THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 22, pp. 16362–16368, June 1, 2007

Molecular Basis for Chloronium-mediated Meroterpene Cyclization

*CLONING, SEQUENCING, AND HETEROLOGOUS EXPRESSION OF THE NAPYRADIOMYCIN BIOSYNTHETIC GENE CLUSTER**

Received for publication, November 30, 2006, and in revised form, March 23, 2007 Published, JBC Papers in Press, March 28, 2007, DOI 10.1074/jbc.M611046200

Jaclyn M. Winter[‡1], Michelle C. Moffitt[‡], Emmanuel Zazopoulos[§], James B. McAlpine[§], Pieter C. Dorrestein[¶], and Bradley S. Moore[‡¶2]

From the [‡]Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093-0204, [§]Ecopia BioSciences Inc., Quebec, H4S 2A1, Canada, and [¶]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, California 92093

Structural inspection of the bacterial meroterpenoid antibiotics belonging to the napyradiomycin family of chlorinated dihydroquinones suggests that the biosynthetic cyclization of their terpenoid subunits is initiated via a chloronium ion. The vanadium-dependent haloperoxidases that catalyze such reactions are distributed in fungi and marine algae and have yet to be characterized from bacteria. The cloning and sequence analysis of the 43-kb napyradiomycin biosynthetic cluster (*nap*) from *Streptomyces aculeolatus* NRRL 18422 and from the undescribed marine sediment-derived *Streptomyces* sp. CNQ-525 revealed 33 open reading frames, three of which putatively encode vanadium-dependent chloroperoxidases. Heterologous expression of the CNQ-525-based *nap* biosynthetic cluster in *Streptomyces albus* produced at least seven napyradiomycins, including the new analog 2-deschloro-2-hydroxy-A80915C. These data not only revealed the molecular basis behind the biosynthesis of these novel meroterpenoid natural products but also resulted in the first *in vivo* verification of vanadium-dependent haloperoxidases.

Nature has devised several mechanisms to polarize the terminal olefin of linear terpenes to facilitate the creation of new C–X bonds. For instance, cyclization of the C_{30} hydrocarbon squalene to steroids and hopanoids is initiated, respectively, by epoxidation or protonation of the terminal olefin. Although these biosynthetic strategies are widely distributed, a third mechanism for terpene cyclization has been characterized in marine macroalgae involving bromonium ion-induced ring closure (1–3). Oxidation of the halide is catalyzed by vanadium-dependent bromoperoxidase in the presence of hydrogen peroxide to produce the corresponding hypohalous acid. This species then further reacts with electron-rich organic substrates in a regio- and stereoselective manner, giving rise to brominated terpenes and other halogenated natural products (1, 2, 4). Vanadium-dependent bromoperoxidases are widely distributed in marine algae, and the first enzyme was discovered in 1984 from the brown alga *Ascophyllum nodosum* (5).

Vanadium chloroperoxidases (V-ClPOs),[3] on the other hand, have been isolated primarily from dematiaceous hyphomycete fungi (1). The first enzyme was characterized in 1993 from *Curvularia inaequalis* (6), and even though there are numerous chlorinated marine natural products, V-ClPOs have not been reported from marine organisms to date (1, 2, 7). Although the biological function of V-ClPOs has not yet been elucidated, marine algal vanadium-dependent bromoperoxidases have been shown through *in vitro* chemoenzymatic conversions to catalyze bromonium ion-initiated cyclization of terpenes and ethers (1, 8). These studies not only demonstrated that the enzymes were able to initiate cyclization of a terpene by a bromonium ion but also proved that the halogenation reaction occurred with stereochemical control. To date, all known V-dependent haloperoxidases have been characterized *in vitro* from eukaryotic systems (9).

Structural inspection of the bacterial meroterpenoid antibiotics belonging to the napyradiomycin family of chlorinated dihydroquinones suggests that their terpenoid fragments undergo related chloronium ion-induced cyclization biochemistry. Napyradiomycins A1 and A2, B1–B4, C1 and C2 (10, 11), A80915A–D (**1**) and -G (12), SF2415A1–A3 and B1–B3 (13), and related diprenylated naphthoquinone natural products (**2** and **3**) (14) are produced by several actinomycetes (Fig. 1), including *Streptomyces aculeolatus* NRRL 18422 (12, 13) and the marine sediment-derived *Streptomyces* sp. CNQ-525 (14). Stable isotope tracer experiments established that napyradiomycins A1, A2, B1, C1, and C2 (15) as well as other meroterpenoids, such as naphterpin (16), furaquinocin (17), and neomarinone (18), are biosynthesized from the symmetrical pentaketide 1,3,6,8-tetrahydroxynaphthalene (THN) and iso-

* This work was partially supported by National Institutes of Health Grant AI52445 (to B. S. M.), the University of California at San Diego Skaggs School of Pharmacy and Pharmaceutical Sciences, and the Department of Chemistry and Biochemistry (to P. C. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) EF397638 and EF397639.

[1] Supported by a Ruth L. Kirschstein National Research Service Award from the Marine Biomedicine and Biotechnology Training Program at Scripps Institution of Oceanography (GM067550).

[2] To whom correspondence should be addressed: Ctr. for Marine Biotechnology and Biomedicine, Scripps Inst. of Oceanography, UCSD, 8655 Discovery Way, La Jolla, CA 92037-0204. Tel.: 858-822-6650; Fax: 858-822-6652; E-mail: bsmoore@ucsd.edu.

[3] The abbreviations used are: V-ClPO, vanadium chloroperoxidase; THN, 1,3,6,8-tetrahydroxynaphthalene; LC-MS, liquid chromatography-mass spectrometry; FTMS, Fourier transform mass spectrometry.

