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BRIEF COMMUNICATION

Identification and heterologous expression of the actinoallolide biosynthetic gene cluster

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Abstract

Actinoallolides are anti-trypanosomal macrolides isolated from the secondary metabolites of two endophytic actinomycete strains, *Actinoallomurus fulvus* MK10-036 and K09-0307. A putative actinoallolide biosynthetic gene cluster was predicted from the genome sequence of the strain K09-0307. The gene cluster spans a contiguous 53 kb DNA region that comprises seven genes encoding three PKSs (*aalA1, aalA2*, and *aalA3*), cytochrome P450 (*aalB*), acyl-CoA dehydrogenase (*aalC*), crotonyl-CoA reductase (*aalD*), and TetR family regulator (*aalR*). The entire gene cluster was cloned into a plasmid pYIK1 by assembling DNA fragments, which were obtained from two cosmids containing left and right parts of the gene cluster. Following the introduction of an *ermE** promoter at 100bp upstream from the start codon of *aalA1*, the gene cluster was introduced into *Streptomyces coelicolor* M1152. Subsequent LC-MS analysis revealed production of actinoallolide A in the culture broth. Thus, the actinoallolide biosynthetic gene cluster was identified by heterologous expression in *Streptomyces*.

Neglected tropical diseases are tropical infections that are especially common in low-income populations in developing regions of Africa, Asia, and the Americas. These diseases are caused by a variety of pathogens such as viruses, bacteria, protozoa, and helminths. Of these diseases, Sleeping Sickness and Chagas disease are caused by parasitic flagellated protozoa, *Trypanosoma brucei* and *T. cruzi*, respectively [1, 2]. To treat the diseases, some anti-trypanosomals including suramin, pentamidine, eflornithine, melarsoprol, benznidazole, and nifurtimox have been used [3, 4], but some problems in their use remain to be unsolved; the drugs are expensive, toxic, and difficult to administer, and more seriously, parasite resistance to them is increasing. Therefore, safer and highly effective anti-trypanosomal drugs are urgently required.

Actinoallolides were discovered as anti-trypanosomal macrolides from the culture broth of an endophytic actinomycete, *Actinoallomurus fulvus* MK10-036 [5]. Actinoallolide A displays potent and selective in vitro activity against *T. brucei rhodesiense* STIB900 and *T. cruzi* Tulahuen C4C8 with IC_{50} values similar to those of the therapeutic drugs mentioned above [5]. Actinoallolide A is a 12-membered macrolide, which have a 5-membered hemiacetal moiety inside the ring and a side chain including constitutive asymmetric centers [5]. Although macrolides are a class of natural products consisting of a macrocyclic lactone ring [6] and biosynthesized by the joining together of simple acyl-CoA [7], there has been no published report of the biosynthesis of actinoallolides to date. Here, we describe the identification and heterologous expression of the actinoallolide biosynthetic gene cluster.

We found that another *A. fulvus* strain, K09-0307, also produces actinoallolide A (Scheme S1 and Figure S1). In this study, we used the strain K09-0307 to identify the gene cluster for actinoallolide biosynthesis. We have obtained a

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Table 1 Proposed functions of open reading frames in the actinoallolide biosynthetic gene cluster

Gene	Size (bp)	Homolog (accession number); origin	Identity/similarity (%/%)	Proposed function
orf(−1)	1221	imidazolonepropionase (SFI60323); *Amycolatopsis sacchari*	76/83	imidazolonepropionase
aalD	1416	crotonyl-CoA carboxylase/reductase (WP_073925706); *Streptomyces* sp. CB03911	83/90	crotonyl-CoA carboxylase/reductase
aalA1	16,212	TrdAI (ADY38531); *Streptomyces* sp. SCSIO 1666	50/61	type I PKS
aalA2	19,374	malonyl CoA-acyl carrier protein transacylase (CTQ99072); *Kibdelosporangium* sp. MJ126-NF4	67/76	type I PKS
aalA3	12,885	malonyl CoA-acyl carrier protein transacylase (CEL13384); *Kibdelosporangium* sp. MJ126-NF4	67/76	type I PKS
aalB	1179	cytochrome P450 (WP_075124598); *Actinophytocola xanthii*	78/86	cytochrome P450
aalC	1608	putative acyl-CoA dehydrogenase (SFN54472); *Actinomadura madurae*	81/86	acyl-CoA dehydrogenase
aalR	615	TetR family transcriptional regulator (SCL74088); *Micromonospora peucetia*	71/81	regulator
orf(+1)	609	hypothetical protein (WP_079314293); *Microbispora* sp. GKU 823	68/78	hypothetical protein
orf(+2)	801	CHAP domain-containing protein (WP_030511151); *Microbispora rosea*	53/67	CHAP domain-containing protein

