Identification of a gene cluster for telomestatin biosynthesis and heterologous expression using a specific promoter in a clean host

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Telomestatin, a strong telomerase inhibitor with G-quadruplex stabilizing activity, is a potential therapeutic agent for treating cancers. Difficulties in isolating telomestatin from microbial cultures and in chemical synthesis are bottlenecks impeding the wider use. Therefore, improvement in telomestatin production and structural diversification are required for further utilization and application. Here, we discovered the gene cluster responsible for telomestatin biosynthesis, and achieved production of telomestatin by heterologous expression of this cluster in the engineered Streptomyces avermitilis SUKA strain. Utilization of an optimal promoter was essential for successful production. Gene disruption studies revealed that the tlsB, tlsC, and tlsO–T genes play key roles in telomestatin biosynthesis. Moreover, exchanging TlsC core peptide sequences resulted in the production of novel telomestatin derivatives. This study sheds light on the expansion of chemical diversity of natural peptide products for drug development.

Natural products produced by actinomycetes are used for a variety of drugs and bioprobes for chemical biology and biochemistry research. Telomestatin (1) (Fig. 1A) is a macrocyclic peptide isolated from Streptomyces anulatus 3533-SV41. Owing to specific binding to the telomeric G-quadruplex structure of the 3'-telomere end and strong telomerase inhibitory activity2, it is globally used as the standard substance for in vitro assays3–8. To date, various telomerase inhibitors have been isolated from natural products9–11 or developed in organic synthesis studies12–14, and their activities have been evaluated. However, no compound showing stronger activity than 1 has been found, except for synthetic (S)-isomer15.

Despite the importance for biological research applications, the supply of 1 is limited. The purification process from a culture broth of S. anulatus 3533-SV4 has been extremely difficult, because it produces small amounts of 1 in the presence of a variety of secondary metabolites. Total synthesis studies of 1 and its derivatives have also been performed15–17. However, because of technical difficulties in the isolation process, the yield was insufficient for downstream chemical modification. Therefore, the development of an efficient system for producing 1 has been desired. We adopted a heterologous gene expression system from which any produced compound can be easily recovered. The clean host strain Streptomyces avermitilis SUKA18,19, which disrupts major secondary metabolite biosynthetic gene clusters, represents a breakthrough for the efficient production and isolation of 1. Here, we

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report the identification, by heterologous expression, of the gene cluster that drives 1 biosynthesis, and the deri-
vatization of 1 for future drug development.

Results and Discussion

Exploration of the 1 Biosynthetic Gene Cluster and Promoter-assisted Heterologous Expression. Based on the structure of 1, we first assumed an involvement of non-ribosomal peptide synthetase for biosynthesis. However, genome sequence analyses of S. anulatus 3533-SV4 did not support this assumption. Therefore, we speculated that 1 was derived from the ribosomally synthesized and post-translationally modified peptide (RiPP) biosynthetic machinery, which may catalyse the formation of a single thi-azoline ring from cysteine, two methyloxazole rings from threonines, and five oxazole rings from serines, via dehydration and oxidation. To discover the genes encoding the peptide backbone of 1, we searched the whole-genome sequence of S. anulatus 3533-SV4. As expected, we identified the gene (tlsC) encoding such a core peptide sequence (CTTSSSSS) in a small open reading frame encoding 42 amino acids (Fig. 1B). Importantly, the tlsC gene was also associated with putative genes encoding YcaO-type dehydratase (tlsQ) and flavin-dependent oxidoreductase (tlsP) in the same operon (Table S1). Unexpectedly, the operon harboured a set of genes that are possibly involved in fatty acid biosynthesis by type-II FAS machinery, which is considered unnecessary for 1 biosynthesis.

