous recombination (Fig. 1a), and confirmed the gene-deletion by PCR analysis (Fig. 1b). Analysis of the total DNA of mutant 663d using primer pairs 663V1/663D2, 663V2/663U1, and 663V1/663V2 produced a 1-, 1-, and 1.1-kb bands, respectively, whereas corresponding analysis of the total DNA of wild-type ATCC31267 gave 1.65-, 1.65-, and 1.75 kb bands, confirming the deletion of the *sig6* gene.

To compare the phenotypes of ATCC31267 and 663d, we cultured the strains on YMS and MM media. The morphology was very similar for 663d and ATCC31267, indicating that deletion of the *sig6* gene does not affect this process in *S. avermitilis*.

ECF σ factors are usually involved in modulation of stress responses. Stress tests were conducted to determine which type of stress Sig6 responds to. When serial dilutions of 663d spores were dot-inoculated onto

plates, their growth patterns were similar to those of ATCC31267 (data not shown). The finding indicates that Sig6 is not involved in any of the stress responses examined.

Avermectin production is enhanced by *sig6* deletion

The *sig6*-deletion mutant 663d and wild-type ATCC31267 were each cultured in the fermentation medium for 10 days. HPLC analysis of fermentation products showed considerably higher avermectin production in 663d. After 10 days of culture, avermectin yields of 663d variants, which ranged from 498 μg/ml for 663d-1 to 679 μg/ml for 663d-4, were 2.0- to 2.7-fold higher than the avermectin yield of ATCC31267 (248 μg/ml) (Fig. 2a).

To confirm that the *sig6* deletion was the sole cause of the enhanced avermectin production, we reintroduced a 1,177-bp DNA fragment containing the coding region of *sig6* and its putative promoter into 663d-1 through integration of the pSET152-based complementation plasmid pSEE663 into the chromosome. Avermectin yield of this complementation strain (termed 663d-c) was 224 μg/ml, similar to that of ATCC31267 (Fig. 2a). Thus, the absence of Sig6 was the cause of the increased avermectin production in the *sig6*-deletion mutant 663d.

To determine whether enhanced avermectin production results from improved cell growth, we analyzed these two parameters in 663d-1 and ATCC31267 grown in liquid fermentation medium II. Deletion of the *sig6* gene had no significant effect on cell growth (Fig. 2b) but clearly enhanced avermectin production (Fig. 2c). Because liquid fermentation medium II contains a soluble nitrogen source and does not favor avermectin production, the yield was lower than that in fermentation medium I. These findings indicate that the higher avermectin production in *sig6*-deletion strains was not due to enhancement of cell growth.

sig6 deletion stimulates expression of the pathway-specific activator *aveR*

The gene *aveR* encodes a pathway-specific activator essential for transcription of all avermectin biosynthetic genes (Guo et al. 2010). We performed RT-PCR analysis to determine expression levels of *aveR* and the biosynthetic gene *aveA1* in 663d and ATCC31267. RNA samples were isolated from these cultures after 2 and 6 days in fermentation broth.

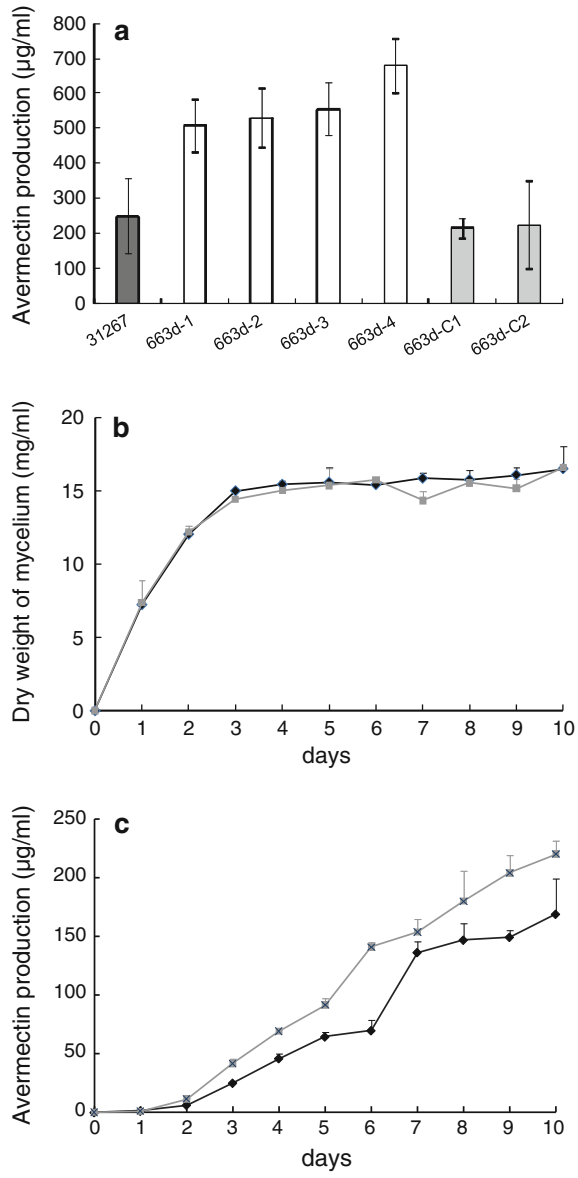

Fig. 2 The relationship between avermectin production and growth in transformed and wild-type *Streptomyces avermitilis*. **a** Avermectin production by wild-type ATCC31267, *sig6*-deletion mutants 663d-1 to -4, and *sig6*-deletion complementation strains 663d-C1 and -C2 (from *left to right*). **b** Growth in 663d (*square*) and ATCC31267 (*diamond*) in liquid fermentation medium II. **c** Avermectin production in 663d (*square*) and ATCC31267 (*diamond*) in liquid fermentation medium II. Values are means (±SD in **a** and +SD in **b** and **c**) from three independent experiments

