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Quinoprotein dehydrogenase functions at the final oxidation step of lankacidin biosynthesis in *Streptomyces rochei* 7434AN4

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Reinvestigation of the metabolite profile in a disruptant of the quinoprotein dehydrogenase (*orf23*) gene revealed that the Orf23 protein catalyzes dehydrogenation of the C23-C25 lactate moiety to pyruvate during lankacidin biosynthesis in *Streptomyces rochei* 7434AN4. The dehydrogenase activity was expressed and detected in a soluble fraction of the *Streptomyces lividans* recombinant harboring *orf23*. The Orf23 protein preferentially converts lankacidinol to lankacidin C in the presence of pyrroloquinoline quinone (PQQ). Other lankacidinol derivatives, lankacidinol A and iso-lankacidinol, were also converted to the corresponding C-24 keto compounds, lankacidin A (=sedecamycin) and iso-lankacidin C. Addition of various divalent metal cations, especially Ca^{2+}, enhanced the dehydrogenase activity, whereas EDTA completely inhibited. These findings confirmed that the quinoprotein dehydrogenase Orf23 functions at the final oxidation step of lankacidin biosynthesis.

[**Key words:** Biosynthesis; Quinoprotein dehydrogenase; Secondary metabolite; Antibiotics; Pyrroloquinoline quinone]

A group of unique 17-membered carbocyclic polyketide antibiotics lankacidins (Fig. 1) are produced by *Streptomyces rochei* 7434AN4 that carries three linear plasmids, pSLA2-L, -M, and -S (1). Lankacidins show various biological activities including antimicrobial action against gram-positive bacteria (2). X-ray crystallographic analysis revealed that lankacidin C (**1**; Fig. 1) and lankamycin (14-membered macrolide antibiotic) inhibit peptide formation synergistically by binding to the neighboring sites in the large bacterial ribosomal subunit (3,4). In addition, lankacidin C enhances stable micro-tubulin assembly and displaces taxoids from their binding sites (5). Nucleotide sequencing and gene inactivation experiments of the largest linear plasmid pSLA2-L revealed that the lankacidin biosynthetic (*lkc*) gene cluster is located on pSLA2-L (210,614 bp) (6). The *lkc* cluster contains a non-ribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) hybrid gene *lkcA*, three multidomain type-I PKS genes (*lkcC*, *lkcF*, *lkcG*), a discrete dehydratase gene *lkcB*, a discrete acyltransferase gene *lkcD*, and an amine oxidase gene *lkcE* (7). Remarkably, the *lkc* cluster carries only five ketosynthase (KS) domains although eight condensation reactions of malonyl CoA are necessary, suggesting a modular-iterative mixed polyketide biosynthesis of lankacidin (8,9). Heterologous expression of the *lkcA-lkcO* genes in *Streptomyces lividans* led to the production of lankacidinol A (**2**), suggesting that a set of *lkc* genes is sufficient to construct the lankacidin skeleton (8). Recently, Dickschat et al. (10) reported that an additional dehydratase activity coded on *lkcC* is required for lankacidin biosynthesis and He et al. (11) speculated the origin of C_3 unit (C23-C25) amide-bound to the lankacidin skeleton.

One of the striking features of the *lkc* gene cluster is the presence of biosynthetic genes (*lkcK-lkcO*) for pyrroloquinoline quinone (PQQ; Fig. S1) at its left end (7). This was the first report of the PQQ cluster in gram-positive bacteria. PQQ, the most characterized quinone cofactor, is known as a third family of redox cofactor in various quinoprotein and quinohemoprotein dehydrogenases (12–15). These quino(hemo)proteins utilize quinone cofactor to catalyze dehydrogenation of primary or secondary hydroxyl groups in sugars and alcohols in the periplasm of gram-negative bacteria. Hence we focused on the PQQ biosynthetic cluster *lkcK-O* (*orf8-4*) and the quinoprotein dehydrogenase (*orf23*) gene coded on pSLA2-L in *S. rochei*. Strain FS7, a disruptant of the *lkcL* (*pqqC*) gene, showed no inhibitory zone around $R_f = 0.5$ ($CHCl_3$-MeOH = 15:1, v/v) on TLC bioautography when compared with the parent strain. Its deficiency was restored by exogenous addition of PQQ (7), indicating its crucial role in lankacidin biosynthesis. Nevertheless, a mutant of the *orf23* gene showed a distinct inhibitory zone around $R_f = 0.5$.