To identify the gene cluster responsible for the biosynthesis of 1, we performed heterologous gene expression using the engineered host strain Streptomyces avermitilis SUKA 18, 19. First, the primary clone (pKU503Dtls_P4-K8) carrying the entire presumptive gene cluster for 1 biosynthesis was obtained from a bacterial artificial chromosome (BAC) library of S. anulatus 3533-SV4. The operon containing 21 genes was subcloned, following NotI restriction endonuclease digestion of pKU503Dtls_P4-K8. Then, a xylose-inducible promoter (xylAp), originating from the xylA (sav7182) promoter of S. avermitilis, was inserted into the construct, upstream of the 1 gene cluster (Fig. 2A). The resulting construct was integrated into a specific locus of the S. avermitilis SUKA17 chromosome via polyethylene glycol-assisted protoplast transformation. The transformants were cultured in the presence of 2% xylose, and their metabolite profiles were analysed by HPLC/MS. However, the production of 1 was not observed (Fig. 2B), indicating that the xylA promoter was not suitable for activation of the 1 gene cluster. Next, we considered that transcription should be controlled during the productive phase of the secondary metabolites. Therefore, we replaced xylAp with olmRp and sav2794p, respectively (Fig. 2A). The olmRp promoter drives expression of the olmRI (sav2902) gene, which encodes the LuxR-family transcriptional regulator controlling the expression of genes required for oligomycin biosynthesis in S. avermitilis 22, and the transcription was found to begin during the late logarithmic phase of growth. The sav2794p promoter drives expression of the sav2794 gene,
which encodes a secreted neutral metalloprotease and was expected to be expressed during the late logarithmic phase of growth. These two promoters were introduced upstream of the \( \text{gene cluster} \) via \( \lambda \)-RED recombination\(^{23} \). The resulting constructs were integrated into \( S. \) \textit{avermitilis} \( \text{SUKA17} \), and their metabolite profiles were analysed using HPLC/MS (Fig. 2B). We detected only a trace amount of \( \text{I} \) under the control of the \( \text{olmRp} \). When the \( \text{sav2794p} \) was used for expression of the \( \text{I} \) \text{gene cluster}, we detected a clear and discrete peak of \( \text{I} \), with a pseudo molecular ion peak having an \( m/z \) ratio of 583 \([M+H]^+\). The yield of \( \text{I} \) reached more than 5 mg/L in \( 0.3 \times \) BPS medium\(^{24} \). This was the first demonstration that this specific promoter (\( \text{sav2794p} \)) is essential for production of this secondary metabolite using heterologous gene expression.

**Gene Inactivation Experiments Determined Essential Genes for 1 Biosynthesis.** Our success with producing \( \text{I} \) at a high yield enabled us to characterize the functions of the genes involved in \( \text{I} \) biosynthesis. The gene cluster contains genes that potentially function as a transporter (\( \text{tlsA} \)), a transcriptional regulator (\( \text{tlsB} \)), and a precursor peptide (\( \text{tlsC} \)). This gene cluster also encodes unidentified enzymes that are homologous to enzymes promoting fatty acid biosynthesis (from \( \text{tlsD} \) to \( \text{tlsN} \)), post-translational peptide modification (\( \text{tlsP} \) and \( \text{tlsQ} \)), and peptidase function (\( \text{tlsS} \) and \( \text{tlsT} \)); furthermore, it also encodes molecules homologous to proteins with unknown functions (\( \text{tlsO} \), \( \text{tlsR} \), and \( \text{tlsU} \)) (Fig. 1B, Table S1). To address the roles of these genes in \( \text{I} \) biosynthesis, we first constructed a \( \Delta \text{tlsC} \) core disruptant (\( \Delta \text{tlsC} \) core), which lacks the core peptide sequence. When the \( \Delta \text{tlsC} \) core construct was integrated into \( \text{SUKA17} \), it abolished the production of \( \text{I} \), suggesting that the core peptide is modified by the RiPP biosynthetic machinery (Fig. 3).

Next, \( \text{tls} \) gene-deletion mutants were constructed using the pKU592A::sav2794p::\( \text{tls} \) cluster (Fig. S1, Tables S2 and S3). Each deletion construct was introduced into \( S. \) \textit{avermitilis} \( \text{SUKA22} \) (isogenic to \( \text{SUKA17} \)), and the resulting metabolite profiles were analysed by HPLC/MS (Fig. 3). To examine the role of the putative transporter, we also evaluated transformants lacking the \( \text{tlsA} \) gene (\( \Delta \text{tlsA} \)). Contrary to our expectation, wild type \( \text{tls} \) and \( \Delta \text{tlsA} \) transformants accumulated \( \text{I} \) in cells, and we observed no alterations in production of \( \text{I} \). However, we could not rule out involvement of the \( \text{tlsA} \) gene in the biosynthesis of \( \text{I} \), because the growth of the \( \Delta \text{tlsA} \) transformants was slower than that of the wild type. Transformants lacking the \( \text{tlsB} \) gene (\( \Delta \text{tlsB} \)) showed complete abolishment of \( \text{I} \) production, suggesting that \( \text{TLSB} \) is involved in \( \text{I} \) biosynthesis as a positive transcriptional
The C-terminal sequence of TlsB contains a helix-turn-helix motif, which is widely observed in the LuxR family of transcriptional regulators. Moreover, TlsB showed homology to AviC1, which regulates avilamycin biosynthesis, and Lipreg1, which regulates lipomycin biosynthesis. Consistent with our finding, deletion of the \textit{aviC1} and \textit{lipreg1} genes resulted in a significant loss in production of the corresponding metabolites. Compared to cells with wild type \textit{tls}, the transformants lacking the genes \textit{tlsD} through \textit{tlsN} (Δ\textit{tlsD–N}) showed diminished production of 1 (Fig. 3 and Fig. S2). Metabolite analysis revealed that the production of a compound with a pseudo molecular ion peak at \textit{m/z} 298 [M + H]+, termed FA297, was abolished in Δ\textit{tlsD–N} transformants (Fig. S3). In addition, the production of 1 and FA297 was concomitantly abolished in Δ\textit{tlsB} mutants (Fig. S3).

