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ORIGINAL PAPER

Mi-Yeon Lee · Ji Seon Myeong · Hyun-Joo Park
Kyuboem Han · Eung-Soo Kim

Isolation and partial characterization of a cryptic polyene gene cluster in *Pseudonocardia autotrophica*

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Abstract The polyene antibiotics, a category that includes nystatin, pimaricin, amphotericin, and candicidin, comprise a family of very promising antifungal polyketide compounds and are typically produced by soil actinomycetes. The biosynthetic gene clusters for these polyenes have been previously investigated, revealing the presence of highly similar cytochrome P450 hydroxylase (CYP) genes. Using polyene CYP-specific PCR screening with several actinomycete genomic DNAs, *Pseudonocardia autotrophica* was determined to contain a unique polyene-specific CYP gene. Genomic DNA library screening using the polyene-specific CYP gene probe identified a positive cosmid clone, which contained a DNA fragment of approximately 34.5 kb. The complete sequencing of this DNA fragment revealed a total of seven complete and two incomplete open reading frames, which were found to be highly similar, but still unique, when compared to previously known polyene biosynthetic genes. These results suggest that the polyene-specific screening approach may constitute an efficient method for the isolation of potentially valuable cryptic polyene biosynthetic gene clusters from various rare actinomycetes.

Keywords Polyene · Polyketide · Antifungal · Cryptic gene cluster · *Pseudonocardia* · Cytochrome P450 hydroxylase

M.-Y. Lee · J. S. Myeong · E.-S. Kim (✉)
Department of Biotechnology, Inha University,
402-751 Incheon, Korea
Tel: +82-32-8608318
Fax: +82-32-8724046
E-mail: eungsoo@inha.ac.kr

H.-J. Park
SongWon Envichem Inc., Kangnam-gu, 135-010 Seoul, Korea

K. Han
Hanson Biotech Co., Ltd 201 IACRI, Han Nam University,
Daeduk-gu, 306-791 Daejeon, Korea

Introduction

The polyene antifungal antibiotics, which are produced principally by Gram-positive soil actinomycetes, comprise a family of type I polyketide macrolide ring compounds with 20–40 carbon backbones, containing 3–8 conjugated double bonds [6, 14]. The primary antifungal mechanism by which these polyene antibiotics exert their effects is believed to involve specific binding to the ergosterol present in the fungal membrane and the formation of channels which allow for the leakage of cellular K^+ and Mg^{2+}, eventually culminating in the death of the fungal cell [4, 6, 16]. Although polyene compounds are limited with regard to their clinical use, due largely to their high toxicity and side effects, the superior antifungal activities of polyene compounds are still being considered in the further development of improved antifungal drugs [3, 9, 14]. Recently, the polyene biosynthetic gene clusters from nystatin, amphotericin, pimaricin, and candicidin have been cloned and characterized [1–3, 5, 7]. Based on the complete sequences of polyene biosynthetic genes, highly similar polyketide synthase (PKS) genes and post-polyketide modification genes have been identified in the clusters [3, 8, 15, 18]. In order to completely biosynthesize polyene compounds, successive carbon condensation steps for the formation of the polyketide backbone are followed by post-PKS modifications including regiospecific hydroxylation with cytochrome P450 hydroxylases (CYPs) [11–13]. Genes encoding polyene-specific CYPs have been located in all the previously characterized polyene gene clusters including those for nystatin, amphotericin, pimaricin, and candicidin [11–13]. Based on amino acid sequence alignment of polyene CYPs including those encoded by *amphN* (amphotericin-producing *Streptomyces nodosus*), *nysN* (nystatin-producing *S. noursei*), *pimG* (pimaricin-producing *S. natalensis*), and *canC* (candicidin-producing *S. griseus*) [2, 5, 7, 8, 18], highly conserved regions have been identified, which are specific to polyene CYPs. These regions have also proven to be different from the

previously characterized oxygen-binding site and heme ligand pocket. Here, in brief, we report a polyene-specific PCR screening approach that was used for the isolation of the cryptic polyene gene cluster from *Pseudonocardia autotrophica*, followed by a partial characterization of the nine open reading frames (ORFs), which are presumably involved in biosynthesis of the novel cryptic *Pseudonocardia* polyene metabolite.