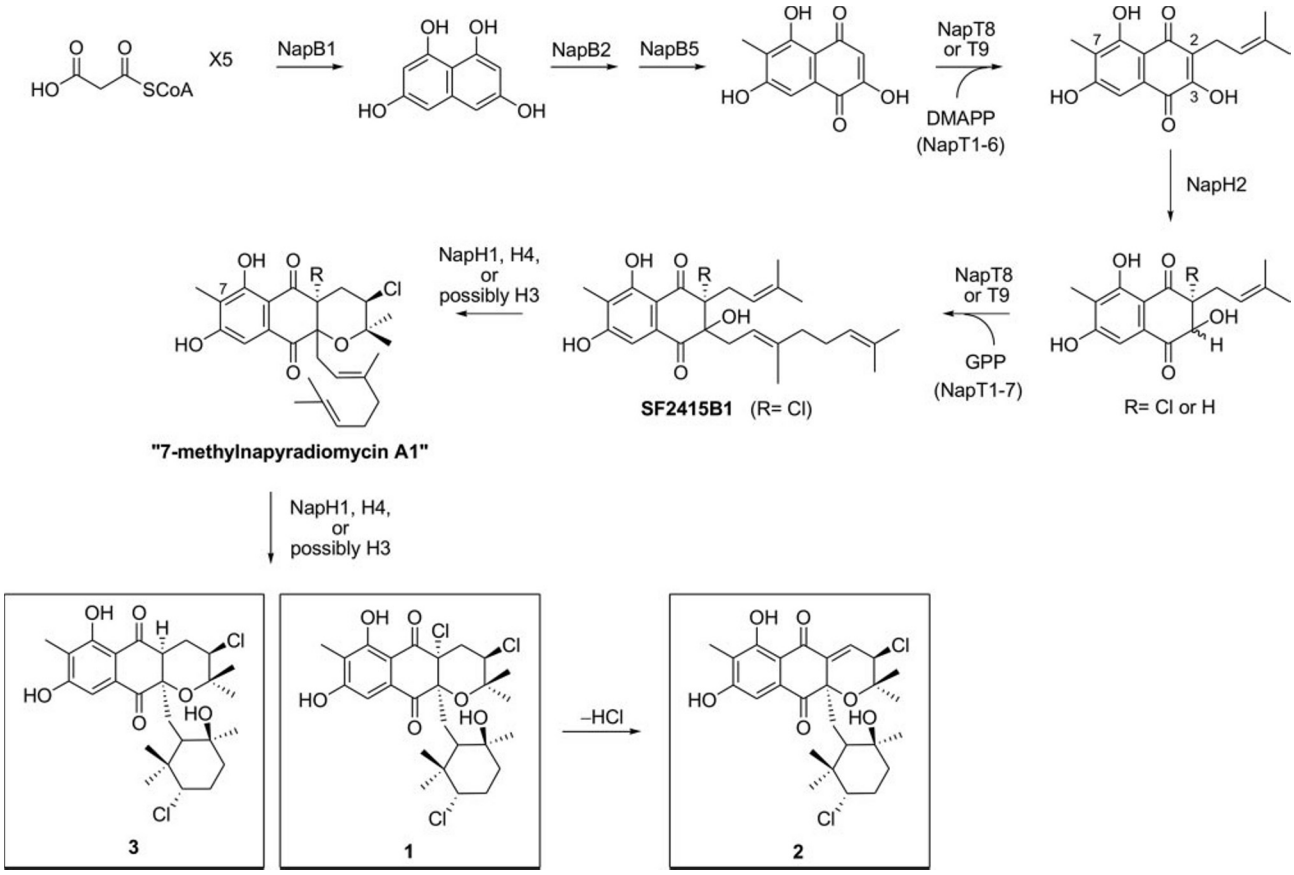

FIGURE 1. **Structures and proposed biosynthetic pathway of the chlorinated dihydroquinones 1–3**. The numbering scheme for the napyradiomycin analogs has been adopted from Ref. 13.

prenoid units. In most cases, the isoprenoid building blocks are derived from the mevalonic acid biosynthetic pathway.

Recent biosynthetic gene cluster analysis of the hybrid monoterpene-polyketides furaquinocin from *Streptomyces* sp. strain KO-3988 and furanonaphthoquinone I from *Streptomyces cinnamonensis* DSM 1042 revealed a clustering of genes coding for the type III polyketide synthase THN synthase, a prenyltransferase, and a complete set of mevalonic acid-specific geranyl diphosphate biosynthetic enzymes (19, 20). Herein we report the cloning and sequence analysis of the napyradiomycin biosynthetic gene cluster (*nap*) from *Streptomcyces* sp. CNQ-525 and *S. aculeolatus* NRRL 18442, its functional expression in the heterologous host *Streptomyces albus*, and the structure elucidation of a new napyradiomycin analog. The bioinformatics analysis of the *nap* cluster, which contains the unprecedented distribution of three V-ClPOs, further revealed the molecular basis for the chlorination and cyclization of the terpene units in the napyradiomycin family of antibiotics.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions—*Streptomyces* sp. CNQ-525 (14) was a gift from Dr. William Fenical (Scripps Institution of Oceanography). For sporulation, it was grown in A1 growth medium for nine days at 30 °C with shaking (250 revolutions/min). pOJ446 (21) was used for cosmid library construction and was grown in LB liquid medium. The expression host *S. albus* was provided by Dr. Joern Piel (University of Bonn). Strain and DNA manipulations as well as southern hybridization experiments were performed according to standard procedures (22, 23).

Cosmid Library Construction and Sequencing—pOJ446 was digested with HpaI, dephosphorylated with calf intestinal phosphatase, and digested with BamHI. *Streptomyces* sp. CNQ-525 genomic DNA was partially digested with Sau3AI. DNA fragments between 35 and 40 kb were isolated by agarose gel electrophoresis and purified for ligation. Before adding the ligase, the mixture containing plasmid and genomic DNA was incubated at 50 °C for 5 min and then placed on ice for 5 min. Ligation was carried out overnight at 16 °C using T4 DNA ligase. Packaging and titration were performed using XL-1 blue MRF cells (Stratagene) and MaxPlax λ packaging extracts (Epicenter) according to the manufacturer's instructions. After selection with apramycin (100 μg/ml), ~2000 colonies were picked and grown overnight in 96-well plates. Cultures were transferred to a Hybond-N membrane (Amersham Biosciences) and grown overnight on LB agar containing apramycin (100 μg/ml). Colonies were lysed and DNA denatured and fixed according to standard procedures (22). The library was screened using *Streptomyces* sp. CNH-099-based type III polyketide synthase and prenyltransferase genes as heterologous probes.[4] Peroxidase labeling and detection of enzyme-la-

[4] M. C. Moffitt and B. S. Moore, unpublished data.