The compositional formula of FA297 was determined to be C_{20}H_{43}N by HRESIMS (Fig. S4). As FA297 was turned out to be a mixture, further characterization of chemical entity was attempted using GC/MS (Fig. S5). Based on the molecular formula and the GC/MS data, aliphatic amine structures were suggested. However, the chemical structure could not be determined because of low abundance. Next, we constructed Δ\textit{tlsS}, Δ\textit{tlsT}, and Δ\textit{tlsST} transformants to examine whether they accumulated biosynthetic precursors, because TlsS and TlsT are annotated as peptidases that might be involved in cyclic peptide formation by cleavage of the leader peptide moiety of precursor peptides. Contrary to our expectation, each mutant showed production of 1, although their production levels were significantly reduced (Fig. 3, Fig. S2); this indicated that peptide precursors of 1 were partially modified by intrinsic peptidases, even in the absence of TlsS and TlsT. To address the roles of TlsD–N during peptide modification, we also constructed a transformant lacking the \textit{tlsD–N} and \textit{tlsSTU} genes. Interestingly, the mutant showed completely abolished production of 1. Based on these observations, we speculated that a presence of FA297 may be involved in correct processing of biosynthetic intermediates. In addition, based on translational coupling between the \textit{tlsT} and \textit{tlsU} genes, we expected the \textit{tlsU} gene to be involved in 1 production. However, single deletion of the \textit{tlsU} gene did not affect the production of 1.

To analyse the biosynthetic mechanism of peptide modification, transformants lacking the \textit{tlsO}, \textit{tlsP}, \textit{tlsQ}, or \textit{tlsR} genes (Δ\textit{tlsO}, Δ\textit{tlsP}, Δ\textit{tlsQ}, or Δ\textit{tlsR}) were constructed, and the resulting metabolites were analysed by HPLC/MS (Fig. 3). TlsQ shared homology with the YcaO protein, which is generally involved in formation of the azoline ring in RiPP biosynthesis. TlsP is homologous to FMN-dependent oxidoreductase, which generally catalyses azole ring formation. Therefore, we predicted that the Δ\textit{tlsP} and Δ\textit{tlsQ} transformants would accumulate azoline and precursor peptide intermediates, respectively (Fig. 4). However, both transformants showed completely abolished production of 1 and its biosynthetic intermediates (Fig. 3). Next, we investigated TlsO and

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**Figure 4.** Proposed biosynthetic pathway for 1.
TlsO, which showed no homologies to functionally annotated proteins (Table S1). Because the ΔtlsO and ΔtlsR transformants displayed completely abolished production of 1, and no biosynthetic intermediates accumulated, we were unable to make informed speculations regarding their respective functions (Fig. 3). In vitro analysis using purified enzymes is key for elucidating the functions of novel enzymes such as TlsO and TlsR. Based on a report that McbB-D is involved in microcin B17 biosynthesis, a possible explanation is that TlsO, TlsP, TlsQ, and TlsR might form a heterooligomer, and that all four enzymes are indispensable for the required catalytic activities.

To gain insight into the catalytic functions of TlsO and TlsR, we compared the homologous protein sequences involved in the biosynthesis of YM-216391, which also has a head-to-tail heterocyclic ring (Fig. S6), as found in 1. Blast analysis showed that the C-terminal sequence of TlsO shares moderate homology with the N-terminal sequence of YmBC, which is annotated to be a cyclodehydratase domain. In addition, TlsR is homologous to YmB1, which is also reported to be a cyclodehydratase. These data suggested that future characterizations of TlsO and TlsR will disclose a common modification mechanism for natural macrocyclic peptide products.