draft genome sequence of the strain K09-0307. The total size of the sequence was 8.7 Mb and a putative actinoallolide biosynthetic gene cluster was predicted by the antiSMASH web server [8]. The gene cluster spans a contiguous 53 kb DNA region (DDBJ accession number LC326402) that comprises seven genes encoding three polyketide synthases (PKSs) (*aalA1, aalA2*, and *aalA3*), cytochrome P450 (*aalB*), acyl-CoA dehydrogenase (*aalC*), crotonyl-CoA reductase (*aalD*), and TetR family regulator (*aalR*) (Table 1, Fig. 1a). The PKSs are composed of one loading module and 10 extension modules (Fig. 1b). The signature amino acid residues of acyltransferase (AT) domains predicted the substrates (Figure S2); malonyl-CoA in the modules 6 and 10, methylmalonyl-CoA (or propionyl-CoA) in the loading module and modules 1, 2, 3, 4, 5, 7, and 8, and ethylmalonyl-CoA in module 9. The PKSs have some modification domains in each module; eight ketoreductase (KR) domains in modules 1, 2, 3, 4, 5, 6, 7, and 8, four dehydratase (DH) domains in modules 3, 4, 7, and 8, and two enoyl reductase (ER) domains in modules 4 and 8 (Fig. 1b). Following the fingerprint rule of the KR domain [9], KR2 and KR6 were classified as the A1-type, which give L-configuration at the beta position of the thioester (Figure S3). KR3, KR4, KR5, KR7, and KR8 were classified as the B1-type, which give a D-configuration. We confirmed that the stereochemistry deduced from the fingerprint is fully identical to that of actinoallolide A (Fig. 1b). KR1 could not be classified into any type because the fingerprint of KR1 was similar to both the A1 and B1 types (Figure S3). Although KR1 has catalytic amino acids and the NADPH binding motif, it seems to be inactive, as predicted from the structure of actinoallolide A that has a ketone at C-21. The proposed pathway for actinoallolide biosynthesis is described in Fig. 1b. Following the formation of polyketide backbone by AalA1, AalA2, and AalA3, the macrolactone is formed by a thioesterase (TE) domain of AalA3. The cytochrome P450 AalB might be involved in hydroxylation at C-6 and subsequent nucleophilic addition to a ketone at C-3 presumably gives a unique 5-membered hemiacetal in the macrolactone.

To clone the actinoallolide biosynthetic gene cluster, we screened a cosmid library of the *A. fulvus* K09-0307 genomic DNA by PCR. Consequently, cosmids 2D5 and 5C5, which carried left and right parts of the gene cluster, were isolated (Fig. 1a). In order to assemble both parts of the gene cluster, four DNA fragments were prepared (Figure S4); 14 kb XhoI fragment of 2D5 (fragment 1), 31 kb BstBI and SpeI fragment of 5C5 (fragment 2), 12 kb BstBI fragment of 2D5 containing 40 bp overlapping sequences with fragments 1 and 2, respectively (fragment 3), and 7 kb PCR product of pYIK1 containing 40 bp overlapping sequences with fragments 1 and 2, respectively (fragment 4). The four DNA fragments were assembled by Gibson

