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Characterization of a Gene Cluster Responsible for the Biosynthesis of Anticancer Agent FK228 in *Chromobacterium violaceum* No. 968[▽]

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A gene cluster responsible for the biosynthesis of anticancer agent FK228 has been identified, cloned, and partially characterized in *Chromobacterium violaceum* no. 968. First, a genome-scanning approach was applied to identify three distinctive *C. violaceum* no. 968 genomic DNA clones that code for portions of nonribosomal peptide synthetase and polyketide synthase. Next, a gene replacement system developed originally for *Pseudomonas aeruginosa* was adapted to inactivate the genomic DNA-associated candidate natural product biosynthetic genes in vivo with high efficiency. Inactivation of a nonribosomal peptide synthetase-encoding gene completely abolished FK228 production in mutant strains. Subsequently, the entire FK228 biosynthetic gene cluster was cloned and sequenced. This gene cluster is predicted to encompass a 36.4-kb DNA region that includes 14 genes. The products of nine biosynthetic genes are proposed to constitute an unusual hybrid nonribosomal peptide synthetase-polyketide synthase-nonribosomal peptide synthetase assembly line including accessory activities for the biosynthesis of FK228. In particular, a putative flavin adenine dinucleotide-dependent pyridine nucleotide-disulfide oxidoreductase is proposed to catalyze disulfide bond formation between two sulfhydryl groups of cysteine residues as the final step in FK228 biosynthesis. Acquisition of the FK228 biosynthetic gene cluster and acclimation of an efficient genetic system should enable genetic engineering of the FK228 biosynthetic pathway in *C. violaceum* no. 968 for the generation of structural analogs as anticancer drug candidates.

FK228 ($C_{24}H_{36}N_4O_6S_2$; molecular weight, 540.2) (Fig. 1), also known as FR901228 or depsipeptide and registered as NSC 630176 or romidepsin, is a natural product discovered in the fermentation broth of *Chromobacterium violaceum* no. 968 in a screening program for agents that reverse the malignant phenotype of a Ha-*ras* oncogene-transformed NIH 3T3 cell line (51, 52). It exhibited outstanding anticancer activities against an array of tumor cell lines, including many members of a standard panel of 60 cell lines from the U.S. National Cancer Institute (18, 53). FK228 has entered extensive clinical trials and has shown promising properties as a new type of anticancer drug (5, 30, 35, 36, 41). A multinational pivotal trial of FK228 for the treatment of cutaneous T-cell lymphoma has been launched by Gloucester Pharmaceuticals, Inc., and the company plans to file for U.S. Food and Drug Administration approval in late 2007.

Structurally, FK228 is a bicyclic depsipeptide that features a 16-membered macrolactone ring containing an ester linkage and a 17-membered ring containing the same ester linkage and a disulfide bond, the latter of which endows FK228 with an unprecedented molecular scaffold (Fig. 1). Its structure was determined by spectroscopic and X-ray crystallographic analyses (45) and was confirmed by total synthesis (27). A close examination of the FK228 structure identified building blocks of three amino acids (D-cysteine, D-valine, and L-valine), an amino acid derivative (2,3-dehydro-2-aminobutanoic acid; also called 2,3-dehydrothreonine), and a complex L-(*S*,*E*)-3-hydroxy-7-mercaptohept-4-enoic acid moiety that is likely built from one Cys and two C_2 units derived from malonyl coenzyme A (MCoA). These observations suggest a hybrid nonribosomal peptide (NRP)-polyketide (PK)-NRP nature for FK228.

Mechanistically, FK228 was originally discovered as an anti-*ras* agent (51, 52); later, it was found to interfere with mitogen-induced signaling pathways (38, 42, 43), and more recently it has been identified as a potent histone deacetylase (HDAC) inhibitor (17, 33). Histone acetylation catalyzed by histone acetyltransferases is an important component of chromatin remodeling and gene expression regulation; histone hypoacetylation mediated by HDACs is often associated with the onset and progression of cancer (24, 57). HDAC inhibitors are a diverse group of molecules that can induce growth arrest, differentiation, apoptosis, and autophagocytic cell death of cancer cells (10, 24, 31, 57). Interestingly, FK228 has an intramolecular disulfide bond, which makes it structurally distinct from other known HDAC inhibitors, such as hydroxamic acids, apicidin, and trapoxin. This disulfide bond has been postulated to mediate a novel mechanism of cytotoxic action of FK228 (Fig. 1). Furumai and coworkers showed that FK228 serves as a stable prodrug and is activated by intracellular reduction of the disulfide bond after uptake into cells or organisms. The freed sulfhydryl group on the longer aliphatic tail of reduced FK228 fits inside the catalytic pocket of preferred class I HDACs, chelating Zn^{2+}, and thus inhibits the enzyme activities (17). Xiao and coworkers also independently detected more active metabolites in rat plasma and human plasma following their incubation with FK228 in the presence of glutathione (56). The 50% inhibitory concentration of FK228 was found to be nanomolar for inducing apoptosis in cells from patients with chronic