Finally, the series of tls gene-deletion experiments suggested that eight genes (tlsB, C, O, P, Q, R, S, and T) are essential for 1 biosynthesis (Fig. 4). The core structure of 1 was derived from the tlsC gene encoding the small peptide sequence. We speculate that the core peptide sequence is cyclized by TlsP and TlsQ, resulting in oxazoline/oxazole formation. TlsO and TlsR are presumably involved in formation of the last thiazoline moiety, which might occur after macrocyclisation. TlsS and TlsT may excise the N-terminal leader peptide and the C-terminal follower peptide, to form 1.

Creation of Novel 1 Derivatives by Amino Acid Substitution of TlsC Core Peptide Sequence. It is extremely important to expand the chemical diversity of 1, and/or to optimize production of the natural peptide itself, for future drug development. Since a binding to a telomeric G-quadruplex structure requires an isomeric macrocyclic product, we established a system to produce 1 derivatives by introducing mutations into the tlsC gene. We introduced mutations in the same gene locus to ensure optimum expression of the tlsC gene cluster. Then, we constructed SUKA17 transformants that harboured T26S, T27S, and T26S/T27S mutations in the tlsC gene, and successfully produced metabolites showing m/z 569 [M + H]+ and m/z 555 [M + H]+ (Fig. 5). The analysis of collision-induced dissociation (CID) MS/MS spectra (Figs S7–13, Tables S4–5) supported the biogenetically deduced structures of 2, 3, and 4. Briefly, characteristic sulphur-containing product ions were observed at m/z 168

Figure 5. HPLC/MS analysis of metabolites produced in SUKA17, which was transformed with the tls gene cluster containing a mutation in tlsC.
from 1 and m/z 154 from T26S variant, whereas virtually no m/z 168 or m/z 154 peaks were observed in the CID spectra of T27S variant or T26S/T27S variant due to the loss of methyl group on second oxazole adjacent to the possible initial cleavage site (Fig. S13, more detailed discussions in the supplementary information).

Ruffner et al. constructed a random trurnamide core-peptide library29 and generated a vast number of trurnamide derivatives; however, the amount of each product was very limited, which impeded evaluations of their biological activities. Tianero et al. recently succeeded in producing patellins, with a yield of ~27 mg/L, from E. coli by feeding the bacteria cysteine and mevalonate. Biosynthetic production of 1 via genetic engineering has been a challenging project, which we know because we were the group to first identify 1. Here, we significantly improved the method of producing 1 and its derivatives by selecting an optimum host and promoter. Since we found a method of creating 1 derivatives efficiently, our future research will focus on evaluations of the biological activities of those derivatives.

In summary, we succeeded in the heterologous expression of the 1 gene cluster in the versatile host S. avermitilis SUKA, under the control of the sav2794 promoter. When 1 was produced, cell grew slowly because of a stress. Our strategy of using this specific promoter provided insights into the 1) optimum expression of a gene cluster to achieve secondary metabolite biosynthesis, and 2) production of metabolites after normal cell growth in the heterologous expression host strain. Moreover, we demonstrated the efficient use of this system for expanding the chemical diversity of valuable, natural peptide products.

Methods

Chemicals and reagents. Ampicillin, kanamycin, and chloramphenicol were purchased from Nacalai (Kyoto Japan). Neomycin, gentamicin, and apramycin were purchased from Sigma-Aldrich Co. (MO, USA). All other chemicals were of analytical grade. Fortimicin sulphate was kindly supplied from Kyowa Hakko Co. (Tokyo, Japan). Restriction enzymes were purchased from Takara Bio, Inc. (Shiga, Japan) and New England BioLabs (MA, USA).

DNA manipulation. The vectors and strains used in this study are summarized in Table S2. General DNA manipulations in E. coli were performed according to standard protocols31. The hot alkaline method was followed for low-copy plasmid isolation32. Polymerase chain reactions (PCRs) were performed using a C1000 or T100 thermocycler (Bio-Rad). When performing λ-RED recombination, In Fusion cloning (Takara Bio, Inc.) and Gibson assembly (New England BioLabs), DNA fragments were obtained using either Phusion DNA polymerase (New England BioLabs), the Expand High Fidelity PLUS PCR system (Roche Diagnostics, Tokyo, Japan), or Pfu Ultra II Fusion HS DNA polymerase (Agilent Technologies). PCR reaction mixtures were prepared based on the manufacturers’ protocols, with minor modifications (see supplementary methods).