TABLE 1
Deduced function of the open reading frames in Fig. 2

Gene Product	Amino acids (no)	Proposed function	Sequence similarity (protein, origin)	Similarity/identity (%)	Protein accession number	Reference
NapB1	355	THN synthase	SarppA, *S. antibioticus*	92/84	BAD89289	28
NapB2	184	MomA	MomA, *S. antibioticus*	86/74	BAD89290	28
NapB3	386	Aminotransferase	Fur3, *Streptomyces* sp.	85/76	BAE78971	19
NapB4	529	Acyl-CoA synthase	Fur5, *Streptomyces* sp.	85/74	BAE78973	19
NapB5	337	Methyltransferase	Fur4, *Streptomyces* sp.	85/69	BAE78974	19
NapH1	509	V-ClPO	TioM, Micromonospora sp.	33/21	CAJ34369	34
NapH2	425	$FADH_2$ dependent halogenase	CalO3, *M. echinospora*	63/43	AAM70353	35
NapH3	441	V-ClPO	TioM, *Micromonospora* sp.	38/25	CAJ34369	34
NapH4	523	V-ClPO	TioM, *Micromonospora* sp.	37/27	CAJ34369	34
NapT1	389	HMG-CoA synthase	HmgS, *Streptomyces* sp.	86/78	BAB07795	36
NapT2	353	HMG-CoA reductase	HmgR, *Streptomyces* sp.	93/87	BAA70975	37
NapT3	380	Type 2 IPP isomerase	Fni, *Streptomyces* sp.	89/82	Q9KWG2	36
NapT4	412	Phosphomevalonate kinase	Pmk, *Streptomyces* sp.	68/62	BAD86802	38
NapT5	385	Mevalonate decarboxylase	MdpD, *Streptomyces* sp.	83/77	BAD86801	38
NapT6	262	Mevalonate kinase	OrfA, Streptomyces sp.	81/70	BAB07790	36
NapT7	310	Polyprenyl synthase	Fur19, *Streptomyces* sp.	82/71	BAE78987	19
NapT8	297	Prenyltransferase	CloQ, *Streptomyces* sp.	65/46	BAE78975	19
NapT9	287	Prenyltransferase	Orf2, *Streptomyces* sp.	54/35	BAE00106	39
NapR1	268	Regulatory protein	Orf41, *S. globisporus*	52/38	AAL06696	40
NapR2	423	Sodium transporter	Orf1, *S. carzinostaticus*	68/52	BAD38870	41
NapR3	220	Transcription regulator	TcmR, *S. coelicolor*	53/43	CAD30962	42
NapR4	464	Transporter	PcaK, *B. mallei*	52/33	AAU45502	43
NapR5	535	Efflux protein	Sco0375, *S. coelicolor*	80/68	CAD55439	42
NapR6	535	Efflux protein	MonT, *S. avermitilis*	70/52	NP_824465	44
NapR7	219	Transcription regulator	TcmR, *S. coelicolor*	62/45	NP_629129	42
NapR8	383	Regulatory protein	MarR, *R. palustris*	47/37	NP_947139	42
NapR9	401	Efflux transporter	AraJ, *S. avermitilis*	71/56	NP_823779	44
NapU1	225	Unknown	OvmZ, *Streptomyces* sp.	63/46	BAE78986	19
NapU2	255	Unknown	InfB, *N. farcinica*	35/29	YP_120279	45
NapU3	206	Hypothetical protein	Sru1144, *S. ruber*	50/33	YP_445270	46
NapU4	237	Hypothetical protein	Nfa6880, *N. farcinica*	40/27	BAD55533	45
Transposase 1	67	Transposase	Tra8, *C. glutamicum*	46/38	NP_599434	47
Transposase 2	445	Transposase	FdmX, *S. griseus*	92/87	AAQ08938	48

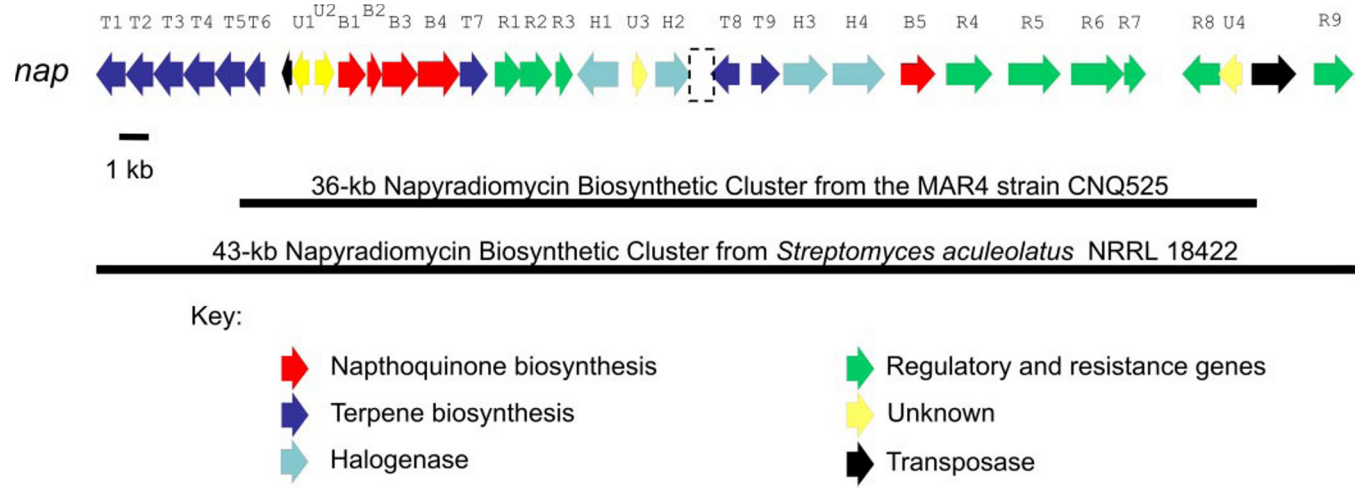

FIGURE 2. **Organization of the napyradiomycin biosynthetic gene cluster (*nap*) in *Streptomyces* sp. CNQ-525 and *Streptomyces aculeolatus* NRRL 18422.** Each *arrow* represents the direction of transcription of an open reading frame. The *dashed box* between *nap*H2 and *nap*T8 represents a ~350-bp unsequenced gap in *Streptomyces* sp. CNQ-525, which correlates to a similarly sized gap in *S. aculeolatus*.

beled positives were performed with the ECL direct nucleic acid labeling and detection systems kit (Amersham Biosciences). Cosmid clone pJW6F11 was sequenced by the shotgun method (Macrogen Inc., Seoul, Korea) and annotated with BLASTP (24) and FRAMEPLOT (25). The genome scanning of *S. aculeolatus* NRRL 18422 was previously reported (26). The GenBank™ accession numbers for the *nap* cluster are EF397639 from *Streptomyces* sp. CNQ-525 and EF397638 from *S. aculeolatus*.