Materials and methods

Bacterial strains, plasmids, and cultivation conditions

All the actinomycete strains were purchased from either the American Type Culture Collection (ATCC, USA) or the Korean Type Cell Collection (KTCT) and were grown routinely on R2YE agar plates at 30°C for sporulation [10]. Actinomycete spores were resuspended and stored in sterile 20% glycerol solution at -20°C. For total DNA isolation, spore suspensions were inoculated into 25 ml of YEME liquid media and cultured for 2 days at 30°C. The total DNA isolation method was previously described elsewhere [10]. The *Escherichia coli* DH5α strain and a streptomycete–*E. coli* shuttle cosmid vector, pOJ446, were used for the cloning experiments, and standard molecular biology procedures were followed, which have been described elsewhere [10].

Cloning and sequence analysis of the 34.5 kb DNA fragment from *P. autotrophica*

The PCR was conducted with polyene CYP-specific primers [Fig. 1; forward primer: 5′-TGGATCGGCGACGACCG(G/C)(A/G/C)(T/C)CGT-3′; reverse primer: 5′-CCG(T/A)A(G/C)AG(G/C)A(T/C)(G/C)CCGTCGTACTT-3′], using genomic DNA from three polyene non-producing strains (*S. coelicolor* M145, *S. avermitilis* ATCC 31267, *S. peucetius* ATCC 29050), two polyene-producing strains (*S. nodosus* KCTC 9035, *S. noursei* KCTC 1083), and two rare actinomycetes (*P. autotrophica* KCTC 9441, *Sebekia benihana* KCTC 9660) as template. The expected size of 350 bp DNA fragments was amplified from the two known polyene-producing strains (*S. nodosus* and *S. noursei*) as well as *P. autotrophica*. In order to clone the cryptic polyene gene cluster which flanks the CYP region, a *P. autotrophica* genomic DNA library was constructed using an *E. coli*–actinomycetes shuttle cosmid vector, pOJ446 [10], followed by colony hybridization screening using the PCR-amplified 350 bp DNA fragment as a probe. The cloned 34.5 kb *P. autotrophica* DNA fragment was then completely sequenced by a commercial DNA sequencing service (Macrogen Co., Korea). In order to compare the deduced amino acid sequences of the ORFs found in the 34.5 kb fragment with those in the public databases, computer-based sequence analyses were conducted using a multiple sequence alignment program with hierarchical clustering (ClustalW, European Bioinformatics Institute).

Results and discussion

As expected, polyene CYP-specific PCR screening indicated the presence of the central region (approximately 350 bp) of the polyene CYP genes in the two polyene producers (*S. nodosus* and *S. noursei*), but this region was not detected in the three polyene non-producers (*S. coelicolor*, *S. avermitilis*, and *S. peucetius*) as well as in *Sebekia benihana*. Interestingly, a cryptic polyene CYP gene was detected in *P. autotrophica*, a rare actinomy-

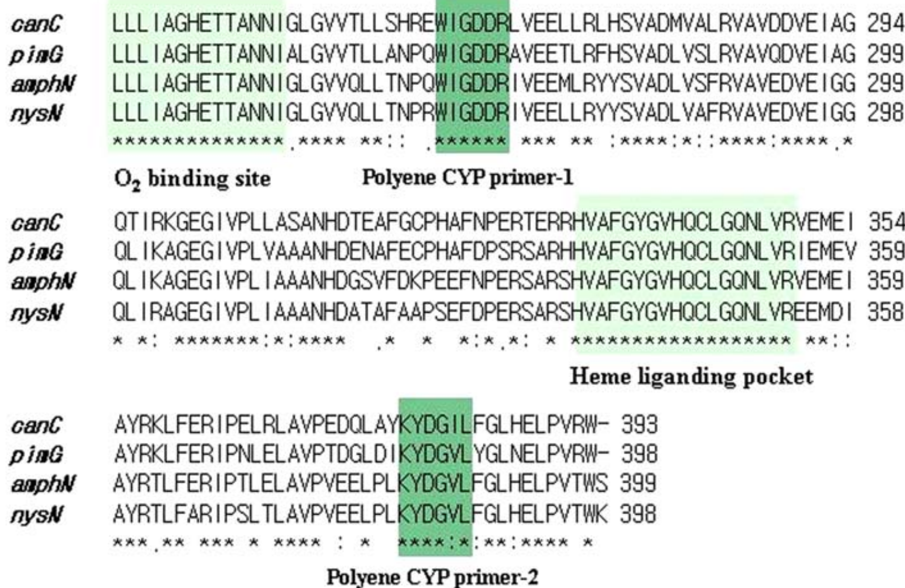

Fig. 1 Degenerate PCR primers for amplification of polyene-specific CYP gene, *canC* (candicidin-producing *S. griseus*), *pimG* (pimaricin-producing *S. natalensis*), *amphN* (amphotericin-producing *S. nodosus*), and *nysN* (nystatin-producing *S. noursei*)