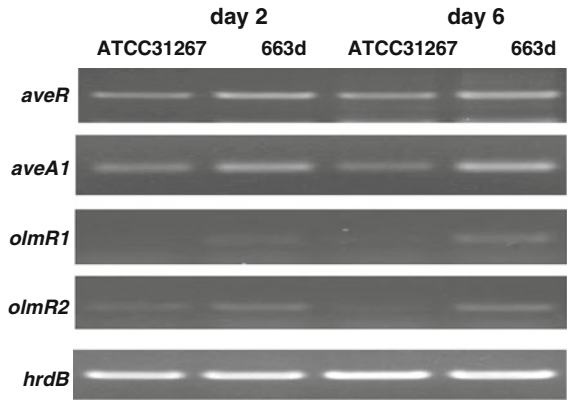

Fig. 3 RT-PCR analysis of *aveR*, *aveA1*, *olmR1*, and *olmR2* transcript levels in ATCC31267 and 663d. *hrdB* was used as the internal control

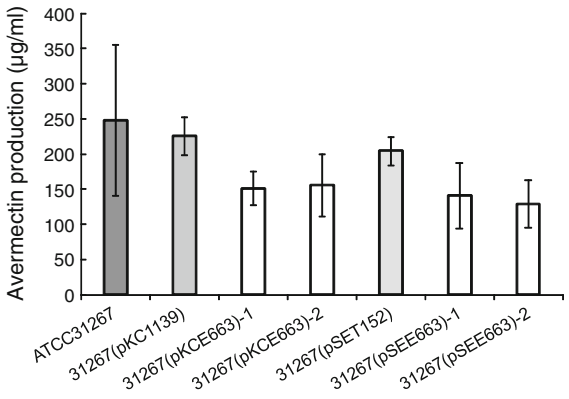

Fig. 4 Effect of *sig6* over-expression on avermectin production in ATCC31267. The strains from *left to right* are wild-type ATCC 31267, vector control strain ATCC 31267 (pKC1139), *sig6*-overexpressing transformants 31267 (pKCE663)-1 and -2, vector control strain ATCC 31267 (pSET152), and *sig6*-overexpressing transformants 31267 (pSEE663)-1 and -2. Values are means (±SD) from three independent experiments

Transcript levels of *aveR* and *aveA1* were much higher in 663d than in ATCC31267 at both times (Fig. 3). These findings suggest that inactivation of Sig6 stimulates transcription of *aveR*, leading to increased transcription of biosynthetic gene *aveA1* and increased avermectin production. In addition, semi-quantitative RT-PCR analyses were performed to determine expression of two putative pathway-specific regulatory genes (*olmRI* and *olmRII*) in the *olm* gene cluster, which is responsible for biosynthesis of another polyketide, oligomycin. Similar to results for *aveR*, transcription levels of *olmRI* and *olmRII* were higher in 663d than in ATCC31267. Thus, Sig6 appears to have a negative regulatory role in secondary metabolism in *S. avermitilis*.

Because of the nature of its regulation, ECF-mediated signaling always up-regulates its target genes and so the effect of Sig6 on antibiotic production is probably indirect. One possibility is that some Sig6 target genes are intermediate transcriptional repressors in a regulatory cascade that in turn modulates expression of pathway-specific regulators associated with antibiotic biosynthesis. Inactivation of Sig6 down-regulates expression of the transcriptional repressors, resulting in increased antibiotic biosynthesis. This possibility remains to be examined.

Over-expression of *sig6* decreases avermectin production

The effect of over-expression of *sig6* on avermectin production in wild-type ATCC31267 was also investigated. The *sig6* integrative expression vector pSEE663 and multi-copy expression vector pKCE663, containing the *sig6* coding region and its putative promoter, were constructed and introduced separately into ATCC31267. The resulting transformants, wild-type strain and vector control strains (containing pKC1139 and pSET152) were each cultured in the appropriate fermentation medium for 10 days, and the fermentation products were analyzed by HPLC. Avermectin production by the vector control strains was similar to that of ATCC31267. In contrast, avermectin yields for the *sig6* over-expressing transformants 31267 (pKCE663) and 31267 (pSEE663) after 10 days were ~63 and 56% that of ATCC31267 (Fig. 4). The growth and morphology of the transformants were essentially the same as those of ATCC31267 (data not shown). These findings, like those from the *sig6*-deletion and complementation experiments, indicate that Sig6 plays a negative regulatory role in avermectin production.

In conclusion, the role of the ECF σ factor Sig6 on avermectin production, stress responses, and morphology in *S. avermitilis* was investigated. Avermectin production was enhanced by Sig6 inactivation, apparently through increased transcription of the pathway-specific factor *aveR*. The findings suggest a strategy for increasing antibiotic production in *Streptomyces* through alteration of the activity of the ECF σ factor.

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