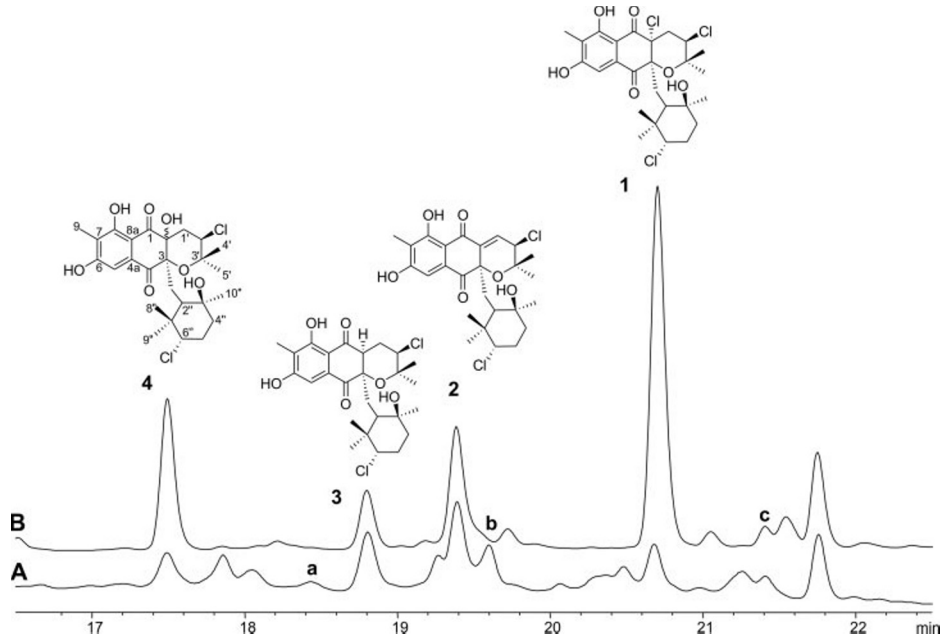

FIGURE 3. LC-MS analysis of the napyradiomycin fraction of *S. albus*/pJW6F11 (*trace A*) and *Streptomyces* sp. CNQ-525 (*trace B*). UV light detection was carried out at 254 nm. Uncharacterized napyradiomycin analogs (***a***, 18.4 min, *m/z* 525; ***b***, 19.6 min, *m/z* 473; and ***c***, 21.4 min, *m/z* 509 (negative ion mode)).

Streptomyces Transformation, Culture Conditions, and MS Analysis of Halogenated Meroterpenoids—pJW6F11 was introduced into *S. albus* protoplasts, apramycin-resistant transformants were selected, and the transformants were cultivated according to standard procedures (23). Several 100-ml cultures of *S. albus*/pJW6F11 were grown in R2YE containing 100 μg/ml apramycin at 30 °C for 9–14 days by rotary shaking (250 revolutions/min). The cells were lysed using 80% MeOH:H_2O, and after extraction with 1:1 MeOH:CH_2Cl_2, the production of *nap*-based compounds was analyzed using a Hewlett Packard 1100 series high performance liquid chromatography system linked to an Agilent ESI-1100 MSD mass spectrometer (gas flow set to 13 ml/min, drying temperature set to 350 °C, and nebulizing pressure set to 40 pounds/square inch). A Luna 4.6 × 150 mm C18 column was used at a flow rate of 0.7 ml/min with a linear solvent gradient of 10–100% acetonitrile in water over a period of 20 min. Fourier transform mass spectral analysis was accomplished with a LTQ-FTMS (ThermoFinnigan). Diluted organic extracts from *S. albus*/pJW6F11 were introduced into the spectrometer via direct infusion at 1–3 μl/min. The sheath gas was set to 4 liters/min, and the capillary inlet was set to 275 °C. The signal was optimized at the 509 *m/z* peak using the autotuning feature in the LTQ portion of the instrument. All FTMS analysis was performed in the negative ion mode, and the data were collected at 200,000 resolution. Extracts from clones harboring pOJ446 without insert did not produce any napyradiomycins. All of the theoretical values were obtained by importing the molecular formulas into the Qual browser software (ThermoFinnigan).

Purification of 2-Deschloro-2-hydroxy-A80915C—Cultures of the wild-type *Streptomyces* sp. CNQ-525 (14) were grown in A1 growth medium for nine days at 30 °C with shaking (250 revolutions/min) and then extracted with EtOAc. Extracts were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude extract (310 mg) from a 1-liter fermentation was subjected to reversed phase C_{18} flash column chromatography (Fisher Scientific, PrepSep C18 1 g/6 ml) with 1:4 MeCN/H_2O, 2:3 MeCN/H_2O, 3:2 MeCN/H_2O, 4:1 MeCN/H_2O, MeCN, and MeOH. Each fraction was analyzed by reversed-phase C_{18} analytical LC-MS as described above, and the fraction eluting with 3:2 MeCN/H_2O was subjected to purification by high performance liquid chromatography using a Waters differential refractometer R401 detector. Compounds were purified on a Luna 250 × 10 mm C8 column employing an isocratic condition of 67% MeCN/H_2O with a flow rate of 2.0 ml/min. 2-Deschloro-2-hydroxyl-A80915C (**4**, 4.3 mg) eluted between 17 and 20 min. NMR spectra were recorded on a Varian Inova 500-MHz spectrometer. ^1H and ^{13}C chemical shifts were referenced to the solvent peak ($CDCl_3$) δ 7.26 and 77.0, respectively. Standard parameters were used for one- and two-dimensional NMR spectra. ^1H NMR δ (multiplicity, assignment, coupling constants (in Hz; HMBCs are in *italic* and nuclear Overhauser effect spectroscopy in **bold**): 0.39 (s, H8″, *C2″*, *C6″*, *C7″*, *C9″*, **H6″**), 0.71 (s, H9″, *C2″*, *C6″*, *C7″*, *C8″*, **H1″$_b$**), 1.25 (s, H10″, *C2″*, *C3″*, *C4″*), 1.29 (s, H4′, *C2′*, *C3′*, *C5′*), 1.34 (m, H1″$_a$, *C2*, *C2″*, *C3″*, *C7″*), 1.50 (d, H4″$_b$, J = 8.8 Hz, *C3″*, *C10″*), 1.51 (d, H2″, J = 8.8 Hz, *C3*, *C1″*, *C3″*, *C4″*, *C7″*, *C8″*, *C9″*, **H6″**), 1.53 (s, H5′, *C2′*, *C3′*, *C4′*, **H2′**), 1.75 (m, H5″$_b$), 1.89 (m, H4″$_a$, *C2″*, *C3″*), 1.90 (m, H5″$_a$, *C3″*, **H6″**), 2.15 (dd, H1′, J = 2.4, 6.9 Hz, *C2*, *C3*, *C2′*, *C3′*, **H2′**), 2.23 (s, H9, *C7*, *C8*, **C8 hydroxyl**), 2.54 (m, H1″$_b$, *C3*, *C4*, *C2″*, *C3″*, **H9″**), 3.55 (dd, H6″, J = 3.5, 12.2 Hz, **H2″**, **H4″$_a$**, **H8″**), 4.16 (br s, C2 hydroxyl), 4.47 (dd, H2′, J = 6.8, 9.5 Hz, *C1′*, *C4′*, *C5′*, **H1′**, **H5′**), 6.33 (br s, C3″ hydroxyl), 7.58 (s, H5, *C4*, *C7*, *C8a*), 9.65 (br s, C6 hydroxyl), 11.66 (s, C8 hydroxyl, *C7*, *C8*, *C8a*, **H9**); ^{13}C NMR ($CDCl_3$) δ: 8.3 (C9), 15.9 (C9″), 22.0 (C4′), 24.4 (C10″), 28.6 (C8″), 29.0 (C5′), 30.2 (C5″). 34.4 (C1″), 40.8 (C4″), 40.8 (C7″), 42.2 (C1′), 58.0 (C2′), 50.9 (C2″), 71.1 (C6″), 71.7 (C3″), 79.2 (C2), 80.8 (C3′), 84.5 (C3), 107.0 (C8a), 108.7 (C5), 119.7 (C7), 132.8 (C4a), 162.4 (C8), 164.2 (C6), 192.5 (C4), 199.0 (C1); LTQ-FTMS m/z = 527.1614 ($C_{26}H_{33}Cl_2O_7$ $[M - H]^+$ 527.1598 calculated).