Here we reinvestigated the metabolic profile of the *orf23* disruptant, and describe an extensive biochemical characterization of the quinoprotein dehydrogenase protein involved in lankacidin biosynthesis in *S. rochei* 7434AN4.

MATERIALS AND METHODS

Strains, reagents, and culture conditions All the strains and plasmids used in this study are listed in Table 1. *S. rochei* strain 51252 that carries only pSLA2-L was used as a parent strain (1). Strain KK23, the *orf23* disruptant of strain 51252, was constructed previously (7). YM medium (0.4% yeast extract, 1.0% malt extract, and

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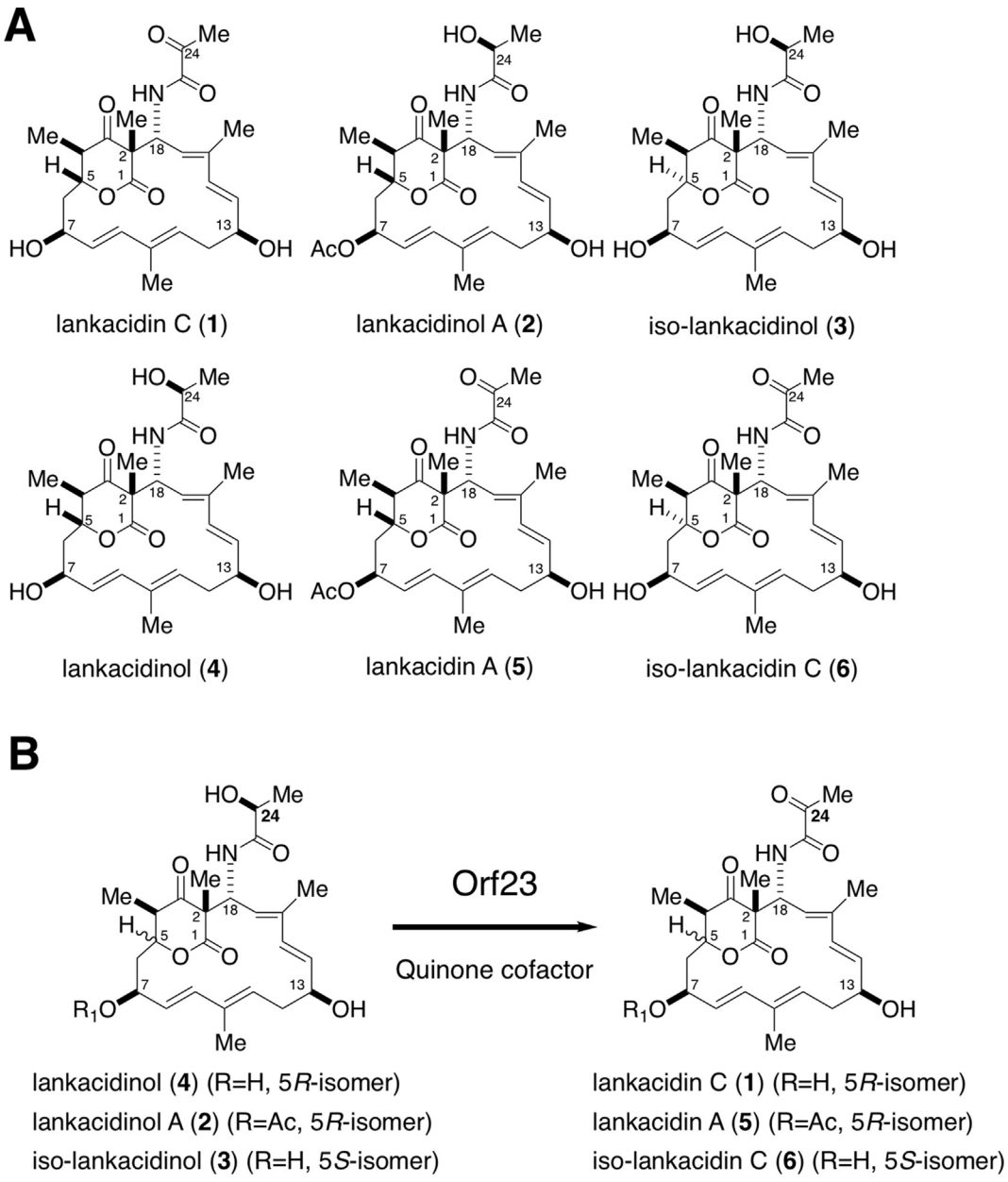