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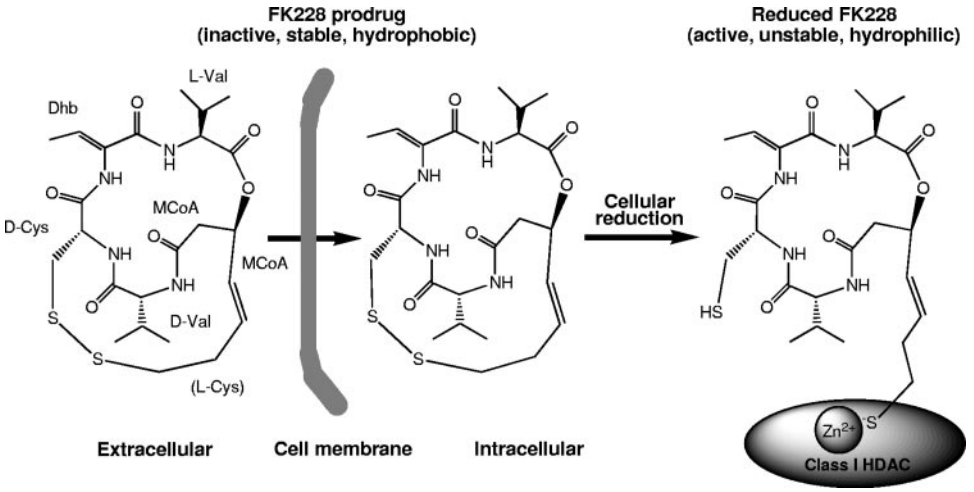

FIG. 1. FK228 structure and mode of action (modified from reference 17 with permission of the publisher). Hydrophobic FK228 can diffuse across the cell membrane. Inside cells, FK228 is activated by cellular reduction, and a freed sulfhydryl group chelates Zn^{2+} inside the catalytic pocket of the preferred class I HDACs and therefore inhibits the enzyme activities.

lymphocytic leukemia (6). Research on FK228 has been expanding rapidly in recent years.

HDAC inhibitors are prime agents for the development of novel anticancer drugs (1, 10, 18, 24, 31, 35, 57). One HDAC inhibitor, Zolinza (vorinostat or suberoylanilide hydroxamic acid), was approved by the U.S. Food and Drug Administration in October 2006, and at least nine other HDAC inhibitors, including FK228, are in various stages of clinical trials as monotherapies or in combinations with other agents (18). Due to its outstanding anticancer activities and novel structural characteristics, as well as certain levels of undesirable cardiac toxicity, FK228 may serve as an excellent molecular scaffold for the generation of structural analogs, from which compounds with improved anticancer properties may be identified. However, chemical synthesis of FK228 has been difficult (27), and derivatization of FK228 by chemical synthesis has not been reported.

We set out to take an alternative approach to making FK228 analogs by means of pathway engineering, combinatorial biosynthesis, or chemoenzymatic synthesis. As the first essential step towards this goal, we identified, cloned, and partially characterized a biosynthetic gene cluster (designated *dep* for depsipeptide) responsible for FK228 biosynthesis. Here we report the identification of candidate biosynthetic genes by a genome-scanning approach, the adaptation of a *Pseudomonas aeruginosa* gene replacement system to create targeted gene-inactivated mutant strains, and the subsequent cloning and characterization of an unusual hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS)-NRPS pathway for FK228 biosynthesis in *C. violaceum* no. 968. Acquisition of the *dep* gene cluster and acclimation of an efficient genetic system should provide a solid foundation for the generation of FK228 analogs by engineered biosynthesis strategies (for recent comprehensive reviews, see references 20 and 54).

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The FK228-producing strain, *C. violaceum* no. 968, was cultured in nutrient broth (1% Difco nutrient broth and 1% glucose) at 30°C for genomic DNA preparation and in fermentation medium (nutrient broth supplemented with 5% Diaion HP-20 resin [Supelco, Pennsylvania]) at 30°C for FK228 production. The vectors pEX18Tc and pPS858, originally developed for *P. aeruginosa* genetics (23), were adopted and applied successfully in *C. violaceum*.