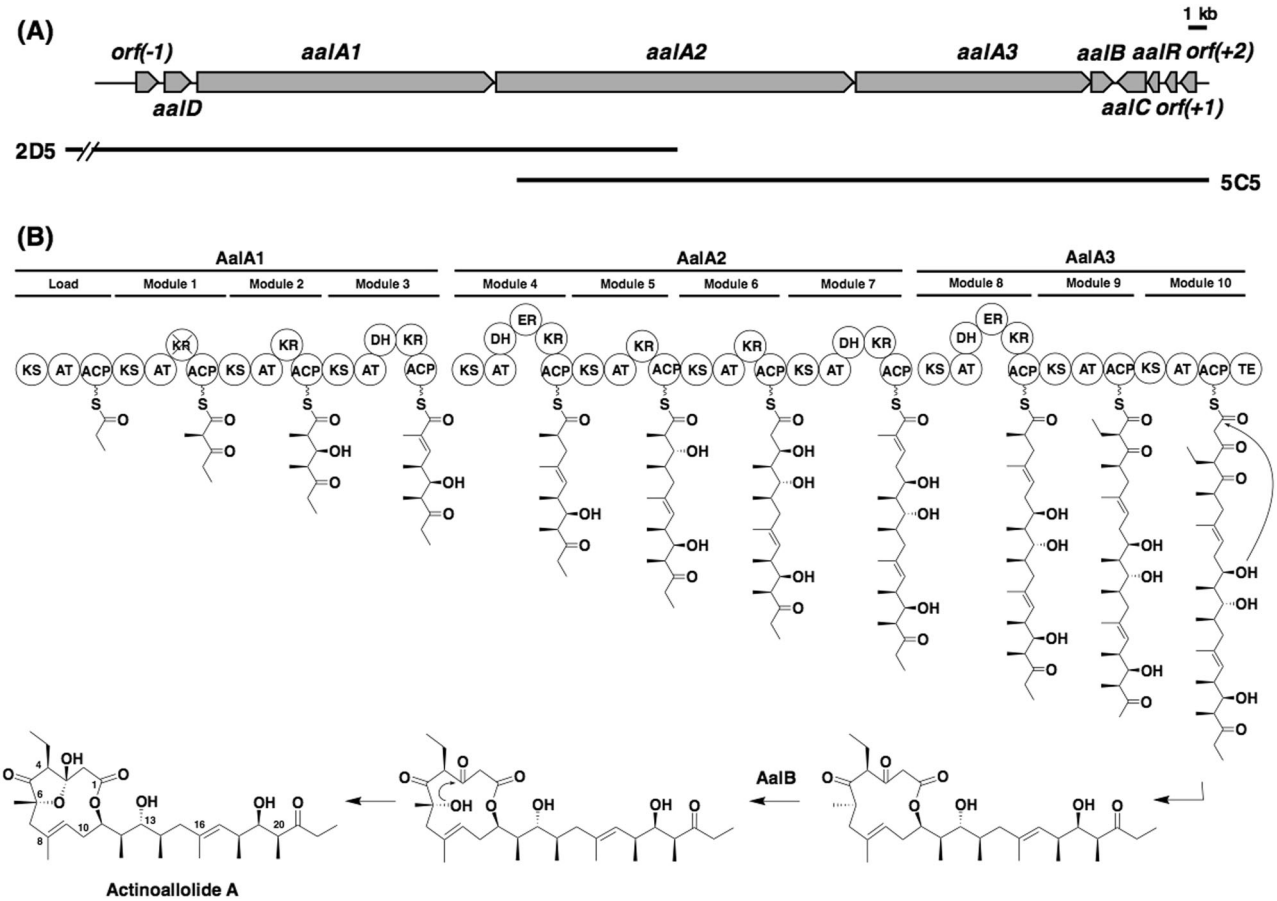

Fig. 1 **a** Actinoallolide biosynthetic gene cluster and relative regions of two cosmids, 2D5 and 5C5. **b** Proposed pathway for actinoallolide biosynthesis