Construction of a BAC library, screening, and minimization of the 1 biosynthetic gene cluster. Genome sequencing of S. avermitilis 3533-SV4 was performed using a PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA), and the sequence data was assembled using HGAP2 (Pacific Biosciences). The BAC library of S. avermitilis 3533-SV4 was constructed as described previously19. The pKU503Dtls_P4-K8 vector, which encodes the CTTSSSSS peptide sequence, was isolated by PCR screening. The putative 1 biosynthetic gene cluster was minimized for heterologous expression in the S. avermitilis SUKA strain. The 5′-terminal 528 bp and the 3′-terminal 549 bp regions found at the ends of the putative cluster were amplified from the pKU503Dtls_P4-K8 vector, using the tls-5frg_F/tls-5frg_R and tls-3frg_F/tls-3frg_R primer sets, respectively (Table S3). The gene fragments were cloned into a vector amplified from pKU492Acos_aac(3)IV, using the 492_F/492xylA_R primer set (Table S3), via In-Fusion® cloning with a SpeI site in the middle of the fragments. The SpeI-digested plasmid and NotI-digested pKU503Dtls_P4-K8 vector were both introduced into E. coli BW25113/pKD78 for λ-RED recombination, as described previously23. After a 1-d incubation at 37°C on an LB plate containing 150 µg/mL apramycin, the desired clone was selected after colony PCR, and was cultured in LB medium containing 25 µg/mL of fortimicin and 150 µg/mL of apramycin. The isolated cosmids were introduced into E. coli DH5α. DNA sequencing was performed to confirm

Construction of promoter cassettes. The 837-bp DNA sequence (olmRp: the promoter sequence consisted of the region from 3,633,163 nt to 3,633,834 nt of the S. avermitilis genome) in the upstream region of the olmRI (sav2902) gene was amplified from a BAC clone (pKU503DoliP15-N14) using the olmRp-aac_1_F and olmRp-aac_1_R primer sets. The fragment (except for the rpsJ promoter) was amplified from pKU460: rpsp-aac(3)I16 using the 460-olmRp-F/aac1-olmRp_R primer set. The DNA fragments were assembled via In-Fusion cloning to obtain pKU460::olmRp-aac(3)I. pKU460::sav2794p (promoter sequence consisted of the region from 3,427,321 to 3,427,705 nt of S. avermitilis genome) -aac(3)I and pKU460::xylAp-p (the promoter sequence of metalloendoprotease in S. cinnamoeus was obtained from the region from 870 to 1,285 nt of sequence accession AB189036, with a point of mutagenesis at C1252T to increase expression levels) -aac(3)I vectors were constructed from pKU460.

Promoter exchange of tls cluster. To evaluate the effects of promoters on 1 production, the xylose-inducible promoter (xylAp) region in pKU492Acos::xylAp-tls was exchanged with the olmR and sav2794 promoters. Cassettes containing the promoters and the aac(3)I gene were amplified from pKU460:olmRp-aac(3)I and pKU460::sav2794p-aac(3)I using either the Univ2_F/olmRp-tls_R primer set for olmRp-aac(3)I, or the Univ2_F/sav2794p-tls_R primer set for sav2794p-aac(3)I. Each promoter cassette was introduced into E. coli BW25113/pKD78 harbouring pKU492Acos::xylAp-tls via electroporation, and was cultured overnight on LB plates containing 25 µg/mL of fortimicin and 150 µg/mL of apramycin. The isolated cosmids were introduced into E. coli DH5α. DNA sequencing was performed to confirm
that the correct promoter exchanges had occurred. The cosmids were designated as pKU492Acoss::olmRp- tls and pKU492Acoss::sav2794p-tls.