DNA manipulations, genome library construction, and DNA sequencing. General DNA manipulations, including plasmid preparation, restriction enzyme digestion, agarose gel electrophoresis, subcloning, and bacterial transformation, were done according to standard protocols (40) or the manufacturer's instructions (New England BioLabs; QIAGEN). Genomic DNA of a *C. violaceum* wild-type or mutant strain was prepared from an overnight culture with a Genomic-tip 500/G kit (QIAGEN) or with an UltraClean microbial DNA isolation kit (MO BIO Labs). For construction of a genome sampling library (58), high-molecular-weight *C. violaceum* genomic DNA was mechanically sheared with a nebulization device (Invitrogen). DNA molecules that were 2 to 4 kb long were recovered from an agarose gel and end repaired with T4 DNA polymerase and Klenow enzyme in the presence of deoxynucleoside triphosphates (1 mM each). The ends of resultant DNA molecules were adenylated using *Taq* DNA polymerase with dATP, ligated to the pGEM-T Easy vector, and transformed into *Escherichia coli* DH5α cells. Four 96-well plates of clones were subjected to template DNA preparation by PCR amplification and purification with a PerfectPrep PCR Cleanup 96 kit (Eppendorf), and end sequencing with BigDye chemistry and SP6 as the primer was performed with an ABI 3730 automated DNA sequencer (Applied Biosystems) at the University of Wisconsin-Madison Biotechnology Center. DNA oligonucleotides were synthesized by Operon Biotechnologies, Inc., and DNA sequencing by primer walking was performed by standard procedures (40). A cosmid library was constructed in the SuperCos 1 vector using previously described procedures (8). Southern blotting, labeling of DNA as a probe, hybridization, and detection were performed according to the manufacturer's protocols (Roche). Shotgun sequencing of cosmid 18 and contig assembly were performed by a service company (ACGT Inc.). Local sequence analysis was performed with the Lasergene program package (DNASTAR, Inc.) and by a homology search against the GenBank database using the BLAST algorithms (2). The domain organization of biosynthetic enzymes was analyzed as described by Ansari et al. (3), with manual intervention.

General strategy for the construction of targeted gene-inactivated mutants of *C. violaceum* no. 968. To mutate a candidate gene by a gene replacement strategy, an internal part of the DNA of a genomic DNA clone (ampicillin resistant) was replaced by a 1.8-kb *FRT* cassette (gentamicin resistant) from pPS858 to make an intermediate construct (Ap^r Gm^r). The *FRT* cassette, along with two flanking genomic DNAs for homologous DNA recombination, was excised and subcloned into pEX18Tc to make a final conjugation construct (Gm^r and tetracycline resistant). The conjugation construct was introduced into *E. coli* S17-1 cells and subsequently transferred into *C. violaceum* cells by conjugation as follows. Two bacterial strains were grown in LB media