RESULTS AND DISCUSSION

The napyradiomycin biosynthetic cluster (*nap*) was discovered from each bacterium using distinct methods. In *Streptomyces* sp. CNQ-525, PCR-amplified THN synthase and prenyltransferase gene fragments were used as probes for the identification of a single pOJ446 cosmid clone (pJW6F11) containing a 36-kb genomic insert, which was sequenced by a shotgun approach. Identification of the complete 43-kb *nap* locus was alternatively achieved in *S. aculeolatus* NRRL 18422 by genome scanning (26). When aligned, the two clusters are similarly organized and 97% iden-

tical at the nucleotide level (Fig. 2). The DNA sequence is interrupted by a ~350-bp gap between the convergent genes *nap*H2 and *nap*T8 that proved impervious to our sequencing efforts in CNQ-525. Analysis of the 43-kb *nap* cluster revealed 33 open reading frames, which included five genes putatively involved in the construction of the naphthoquinone polyketide core (*nap*B1–B5), nine genes associated with the biosynthesis (*nap*T1–T7) and attachment (*nap*T8–T9) of the terpenoid units, four halogenases (*nap*H1–H4), nine putative regulatory and resistance proteins (*nap*R1–R9), four open reading frames of unknown function (*nap*U1–U4), and two transposases that suggest this cluster may have been acquired via horizontal gene transfer (Table 1 and Fig. 2). Of the four *nap* halogenases, three show striking similarity to fungal V-ClPOs and a hypothetical protein TioM from *Micromonospora* sp. ML1, which is unprecedented in prokaryotic gene clusters.

To verify that all of the genetic information for the production of chlorinated dihydroquinones, such as A80915C (**1**), 3″-hydroxy-7-methylnapyradiomycin B2 (**2**), and 2-deschloro-A80915C (**3**), is contained on the *nap* gene cluster, the *Streptomyces* sp. CNQ-525 cosmid clone pJW6F11 that contained the truncated 36-kb *nap* cluster was heterologously expressed in *S. albus.* Although the mevalonic acid pathway genes *nap*T1–T4 and part of *nap*T5 are absent in pJW6F11, the host strain was reasoned to provide the isopentenyl pyrophosphate and dimethylallyl pyrophosphate building blocks from primary metabolism. Co-injection with authentic napyradiomycin standards, high performance liquid chromatography-mass spectrometry, and FTMS analysis of the organic extracts from *S. albus*/pJW6F11 unequivocally demonstrated that the transformant yielded the *nap*-based chlorinated dihydroquinones **1**–**3** as well as a series of related analogs that exhibited the characteristic napyradiomycin chromophore (14) with high UV light absorption at 258, 300, and 356 nm (Fig. 3). The observed masses for two of these dichlorinated analogs at *m*/*z* 527 and 509 (negative ion mode) were observed in both the wild type strain CNQ-525 and the *S. albus*/pJW6F11 transformant, whereas a monochlorinated analog (*m*/*z* 473) and a dichlorinated analog (*m*/*z* 525) were only identified in the transformant.

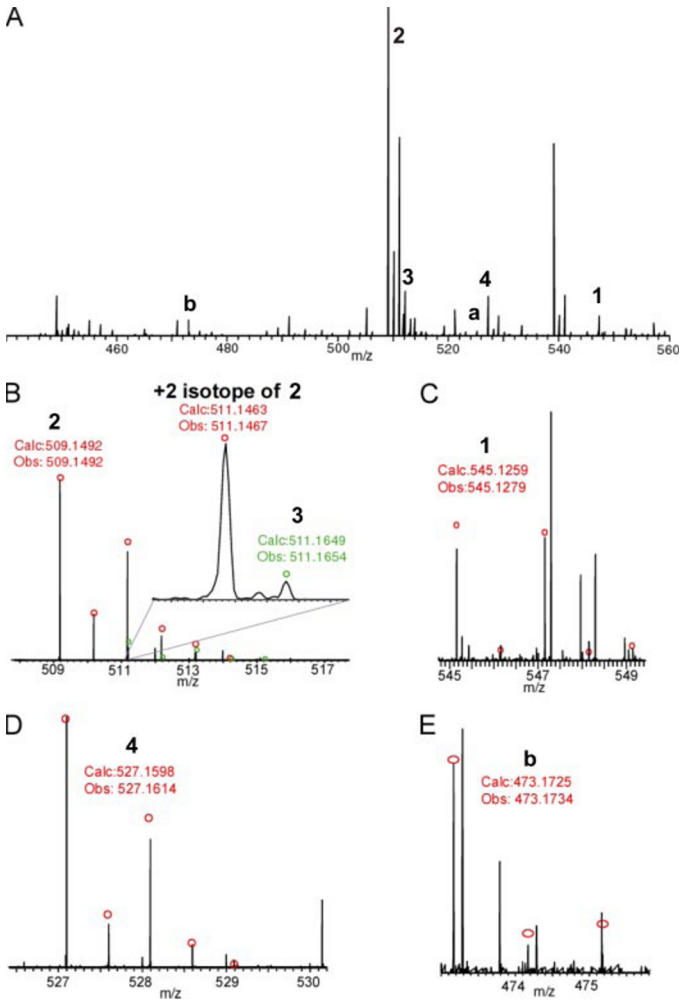

FIGURE 4. *A*, FTMS broadband mass spectrum of the *S. albus*/pJW6F11 organic extract from 440 to 560 *m*/*z* (negative ion mode). *B*, FTMS trace for **2** and **3**. The *red circles* are theoretical isotopic distribution for **2**, whereas the *green circles* correspond to the theoretical isotopic distribution of **3**. *C*, FTMS trace for **1**. The *red circles* indicate the theoretical mass and isotopic distribution. *D*, FTMS signal for the 527 species **4**. The *red circles* indicate the theoretical mass and isotopic distribution. *E*, the observed FTMS signal for the 473 species **b**. The theoretical distribution for the molecular ions associated with $C_{26}H_{30}ClO_6^-$ is indicated with *red circles*.