Deletion of the tls genes. pKU492Acoss::sav2794p-tls was digested with Nhel and HindIII, and was then ligated into the low-copy vector pKU592A, which possesses a 15A origin of replication and the aph(3)I of Tn5 as a selectable marker, to obtain pKU592A::sav2794p-tls. The aph(3)I gene in pKD13 was replaced with the aac(3)IV gene from pKU492Acoss::aac(3)IV via λ-RED recombination. The pKD13-Apr_F and pKD13-Apr_R primer set was used to obtain pKD13::aac(3)IV, which contains the FRT-aac(3)IV-FRT cassette. The aac(3)I gene in the pKU460::sav2794p-aac(3)I and pKU460::ssmp- aac(3)I vectors was replaced with the FRT-aac(3)IV-FRT gene cassette, which was amplified using the 460aacaacIV_F and 460aacaacIV_R primer set (Table S3). The resulting vectors [pKU460::sav2794p-FRT-aac(3)IV-FRT and pKU460::ssmp- FRT-aac(3)IV-FRT] were used to obtain PCR fragments for tls gene deletion. The sets of deletion primers used are also listed in Table S3. During the process of tls gene deletion, the constitutively-expressed ssmp promoter was inserted into downstream genes to assure transcription of the tls operon (Fig. S1A). The resulting plasmid was designated as pKU592A::sav2794p-tls Δtls (Table S2). For the deletion of the genes from tlsD through tlsN, they were replaced with the ampicillin-resistance gene amplified by PCR from pGEM-3zf, using the delFAS-bla_F and delFAS-bla_R primer set, via λ-RED recombination. After SpeI digestion, a synthetic gene fragment encoding the 3′-UTR of tlsC and the 5′-UTR of tlsO (generated by annealing NoScar-delFAS_F and NoScar-delFAS_R) (Table S3) was joined by Gibson assembly (Fig. S1B).

Transformation of S. avermitilis SUKA and isolation of metabolites. Desired plasmids harbouring the 1 biosynthetic gene cluster were prepared from E. coli GM2929 hisDS::Tn10 to obtain unmethylated DNA constructs. The unmethylated plasmids were introduced into avermilis SUKA17 or SUKA22 by polyethylene glycol-assisted protoplast transformation18. Transformants were screened by colony PCR. The desired clones were cultured in 10 mL of production medium (TSB, GY 33, Q, or 0.3 × butanol (n-BuOH)). The organic layer was concentrated by centrifugal evaporation using a GeneVac EZ-2 plus evaporator, and the resultant residues were dissolved in 1 mL of methanol (MeOH). After removing insoluble particles by centrifugation (20,630 × g, 10 min), the supernatant was filtered through a Millex syringe-driven filter unit (0.20 μm, 4 mm, Millipore). The filtrate was analysed by high-performance liquid chromatography (HPLC)/MS using an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm) with CH3CN (30–99%), in 0.1% TFA (0–5 min) as the mobile phase, and with a flow rate of 0.65 mL/min (Figs S2 and S3).

Creation of 1 Derivatives. pKU492Acoss::sav2794p-tls was digested with Nhel and HindIII, and was then ligated into pKU592A_aac(3)IV to obtain pKU592A_aac(3)IV::sav2794p-tls (Table S2). The β-lactamase gene (bla) with flanking SpeI sites was amplified from pGEM-3zf, using the primers blac-tlsCc::F and blac-tlsCc::R (Table S3), and the tlsC gene was replaced with the ampicillin-resistance gene, via λ-RED recombination, to form pKU592A_aac(3)IV::sav2794p-tls ΔtlsC::bla. The synthetic DNAs, which encoded the TlsC_C265, TlsC_T275, and TlsC_C265/T275 mutations, were amplified by annealing each primer set (T265_F + T265_R, T275_F + T275_R, and T265/T275_F + T265/T275_R) (Table S3). After pKU592A_aac(3)IV::sav2794p-tls ΔtlsC::bla was digested with SpeI, the mutated tlsC fragments were assembled by Gibson assembly. After confirmation of the DNA sequences, the resulting plasmids were designated as pKU592A_aac(3)IV::sav2794p-tls ΔT265, pKU592A_aac(3)IV::sav2794p-tls ΔT275, and pKU592A_aac(3)IV::sav2794p-tls ΔT265/T275. pKU592A_aac(3)IV::sav2794p-tls ΔtlsCcore was also constructed during this process (Table S2). The plasmids were introduced into S. avermitilis SUKA 17 or SUKA22, and metabolites from SUKA transformants were analysed as described in the supplementary methods.

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Author Contributions
K.A., H.I., S.T. and K.S. designed the experiments. H.I. performed promoter design. K.A. and H.I. constructed vectors for gene expression. K.A. also performed heterologous gene expression, and product analysis. T.N. performed high resolution MS analysis. J.H., I.K. and M.I. performed genome sequencing, BAC vector construction, and product analysis. F.K. performed searches for candidate genes. H.O. and T.E. provided valuable discussions. K.A., H.I., S.T. and K.S. wrote the manuscript. S.T. and K.S. integrated all components of the research. All authors discussed the results and commented on the manuscript.

Additional Information
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