TABLE 1. Bacterial strains and plasmids used in this study

Strain(s) or plasmid(s)	Description	Source or reference(s)
Chromobacterium violaceum strains		
No. 968 (= FERM BP-1968)	Wild type, FK228 producing, Ap^r $Thio^r$[a]	IPOD[b]
Cv56a/b/c	Serial mutants with an internal part of pP3-B6 DNA replaced by the *FRT* cassette (Gm^r GFP^+) from pPS858, FK228 producing	This study
Cv57a/b/c	Serial mutants with an internal part of pP4-B4 DNA (on *depD* gene) replaced by the *FRT* cassette (Gm^r GFP^+) from pPS858, non-FK228 producing	This study
Cv58a/b/c	Serial mutants with an internal part of pP4-G7 DNA (on *depD* gene) replaced by the *FRT* cassette (Gm^r GFP^+) from pPS858, non-FK228 producing	This study
Escherichia coli strains		
DH5α	General cloning host	40
XL1-Blue MR	Host strain for cosmid library construction	Stratagene
S17-1	Host strain for interspecies conjugation	46
ET12567(pUZ8002)	Alternative host strain (methylation deficient) for conjugation	25, 28
MT670(pRK600)	Alternative host strain for conjugation	14
Plasmids		
pGEM-3Zf	Ap^r, general cloning vector	Promega
pGEM-T Easy	Ap^r, general cloning vector	Promega
pP3-A6	2.8-kb random genomic DNA of *C. violaceum* cloned into pGEM-T Easy, sequenced	This study
pP4-B4	3.6-kb random genomic DNA of *C. violaceum* cloned into pGEM-T Easy, sequenced	This study
pP4-G7	2.6-kb random genomic DNA of *C. violaceum* cloned into pGEM-T Easy, sequenced	This study
pPS858	Ap^r Gm^r GFP^+, source of the *FRT* cassette	23
pYC03-56a	Ap^r Gm^r GFP^+, replacement of an internal 1.8-kp EcoRV fragment on pP3-A6 by a 1.8-kb SmaI fragment (containing the *FRT* cassette) from pPS858	This study
pYC03-57a	Ap^r Gm^r GFP^+, replacement of an internal 1.1-kb BglII/NruI fragment on pP4-B4 (blunt ended) by a 1.8-kb SmaI fragment (containing the *FRT* cassette) from pPS858	This study
pYC03-58a	Ap^r Gm^r GFP^+, replacement of two adjacent internal NruI fragments (456 and 489 bp) on pP4-G7 (blunt ended) by a 1.8-kb SmaI fragment (containing the *FRT* cassette) from pPS858	This study
pEX18Tc	Tc^r $oriT^+$ $sacB^+$, gene replacement vector, conjugative	23
pYC03-56b	Conjugative construct with a 2.8-kb NotI fragment (blunt ended, containing the *FRT* cassette and flanking DNAs) from pYC03-56a ligated into the SmaI site of pEX18Tc	This study
pYC03–57b	Conjugative construct with a 4.3-kb NotI fragment (blunt ended, containing the *FRT* cassette and flanking DNAs) from pYC03-57a ligated into the SmaI site of pEX18Tc	This study
pYC03-58b	Conjugative construct with a 3.7-kb PstI/SphI fragment (blunt ended, containing the *FRT* cassette and flanking DNAs) from pYC03-58a ligated into the SmaI site of pEX18Tc	This study
SuperCos 1	Ap^r Kan^r, cosmid vector	Stratagene
Cosmid 18	Cosmid clone containing the FK228 biosynthetic gene cluster (*dep*) and flanking DNAs, shotgun sequenced	This study
Cosmid 2	Cosmid clone containing a partial *dep* gene cluster	This study
pCos2S1 to pCos2S5	BamHI fragments (4.0, 0.8, 6.2, 4.5, and 7.7 kb, respectively) of cosmid 2 inserted into the same site of pGEM-3Zf, sequenced by the primer walking method	This study

[a] $Thio^r$, thiostrepton resistance.
[b] IPOD, International Patent Organism Depositary, Tsukuba, Japan.

supplemented with appropriate antibiotics (10 μg/ml gentamicin and 10 μg/ml tetracycline for *E. coli* S17-1 [a conjugation construct] and 200 μg/ml ampicillin for *C. violaceum*, which is naturally resistant to ampicillin) at 37 or 30°C with shaking until the late mid-log phase (6 to 8 h). Cells from 1 ml of each culture were collected by centrifugation at 4,000 × *g* for 15 min at 4°C, and the cell pellets were washed once with 1 ml LB medium. Cells were collected again by centrifugation and resuspended in 100 μl LB medium. Cell suspensions of two bacterial strains were pooled and spread evenly on a wet 0.45-μm nitrocellulose membrane (Whatman) on LB agar. After the plate had been incubated at 30°C for 12 to 16 h, the membrane seeded with bacteria was used to print several LB agar plates containing 200 μg/ml ampicillin, 50 μg/ml gentamicin, and 5% sucrose to select for exconjugants.

FK228 production and detection by LC-MS. Wild-type and mutant strains of *C. violaceum* were grown in 25 ml of fermentation medium at 30°C for 3 days with constant agitation (200 rpm). Cells and resins were then collected together by centrifugation at 4,000 × *g* for 20 min at the ambient temperature and lyophilized to dryness. A crude FK228 preparation was obtained by eluting the dried cell debris and resins with 10 ml ethyl acetate. Twenty microliters of this preparation was injected into an Agilent 1100 series LC/MSD Trap mass spectrometer (MS) (Agilent) for detection of the positive ion signals of FK228. The liquid chromatography (LC) program included a linear gradient from buffer A (20% methanol with 0.1% formic acid) to buffer B (80% methanol with 0.1% formic acid) in 15 min and constant elution in buffer B for 5 min, followed by a linear return to buffer A in 5 min. Samples were fractionated by using a Zorbax Eclipse XDB-C_{18} column (2.1 by 110 mm; Agilent) with a flow rate of 0.25 ml/min.

Nucleotide sequence accession numbers. The nucleotide sequences of the inserts in pP3-B6, pP4-B4, pP4-G7, and cosmid 18 have been deposited in the GenBank database under accession numbers EF015612, EF015613, EF015614, and EF210776, respectively.