Table 1 Sizes, probable functions, and similarities of seven complete ORFs present in a cryptic polyene gene cluster in *P. autotrophica*

Gene	ORF size (nt)	Probable function	Similar genes	Source	Identity[a]	Reference[b]
cppJ	16278	Polyketide synthase	*nysJ*	*S. noursei*	60	AAF71767
			amphJ	*S. nodosus*	61	AAK73502
			pimS1	*S. natalensis*	42	CAC20931
cppK	6096	Polyketide synthase	*nysK*	*S. noursei*	64	AAF71768
			amphK	*S. nodosus*	62	AAK73503
			pimS4	*S. natalensis*	46	CAC20919
cppL	1212	Cytochrome P450	*nysL*	*S. noursei*	68	AAF71769
			amphL	*S. nodosus*	64	AAK73504
			pimD	*S. natalensis*	55	CAC20932
cppN	1242	Cytochrome P450	*nysN*	*S. noursei*	74	AAF71771
			amphN	*S. nodosus*	75	AAK73509
			pimG	*S. natalensis*	63	CAC20928
cppDII	1059	Aminotransferase	*nysDII*	*S. noursei*	82	AAF71772
			amphDII	*S. nodosus*	82	AAK73510
			pimC	*S. natalensis*	73	CAC20927
cppDI	1470	Glycosyltransferase	*nysDI*	*S. noursei*	79	AAF71773
			amphDI	*S. nodosus*	75	AAK73512
			pimK	*S. natalensis*	62	CAC20918
cppA	3294	Polyketide synthase	*nysA*	*S. noursei*	62	AAF71774
			ampJ	*S. nodosus*	50	AAK73502
			pimS4	*S. natalensis*	52	CAC20919