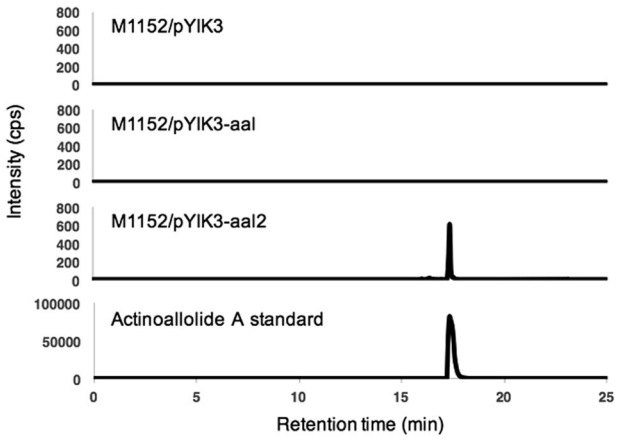

Fig. 2 LC-HRESIMS analysis of actinoallolide production. Extracted ion count chromatoghram from *m/z* 587.3 to 587.4. A compound with *m/z* 587.3538 $[M + Na]^+$, which exactly matched the *m/z* value of actinoallolide A (calcd for $C_{32}H_{52}O_8Na^+$: 587.3554), was detected at 17.4 min in the ethyl acetate extract of culture broth of *S. coelicolor* M1152/pYIK3-aal2

assembly [10] to yield pYIK1-aal, which carried the entire actinoallolide biosynthetic gene cluster (Figures S4 and S5).

An integrase gene, *attP*, *oriT*, and an apramycin resistance gene were inserted into pYIK1-aal. The resulting vector pYIK3-aal was introduced into *S. coelicolor* M1152 by conjugation. *S. coelicolor* M1152/pYIK3-aal was incubated in YD medium (yeast extract 1% and glucose 1%) for 7 days and the ethyl acetate extract of the culture broth was analyzed by LC-HRESIMS. However, none of the actinoallolides were produced by *S. coelicolor* M1152/pYIK3-aal. The gene cluster contains a TetR family regulator gene, *aalR*. TetR is known as a repressor that controls the expression of tetracycline-resistance genes [11]. It seems likely, therefore, that expression of the actinoallolide biosynthetic gene cluster is controlled by AalR.

In order to express the gene cluster, we used the constitutive promoter *ermE** of pYIK1. The 55 kb MfeI and SpeI fragment of pYIK1-aal was assembled with the PCR product of pYIK1 to yield pYIK1-aal2, which had *ermE** promoter at 100 bp upstream from the start codon of *aalA1* (Figure S5). Following insertion of an integrase gene, *attP*, *oriT*, and an apramycin resistance gene, the resulting vector pYIK3-aal2 was introduced into *S. coelicolor* M1152 by conjugation. *S. coelicolor* M1152/pYIK3-aal2 was

incubated in YD medium for 7 days and the ethyl acetate extract of the culture broth was analyzed by LC-HRESIMS. The analysis identified a compound with *m/z* 587.3538 ([M + Na]$^+$; retention time 17.4 min), which exactly matched the *m/z* value of actinoallolide A (calcd for $C_{32}H_{52}O_8Na$: 587.3554; Fig. 2). Furthermore, its MS/MS fragmentation pattern also matched that of actinoallolide A (Figure S6). These results confirmed that the gene cluster is responsible for the biosynthesis of actinoallolide. However, the concentration of actinoallolide A in the culture broth of *S. coelicolor* M1152/pYIK3-aalM (0.012 mg L^{-1}) was much lower than that in the culture broth of *A. fulvus* K09-0307 (1.8 mg L^{-1}). To improve the heterologous production, optimization of the culture condition and/or the promoter would be needed.

The plasmid pYIK3-aal2 does not contain the crotonyl-CoA carboxylase/reductase gene *aalD*. Crotonyl-CoA carboxylase/reductase catalyzes conversion of crotonyl-CoA to ethylmalonyl-CoA, which is one of the polyketide synthase extender units [12]. However, AalD is dispensable for the biosynthesis of actinoallolide via heterologous expression in *S. coelicolor* M1152 (M145 *Δact Δred Δcpk Δcda rpoB [C1298T]*). The crotonyl-CoA carboxylase/reductase SCO6473, a homologous protein of AalD (identity/similarity: 75%/84%), was found in the genome of *S. coelicolor* M145 by a BLAST search. SCO6473 might work as an alternative to AalD.

In conclusion, we first identified the actinoallolide biosynthetic gene cluster using a combination of genome analysis and a heterologous expression approach. More promising actinoallolide analogs may be obtained by genetic engineering of the gene cluster.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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