RESULTS AND DISCUSSION

Identification of candidate natural product biosynthetic genes in *C. violaceum* no. 968. The hybrid NRP-PK-NRP nature of FK228 (Fig. 1) suggests that FK228 is likely biosynthesized by a hybrid NRPS-PKS-NRPS assembly line, probably

TABLE 2. Properties of three sequence tags and their associated candidate (partial) genes

Sequence tag	Recombinant plasmid	Insert size (bp)	Associated gene(s)[a]	Protein homolog(s) (accession no.)	Domain organization[b]	Protein classification	Signature motif(s) or substrate specificity	Predicted substrate specificity	Necessary for FK228 biosynthesis
P3-A6-SP6	pP3-A6	2,826	NN[c]	JamL (AAS98783)	KS-AT^{i}	Type I PKS	QTRTAQ, GHSYG, and AAFH in AT domain	MCoA	No
P4-B4-SP6	pP4-B4	3,612	*depD*	BmyB (CAE11249)	C^{i}-A-PCP-E	Type A NRPS	DLFEMSLIWK in A domain	L-Cys	Yes
P4-G7-SP6	pP4-G7	2,599	*depC*, *depD*	AmphI (AAK73501), NosC (AAF17280)	KR^{i}-ACP, C-A^{i}	PKS, NRPS	NA[d]	NA	Yes

[a] See Fig. 2.
[b] A superscript i indicates incomplete. KS, β-ketoacyl synthase; E, epimerase.
[c] NN, not named.
[d] NA, not available.

with an additional enzymatic activity for the formation of an intramolecular disulfide bond. The biosynthesis of NRPs, PKs, and hybrid NRP-PK or PK-NRP natural products via successive condensation of simple building blocks, such as amino acids, amino acid derivatives, and short carboxylic acids, catalyzed by NRPSs, PKSs, and hybrid NRPS-PKS or PKS-NRPS systems, respectively, has been well studied (for recent comprehensive reviews, see references 15, 16, and 22). For ester bond formation in depsipeptide natural products, the involvement of a discrete D-hydroxyisovalerate dehydrogenase in enniatin biosynthesis by *Fusarium sambucinum* (26) or a novel NRPS module containing an adenylation (A) domain to activate an α-keto acid and an embedded α-ketoreductase (KR) to reduce the tethered substrate to an α-hydroxyacyl intermediate (and presumably a downstream condensation [C] domain acting as a chiral ester synthase rather than an amide synthase) in

TABLE 3. Deduced functions of open reading frames and genes in the *dep* gene cluster and flanking regions

Open reading frame or gene	Protein size (amino acids)	Protein homolog	Accession no.	% Identity/ % similarity	Origin	Proposed function[a]
orf1[b]	150[c]	CV_3386	AAQ61050	87/93	*C. violaceum* ATCC 12472	16S rRNA pseudouridine synthase
orf2	163	CV_3385	AAQ61049	66/76	*C. violaceum* ATCC 12472	MutT/nudix family phosphohydrolase
orf3	190	CV_3384	AAQ61048	88/94	*C. violaceum* ATCC 12472	Transcription elongation factor GreB
depK	85	CCO_1235	EAL57087	36/52	*Campylobacter coli* RM2228	Conserved hypothetical protein, function unknown
depL	155	CV_3383	AAQ61047	68/78	*C. violaceum* ATCC 12472	Helix-turn-helix transcriptional regulator, MarR family
depM	389	PFL_4362	AAY93617	59/73	*Pseudomonas fluorescens* Pf-5	Aminotransferase, class I and II family protein
depN	65					PCP^{n}
depA	1,697	SafB	AAC44128	31/45	*Myxococcus xanthus* strain Mx ×48	NRPS: AL^{i}-C^{n}-A$_{Cys}$-PCP
depB	1,553	CurG	AAT70102	45/61	*Lyngbya majuscula*	PKS: KS-AT^{n}-DH^{n}-KR^{i}-ACP
depC	1,183	CrpB	ABM21570	44/64	*Nostoc* sp. strain ATCC 53789	PKS: KS-DH^{n}-KR^{i}-ACP
depD	3,057	PvdI	AAX16361	36/51	*Pseudomonas aeruginosa*	NRPS: C-A$_{Val}$-PCP-E-C-A$_{Cys}$-PCP-E
depE	1,892	McyB	BAA83993	35/52	*Microcystis aeruginosa*	NRPS: C-A$_{Dhb}$-PCP-C-PCP-TE
depF	390	PP_2437	AAN68049	38/56	*Pseudomonas putida* KT2440	FadE2-like acyl coenzyme A dehydrogenase
depG	321	PSPTO_2724	AAO56225	32/53	*Pseudomonas syringae* pv. tomato DC3000	Phosphotransferase
depH	319	PA4170	AAG07557	56/70	*Pseudomonas aeruginosa* PAO1	Flavin adenine dinucleotide-dependent pyridine nucleotide-disulfide oxidoreductase
depI	304	RRSL_03772	EAP73858		*Ralstonia solanacearum* UW551	Putative esterase/lipase
depJ	254	LnmN	AAN85527	43/58	*Streptomyces atroolivaceus* S-140	Type II thioesterase
orf18	312	CV_3378	AAQ60142	87/93	*C. violaceum* ATCC 12472	Hydrogen peroxide-inducible gene activator OxyR
orf19	85	CV_3377	AAQ60141	92/98	*C. violaceum* ATCC 12472	Cell division topological specificity factor MinE
orf20	270	CV_3376	AAQ61040	92/98	*C. violaceum* ATCC 12472	Septum site-determining protein MinD
orf21[b]	107[c]	CV_3375	AAQ61039	93/97	*C. violaceum* ATCC 12472	Septum formation inhibitor MinC