[a]Amino acid identity in percent
[b]Genbank database number

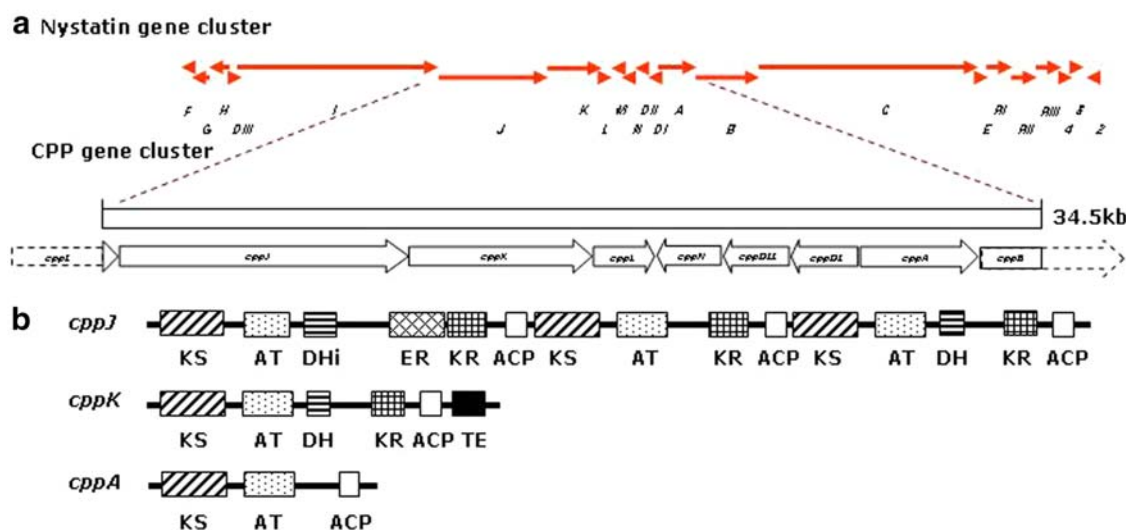

Fig. 2 **a** Genetic organization of the 34.5 kb *P. autotrophica* DNA harboring the cryptic polyene gene cluster and comparison with the *S. noursei* nystatin gene cluster. The approximate position and direction of each gene is indicated by an *open arrow*. *nysF* post-translational PKS modification, *nysG*/*nysH* efflux of nystatin, *nysDIII* mycosamine biosynthesis, *nysI* nystatin PKS (modules 9–14), *nysJ* nystatin PKS (modules 15–17), *nysK* nystatin PKS (module 18 + TE), *nysL* hydroxylation at C-10, *nysM* electron transfer in P450 system, *nysN* oxidation of the methyl group at C-18, *nysDII* mycosamine biosynthesis, *nysDI* attachment of mycosamine, *nysA* nystatin PKS (loading module), *nysB* nystatin PKS (modules 1, 2), *nysC* nystatin PKS (modules 3–8), *nysE* release of the polyketide chain from PKS, *nysRI*/*nysRII*/*nysRIII* regulation of nystatin production, *orf4*/*orf3*/*orf2* putative regulation. **b** The modular domains of *cppJ, cppK*, and *cppA* present in a cryptic polyene gene cluster in *P. autotrophica*. *KS* ketosynthase, *AT* acyltransferase, *ACP* acyl carrier protein, *KR* ketoreductase, *DH* dehydratase, *DHi* inactive dehydratase, *ER* enolyreductase, *TE* thioesterase

cete strain, which exhibits no manifest antifungal activity. Upon sequencing, the PCR-amplified 350 bp DNA fragment from *P. autotrophica* was found to be highly similar to the central region of the other previously known polyene CYP genes (data not shown). In order to clone the cryptic polyene gene cluster which flanks the CYP region, a *P. autotrophica* genomic DNA library was constructed using an *E. coli*–streptomycete shuttle cosmid vector, pOJ446 [10], followed by screening using the PCR-amplified 350 bp DNA as a probe. A positive cosmid clone which contained an insert DNA of approximately 34.5 kb (named pESK601) was isolated and sequenced completely, revealing a total of seven complete and two partial ORFs (Fig. 2). The overall G + C contents of the seven ORFs ranged between 69.4

and 77.7%, and all the ORFs also exhibited a characteristic high G + C content at the third position of the codons, consistent with the structure of most *Streptomyces* ORFs. A DNA database search indicated that these ORFs were components of a unique polyene biosynthetic gene cluster, but were highly similar to the previously characterized nystatin cluster in *S. noursei* (Fig. 2). These ORFs were classified as *cpp* (*C*ryptic *Pseudonocardia P*olyene) genes with a nystatin-like nomenclature, resulting in the designations *cppI*, *cppJ*, *cppK*, *cppL*, *cppN*, *cppDII*, *cppDI*, *cppA*, and *cppB*. The four ORFs including *cppI*, *cppJ*, *cppK*, and *cppA* were determined to be highly similar to the nystatin PKS genes, *nysI*, *nysJ*, *nysK*, and *nysA*, respectively (Table 1). Unlike the situation in the nystatin gene cluster, where the third DH domain in *nysJ* is inactive [5], interestingly, the first DH domain of *cppJ* showed a sequence suggesting that it is inactive. The sequence of *cppA* also suggested that the encoded PKS harbors only three minimal modules, ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). Although *cppL* and *cppN* are believed to be two regiospecific CYP genes that are involved in the post-PKS modification, an ortholog for the *nysM* (ferredoxin)-like ORF present in the nystatin gene cluster was not present in the *cpp* cluster (Fig. 2). *cppDII* and *cppDI* are ORFs believed to encode aminotransferase and glycosyltransferase enzymes, respectively, and are presumably involved in mycosamine biosynthesis. Culture optimization studies as well as structural identification of the putative novel polyene compound produced by *P. autotrophica* are currently in progress. The remaining regions of the cryptic polyene gene cluster in *P. autotrophica* will also be cloned, sequenced, and characterized in future studies. These results suggest that the polyene CYP-specific PCR screening approach may well constitute an efficient method for the isolation of potentially valuable cryptic polyene biosynthetic gene clusters from a variety of microorganisms including rare actinomycete species.

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