FIG. 1. Structures of lankacidin antibiotics and dehydrogenation step in lankacidin biosynthesis. (A) Structures of lankacidin C (**1**), lankacidinol A (**2**), iso-lankacidinol (**3**), lankacidinol (**4**), lankacidin A (**5**), and iso-lankacidin C (**6**). Ac, acetyl; Me, methyl. (B) Dehydrogenation step catalyzed by Orf23 in lankacidin biosynthesis.

0.4% D-glucose, pH 7.3) was used for antibiotic production. For protoplast preparation and protein expression, *S. lividans* TK64 and its recombinants were cultured in YEME liquid medium (16). Protoplasts were regenerated on R1M solid medium (17). PQQ disodium salt was purchased from Mitsubishi Gas Chemical Company, Inc. (Tokyo, Japan).

Isolation of metabolites Strains were cultivated in YM liquid medium at 28 °C for 2 days. The culture broth was extracted twice with equal volume of EtOAc. The combined organic phase was dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The resulting residue was passed through Sephadex LH-20 (GE Healthcare, Chicago, IL, USA) with methanol. The fractions containing lankacidins were combined, and further purified by silica gel chromatography with two different solvent systems of $CHCl_3$-methanol = 50:1–10:1 (v/v) and toluene-EtOAc = 1:3 (v/v). Spectral data including ^1H and ^{13}C NMR assignments for compounds **1**–**4** have already been reported (7,18) (Table S1).

Analysis of metabolites Metabolites were analyzed by high performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and electrospray ionization-mass spectrometry (ESI-MS). The crude extract was dissolved in acetonitrile, applied on a reverse-phase HPLC column (Cosmosil Cholester 4.6 x 250 mm; Nacalai Tesque, Kyoto, Japan), eluted with acetonitrile/phosphate buffer (10 mM, pH 8.2) (3:7, v/v) at a flow rate of 0.7–1.0 ml/min, and then monitored at 230 nm with a JASCO MD-2010 multi-wavelength photodiode array detector. TLC was developed with $CHCl_3$/MeOH (15:1, v/v) and baked after staining with anisaldehyde-H_2SO_4. High resolution ESI-MS spectra were measured by a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Preparation of the recombinant Orf23 protein A 1.6-kb PCR fragment containing *orf23* (nt 41,354-42,985 of pSLA2-L) was amplified using the template cosmid B10 (7) and two primers, 23fNde and 23rHind (Table 1). The PCR fragment was digested with *Nde*I and *Hin*dIII and cloned into pKAR3063H (18), a constitutive expression vector carrying N-terminal $(His)_6$-tag sequence in pHSA81, to give pYY03. The *S. lividans* TK64 recombinant harboring pYY03 was grown at 28 °C for 72 h in YEME liquid medium (34% sucrose) containing 10 μg/ml of thiostrepton. *Streptomyces* cultures were harvested by centrifugation, and the residual cells were resuspended in 50 mM phosphate buffer (pH 8.0) and disrupted by sonication for three cycles of 20-sec with 0.5-min intervals on ice. The cell-free supernatant was dialyzed when necessary.

***In vitro* conversion of lankacidinol in the Orf23 recombinant** A standard assay mixture (1 ml) contains 250 μM lankacidinol (**4**; Fig. 1), 13 μM PQQ, 0.6 mM $CaCl_2$, and the cell-free supernatant of *S. lividans* TK64/pYY03 (200 μl; prepared from 20-ml culture broth) in 50 mM HEPES buffer (pH 7.5). After incubation at

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