[a] Subscripts indicate the substrate specificities of enzymes. Superscripts indicate inactive (i) or nonfunctional (n). Dhb, 2,3-dehydro-2-aminobutanoic acid.
[b] Incomplete.
[c] Truncated.

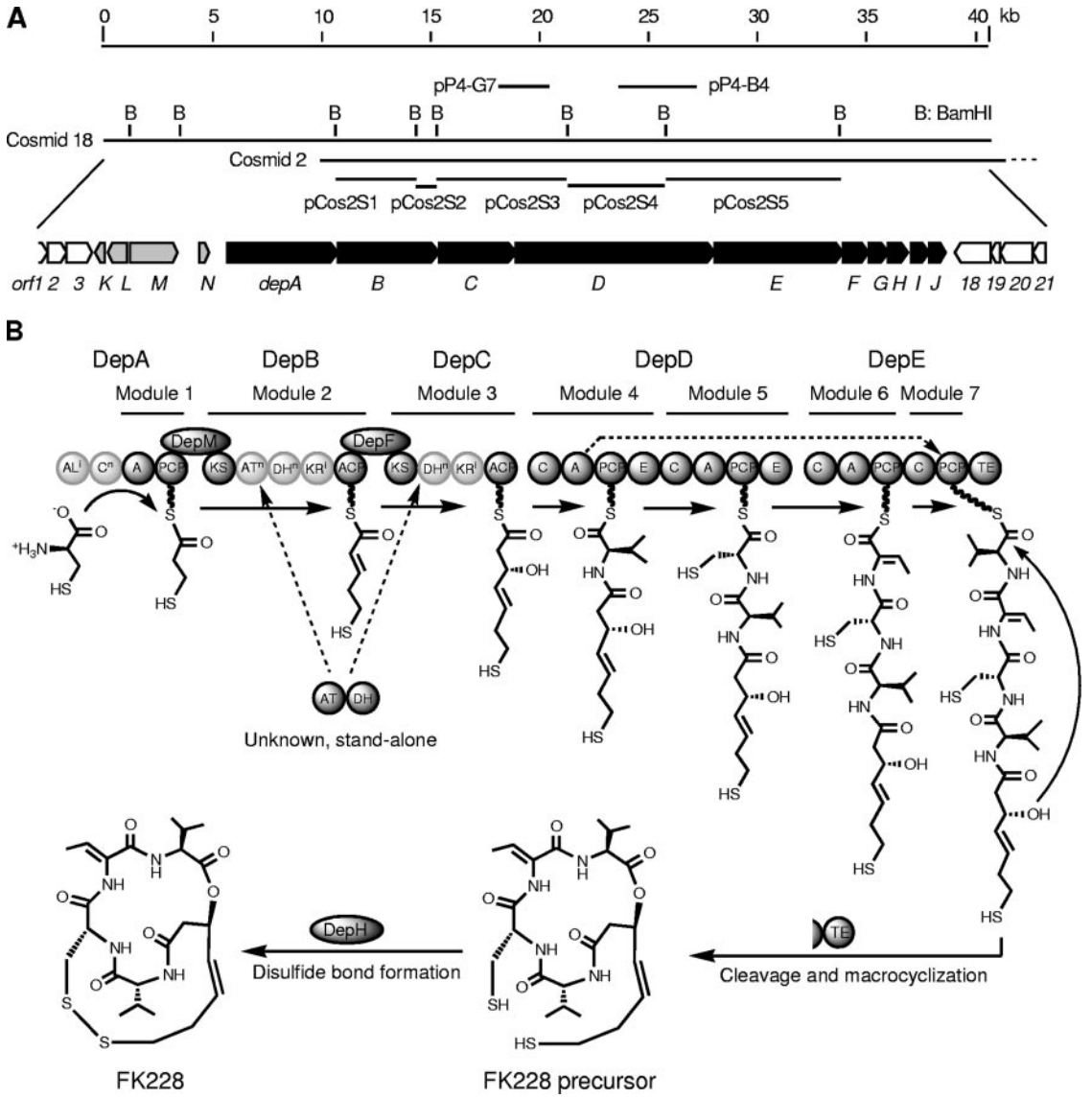

FIG. 2. FK228 biosynthetic (*dep*) gene cluster and a proposed model for FK228 biosynthesis. (A) Physical map of clones and genes. pP4-G7 and pP4-B4 are positively identified genome sampling clones, and each contains part of the *depD* gene. The insert in pP4-G7 was labeled as a DNA probe to obtain cosmids 18 and 2. Cosmid 18 was shotgun sequenced. pCos2S1 to pCos2S5 are subclones of cosmid 2 and were sequenced by a primer walking method. Predicted genes in the *dep* gene cluster are designated *depA* to *depN*, and open reading frames outside the *dep* gene cluster are designated *orf1* to *orf3* and *orf18* to *orf21*. Genes indicated by solid bars (*depA* to *depJ*) were predicted to be in the *dep* gene cluster with confidence; genes indicated by gray bars (*depK* to *depN*) were predicted to be in the *dep* gene cluster with less confidence. (B) Proposed model of FK288 biosynthesis by a hybrid NRPS-PKS-NRPS assembly line, including accessory activities of discrete proteins. PKS and NRPS domains are described in the text. A superscript "i" indicates that a domain is inactive; a superscript "n" indicates that a domain is nonfunctional. Inactive and nonfunctional domains are light gray. AL, acyl coenzyme A ligase; KS, β-ketoacyl synthase; E, epimerase.

cereulide and valinomycin biosynthesis in actinomycetes (29) has been experimentally established. However, whether intramolecular disulfide bond formation in natural products (such as FK228) is an enzymatic reaction or a spontaneous chemical oxidation is unknown. Therefore, our search for candidate FK228 biosynthetic genes focused initially on the genes encoding an obvious NRPS, PKS, or, in particular, hybrid NRPS-PKS or PKS-NRPS system.

Among 360 valid sequence tags obtained from sequencing of the genome sampling library of *C. violaceum* (see Materials and Methods), three distinctive sequence tags, P3-A6-SP6, P4-B4-SP6, and P4-G7-SP6, were identified to be parts of genes encoding PKS, NRPS, and a hybrid PKS-NRPS system, respectively (Table 2). Genes that contain these three tags were considered candidate natural product biosynthetic genes, possibly involved in FK228 biosynthesis. Further primer walking sequencing revealed the complete sequences of the corresponding inserts in pP3-A6, pP4-B4, and pP4-G7. The insert in pP3-A6 contains a 2,826-bp DNA that includes a partial PKS gene (not named), and its translated amino acid sequence has homology to the β-ketoacyl synthase and acyltransferase (AT) domains of type I PKSs (44). Three signature motifs (QTR