High resolution FTMS analysis of the organic extracts from the transformant verified exact masses for napyradiomycins **1** (calculated for $[M - H]^+$: *m*/*z* 545.1259, observed: 545.1279), **2** (calculated for $[M - H]^+$: *m*/*z* 509.1429, observed: 509.1429), and **3** (calculated for $[M - H]^+$: *m*/*z* 511.1649, observed: 511.1654) (Fig. 4). FTMS analysis further provided molecular composition data of three new napyradiomycin analogs, namely the dichlorinated 525 species (**a**) (calculated for $C_{26}H_{30}Cl_2O_7^-$: *m*/*z* 525.1447, observed: 525.1423), the dichlorinated 527 species (**4**) (calculated for $C_{26}H_{33}Cl_2O_7^-$: *m*/*z* 527.1598, observed: 527.1614), and the monochlorinated 473 species (**b**) (calculated for $C_{26}H_{30}ClO_6^-$: *m*/*z* 473.1725, observed: 473.1734) (Fig. 4). The unknown dichlorinated 509 species (**c**) observed by high performance liquid chromatography-mass spectrometry analysis (Fig. 3) likely has the same molecular formula as compound **2** ($C_{26}H_{31}Cl_2O_6^-$), so its exact mass could not be distinguished by FTMS.

Fermentation of *Streptomyces* sp. CNQ-525 followed by extraction and chromatography provided the dichlorinated 527 species (**4**) in 4.3 mg/liter. Analysis of the proton and carbon NMR spectra, with the aid of gradient-enhanced heteronuclear multiple bond correlation data, clearly established that **4** contained the chlorocyclohexyl monoterpenoid unit common to **1**–**3**. NMR comparison with structurally related compounds **1** and **3** indicated that they only differed in the substitution at C-2. The presence of a broad singlet at δ 4.16 in the ^1H spectrum for **4** indicated an additional hydroxyl signal not observed in **1** and **3**, which was confirmed by high resolution FTMS. This new C-2 hydroxyl substitution resulted in subtle differences in the NMR spectra of **4** in comparison to that of the chloro analog **1** at C-1′, in which the methylene protons shift from δ 2.62 and 2.45 in **1** to δ 2.15 in **4**. The relative stereochemistry of **4** was assigned by comparing nuclear Overhauser effect spectroscopy correlations to those previously described for **1** (14, 27).

Expression of the *nap* cluster in *S. albus* unequivocally confirmed that all of the genes required for the production of the

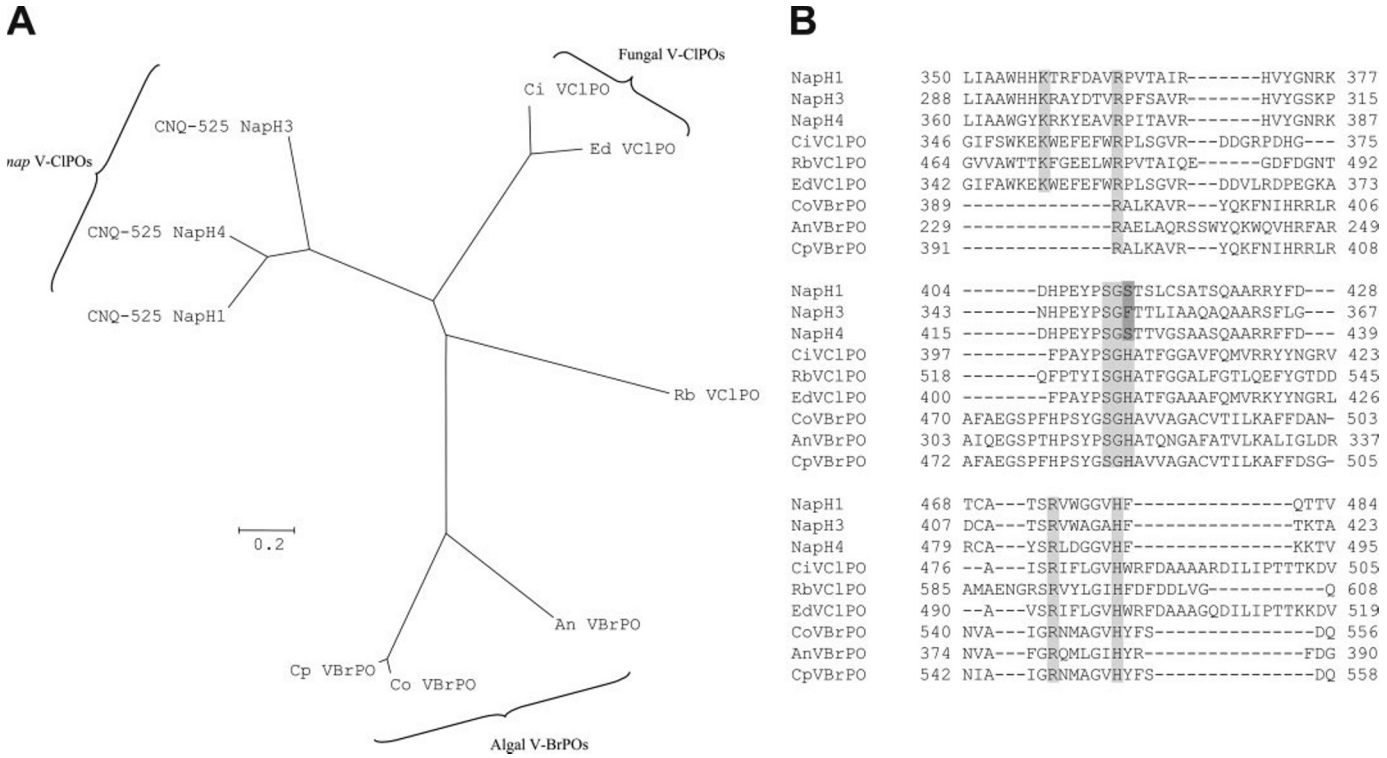

FIGURE 5. *A*, relatedness of NapH1, NapH3, NapH4, and other vanadium haloperoxidases from fungi and algae. Phylogenetic analysis was performed using ClustalW (33), and the unrooted tree was visualized by TreeView. The *scale bar* indicates 0.2 changes/amino acid. *B*, alignment of known vanadium haloperoxidases to NapH1, NapH3, and NapH4 around the conserved active site residues, which are *highlighted* in *light gray*, whereas the differences are *highlighted* in *dark gray*. Sequence identification codes include Ci_VClPO from *C. inaequalis* (GenBank™ accession number CAA59686); Rb_VClPO from *Rhodopirellula baltica* SH1 (CAD72609); Ed_VClPO from *Embellisia didymospora* (CAA72622); An_VBrPO from *A. nodosum* (P81701); Co_VBrPO from *Corallina officinalis* (AAM46061); and Cp_VBrPO from *Corallilna pilulifera* (BAA31261).

7-methylnapyradiomycin family of chlorinated dihydroquinones were contained on the cosmid clone pJW6F11. A biosynthetic scheme for the production of napyradiomycins **1–3** was proposed on the basis of the molecular logic of the *nap* cluster (Fig. 1). As in furaquinocin (19) and furanonaphthoquinone I (20) biosynthesis, assembly of the dihydroquinone core is catalyzed by the type III polyketide synthase homologous THN synthase NapB1, which condenses five malonyl-CoA molecules to THN. The monooxygenase NapB2 putatively oxidizes THN to flaviolin, as is the case in *S. antibioticus* IF013271 (28), which is then methylated by the methyltransferase NapB5. Attachment of the first isoprene unit, using dimethylallyl pyrophosphate as the substrate, occurs through a nucleophilic attack involving one of the two prenyltransferases (NapT8/T9). Hydrogenation or chlorination at C-2 by the $FADH_2$-dependent halogenase NapH2 may facilitate the second prenylation reaction at C-3 with geranyl pyrophosphate by the second *nap* prenyltransferase to yield the diprenylated intermediate SF2415B1 (13). Cyclization of the hemiterpene subunit via a chloronium ion is putatively catalyzed by one of the three *nap* V-ClPOs (NapH1, H3, H4) giving rise to the 7-methyl derivative of napyradiomycin A1. The monoterpene subunit of 7-methylnapyradiomycin A1 putatively undergoes a related V-ClPO-facilitated cyclization to form **1** and **3**, in which a molecule of water is incorporated into the cyclohexanol moiety. Further dechlorination of **1** likely gives rise to **2** (Fig. 1). Although the specifics of the biosynthetic reaction sequence have yet to be confirmed, the successful heterologous expression of the *nap* cluster confirms that all of the genes required for biosynthesis are present.

Phylogenetic analysis of the three *nap* V-ClPOs with characterized fungal V-ClPOs and algal vanadium-dependent bromoperoxidases shows that the three *nap* V-ClPOs form their own clade, with NapH1 and NapH4 being most closely related (Fig. 5*A*). Inspection of the amino acid residues in the active sites of the vanadium haloperoxidases suggests that NapH3 may not be directly involved in the chlorination and cyclization of the terpene units and may instead act as a hydroxylase. Structural and functional comparisons of vanadium haloperoxidases from eukaryotic organisms have shown that six amino acids are required for halogenating activity, whereas two are required for halide selectivity (29–32). All three *nap* enzymes contain His-496 (which covalently binds to the vanadate co-factor) and five of the six residues that participate in hydrogen bonding, Lys-353, Arg-360, Ser-402, Gly-403, and Arg-490. Conserved residue His-404, which is proposed to form a hydrogen bond to the apical oxygen of the co-factor (29), is replaced with Ser (as seen in NapH1 and NapH4) or Phe (as in NapH3). Mutagenesis studies have shown that the V-ClPO mutant H404A from *C. inaequalis* (29) loses chlorinating activity, and a similar natural exchange is observed in NapH3. Hence, NapH3 may not be involved in halogenation but rather may be used to hydrate aliphatic carbons to produce such compounds as 7-methylnapyradiomycin A2 (15). NapH1 and NapH4, on the other hand, contain a hydrophilic Ser residue at this position and are there-

fore predicted to catalyze the chloronium-induced cyclization of the two terpene units. Analysis of the recombinant proteins is presently underway and will help delineate their functions and specificities.

In conclusion, we have demonstrated for the first time the molecular basis for the chlorination and cyclization of terpene units involving novel bacterial V-ClPOs. The isolation of the *nap* biosynthetic cluster from two bacteria provides a powerful toolbox to study these unique halogenating enzymes.

*Acknowledgments—We thank Drs. W. Fenical, P. R. Jensen, and I. Soria-Mercado for the strain Streptomyces sp. CNQ-525, authentic standards **2** and **3**, and helpful discussions.*

REFERENCES

1. Butler, A., and Carter-Franklin, J. N. (2004) *Nat. Prod. Rep.* **21,** 180–188
2. Moore, B. S. (2006) *Nat. Prod. Rep.* **23,** 615–629
3. van Pee, H. K., and Unversucht, S. (2003) *Chemosphere* **52,** 200–312
4. Vaillancourt, F. H., Yeh, E., Vosburg, D. A., Garneau-Tsodikova, S., and Walsh, C. T. (2006) *Chem. Rev.* **106,** 3364–3378
5. Vitler, H. (1984) *Phytochemistry* **23,** 387–390
6. van Schijndel, J. W. P. M., Vollenbroek, E. G. M., and Wever, R. (1993) *Biochim. Biophys. Acta* **1161,** 249–256
7. van Pee, H. K. (1996) *Annu. Rev. Microbiol.* **50,** 275–399
8. Carter, J. N., and Butler, A. (2004) *J. Am. Chem. Soc.* **126,** 15060–15066
9. Hofrichter, M., and Ullrich, R. (2006) *Appl. Microbiol. Biotechnol.* **71,** 276–288
10. Shiomi, K., Nakamura, H., Iinuma, H., Naganawa, H., Isshiki, K., Takeuchi, T., and Umezawa, H. (1986) *J. Antibiot.* (*Tokyo*) **39,** 494–501
11. Shiomi, K., Nakamura, H., Iinuma, H., Naganawa, H., Takeuchi, T., and Umezawa, H. (1987) *J. Antibiot.* (*Tokyo*) **40,** 1213–1219
12. Fukuda, D. S., Mynderse, J. S., Baker, P. J., Berry, D. M., and Boeck, L. D. (1989) *J. Antibiot.* (*Tokyo*) **43,** 623–633
13. Gomi, S., Ohuchi, S., Sasaki, T., Itoh, J., and Sezaki, M. (1987) *J. Antibiot.* (*Tokyo*) **40,** 740–749
14. Soria-Mercado, I. E., Prieto-Davo, A., Jensen, P. R., and Fenical, W. (2005) *J. Nat. Prod.* **68,** 904–910
15. Shiomi, K., Iinuma, H., Naganawa, H., Isshiki, K., Takeuchi, T., and Umezawa, H. (1987) *J. Antibiot.* (*Tokyo*) **40,** 1740–1745
16. Shin-Ya, K., Furihata, K., Hayakawa, Y., and Seto, H. (1990) *Tetrahedron Lett.* **31,** 6025–6026
17. Funayama, S., Ishibashi, M., Komiyama, K., and Ōmura, S. (1990) *J. Org. Chem.* **55,** 1132–1133
18. Kalaitzis, J. A., Hamano, Y., Nilsen, G., and Moore, B. S. (2003) *Org. Lett.* **5,** 4449–4452
19. Kawasaki, T., Hayashi, Y., Kuzuyama, T., Furihata, K., Itoh, N., Seto, H., and Dairi, T. (2006) *J. Bacteriol.* **188,** 1236–1244
20. Haagen, Y., Glueck, K., Fay, K., Kammerer, B., Gust, B., and Heide, L. (2006) *Chembiochem* **7,** 2016–2027
21. Bierman, J., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N., and Schoner, B. E. (1992) *Gene* **116,** 43–49
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich
24. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
25. Ishikawa, J., and Hotta, K. (1999) *FEMS Microbiol. Lett.* **174,** 251–253
26. Banskota, A. H., McAlpine, J. B., Sorensen, D., Aouidate, M., Piraee, M., Alarco, A., Ōmura, S., Shiomi, K., Farnet, C. M., and Zazopoulos, E. (2006) *J. Antibiot.* (*Tokyo*) **59,** 168–176
27. Soria-Mercado, I. E., Jensen, P. R., Fenical, W., Kassel, S., and Golen, J. (2004) *Acta Crystallogr.* **62,** 1627–1629
28. Funa, N., Funabashi, M., Yoshimura, E., and Horinouchi, S. (2005) *J. Biol. Chem.* **280,** 14514–14523
29. Renirie, R., Hemrika, W., and Wever, R. (2000) *J. Biol. Chem.* **275,** 11650–11657
30. Littlechild, J., Garcia-Rodriguez, E., Dalby, A., and Isupov, M. (2002) *J. Mol. Recognit.* **15,** 291–296
31. Barnett, P., Hemrika, W., Dekker, H. L., Muijsers, A. O., Renirie, R., and Wever, R. (1998) *J. Biol. Chem.* **273,** 23381–23387
32. Raugei, S., and Carloni, P. (2006) *J. Phys. Chem. B* **110,** 3747–3758
33. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
34. Lombó, F., Velasco, A., Castro, A., de la Calle, F., Braña, A. F., Sánchez-Puelles, J. M., Méndez, C., and Salas, J. A. (2006) *Chembiochem* **7,** 366–376
35. Ahlert, J., Shepard, E., Lomovskaya, N., Zazopoulos, E., Staffa, A., Bachmann, B. O., Huang, K., Fonstein, L., Czisny, A., Whitwam, R. E., Farnet, C. M., and Thorson, J. S. (2002) *Science* **297,** 1173–1176
36. Takagi, M., Kuzuyama, T., Takahashi, S., and Seto, H. (2000) *J. Bacteriol.* **182,** 4153–4157
37. Takahashi, S., Kuzuyama, T., and Seto, H. (1999) *J. Bacteriol.* **181,** 1256–1263
38. Kawasaki, T., Kuzuyama, T., Kuwamori, Y., Matsuura, N., Itoh, N., Furihata, K., Seto, H., and Dairi, T. (2004) *J. Antibiot.* (*Tokyo*) **57,** 739–747
39. Kuzuyama, T., Noel, J. P., and Richard, S. B. (2005) *Nature* **435,** 983–987
40. Liu, W., Christenson, S. D., Standage, S., and Shen, B. (2002) *Science* **297,** 1170–1173
41. Otsuka, M., Ichinose, K., Fujii, I., and Ebizuka, Y. (2004) *Antimicrob. Agents Chemother.* **48,** 3468–3476
42. Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., Harris, D. E., Quail, M. A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C. W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C. H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, R., Rajandream, M. A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill, J., and Hopwood, D. A.. (2002) *Nature* **417,** 141–147
43. Nierman, W. C., DeShazer, D., Kim, H. S., Tettelin, H., Nelson, K. E., Feldblyum, T., Ulrich, R. L., Ronning, C. M., Brinkac, L. M., Daugherty, S. C., Davidsen, T. J., Deboy, R. T., Dimitrov, G., Dodson, R. J., Durkin, A. S., Gwinn, M. L., Haft, D. H., Khouri, H., Kolonay, J. F., Madupu, R., Mohammoud, Y., Nelson, W. C., Radune, D., Romero, C. M., Sarria, S., Selengut, J., Shamblin, C., Sullivan, S. A., White, O., Yu, Y., Zafar, N., Zhou, L., and Fraser, C. M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 14246–14251
44. Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M., and Omura, S. (2003) *Nat. Biotechnol.* **21,** 526–531
45. Ishikawa, J., Yamashita, A., Mikami, Y., Hoshino, Y., Kurita, H., Hotta, K., Shiba, T., and Hattori, M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 14925–14930
46. Mongodin, E. F., Nelson, K. E., Daugherty, S., Deboy, R. T., Wister, J., Khouri, H., Weidman, J., Walsh, D. A., Papke, R. T., Sanchez-Perez, G., Sharma, A. K., Nesbo, C. L., Macleod, D., Bapteste, E., Doolittle, W. F., Charlebois, R. L., Legault, B., and Rodriguez-Valera, F. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 18147–18152
47. Ideda, M., and Nakagawa, S. (2003) *Appl. Microbiol. Biotechnol.* **62,** 99–109
48. Wendt-Pienkowski, E., Huang, Y., Zhang, J., Li, B., Jiang, H., Kwon, H., Hutchinson, C. R., and Shen, B. (2005) *J. Am. Chem. Soc.* **127,** 16442–16452