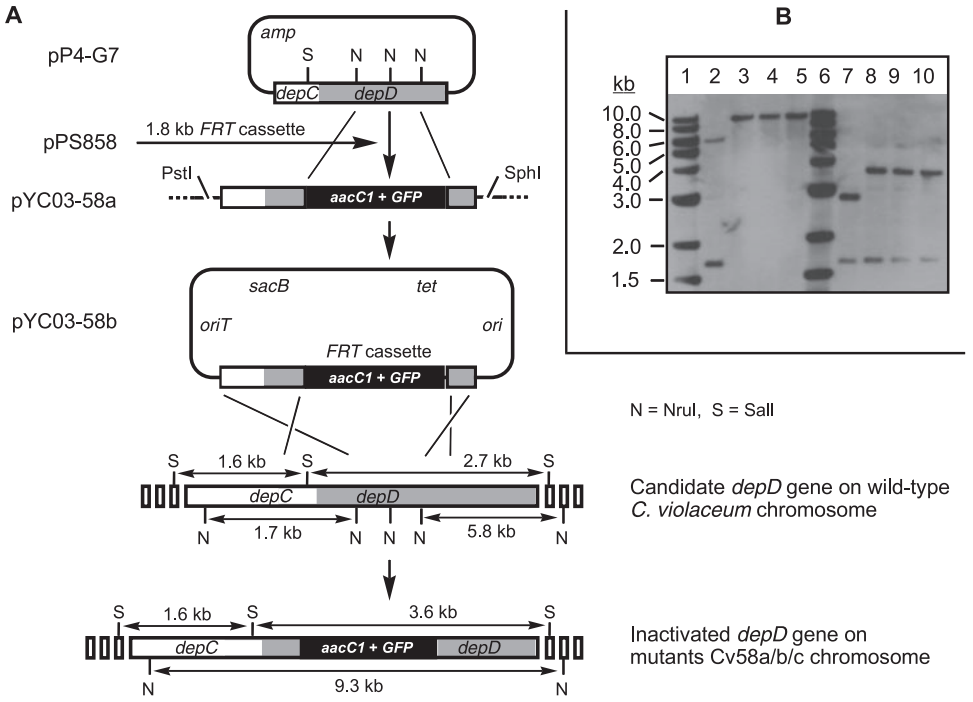

FIG. 3. Creation of *depD*-inactivated mutant strains by targeted gene replacement. (A) Construction of gene replacement vector pYC03-58b and homologous recombination via double crossover between the vector and the bacterial chromosome to generate a mutant genotype. (B) Southern analysis of the genotypes of wild-type and *depD*-inactivated mutant strains of *C. violaceum*, using the labeled 2.6-kb insert DNA of pP4-G7 as a probe. Genomic DNA was digested with NruI (lanes 2 to 5) or SalI (lanes 7 to 10). Lanes 1 and 6, 1-kb DNA ladders (New England Biolabs), hybridized with their digoxigenin-labeled probes; lanes 2 and 7, DNA from the wild-type strain; lanes 3 to 5 and 8 to 10, DNA from independent mutant strains Cv58a, Cv58b, and Cv58c, respectively. A change in the pattern of positively hybridized DNA bands indicates targeted gene inactivation.

TAQ, GHSYG, and AAFH) were identified in the AT domain, and these motifs are similar to the motifs of ATs using MCoA as a substrate (39). The insert in pP4-B4 contains a 3,612-bp DNA that includes a partial gene (designated *depD*) (Table 3 and Fig. 2A), and its translated amino acid sequence has homology to the A, peptidyl carrier protein (PCP), and epimerase domains of type A NRPSs (32). The "NRPS substrate specificity code" of the A domain was identified as DLFEMSLIWK, and this A domain is predicted to activate L-Cys, according to Ansari et al. (3), Challis et al. (7), and Stachelhaus et al. (47). The insert in pP4-G7 contains a 2,599-bp DNA that includes two partial genes (designated *depC* and *depD*) (Table 3 and Fig. 2A), and their translated amino acid sequences have homology to the KR and acyl carrier protein (ACP) domains of PKSs, followed by the C and A domains of NRPSs, indicating that there is a hybrid PKS-NRPS system (11). The A domain is incomplete; therefore, the "NRPS substrate specificity codes" cannot be extracted for prediction of substrate specificity. Inserts in pP4-B4 and pP4-G7 cover different parts of the same *depD* gene.

Adaptation of a *P. aeruginosa* genetic system in *C. violaceum* no. 968 to create targeted gene-inactivated mutant strains. To test whether the identified candidate genes are necessary for FK228 biosynthesis, we inactivated the individual genes (except *depC*, which has only a very short segment on the insert of pP4-G7) in *C. violaceum* no. 968. *C. violaceum* strains belong to the gram-negative β-proteobacteria. Although isolates of *C. violaceum* produce many products with biotechnological and pharmaceutical utility (13) and the genome of a representative strain, *C. violaceum* ATCC 12472, has been sequenced (4), a genetic system for targeted gene inactivation in *C. violaceum* has not been reported prior to this study. Here, a broad-host-range Flp-*FRT* recombination system originally developed for *P. aeruginosa* genetics (23) was adopted and successfully applied to *C. violaceum* no. 968.

To inactivate the P4-G7-SP6-associated *depD* gene (*depD* was chosen as an example for full description here because it encodes part of a hybrid PKS-NRPS system that is of prime interest) (Fig. 3A), two internal NruI fragments (0.46 and 0.49 kb) of the pP4-G7 insert were removed and replaced by a 1.8-kb SmaI fragment of the *FRT* cassette from pPS858 to make an intermediate construct, pYC03-58a. A 3.7-kb PstI/SphI fragment containing the *FRT* cassette with flanking DNAs from pYC03-58a was recovered, end repaired, and inserted into the SmaI site of pEX18Tc to make a final construct, pYC03-58b. Plasmid pYC03-58b was introduced into *E. coli* S17-1 cells and subsequently transferred into *C. violaceum* cells by conjugation. In the designed selection medium (see Materials and Methods), ampicillin at a concentration of 200 μg/ml suppresses the growth of *E. coli* S17-1 cells, gentamicin at a concentration of 50 μg/ml selects for the presence of the *FRT* cassette, and sucrose at a concentration of 5% counterselects for the loss of a functional *sacB*$^+$ gene on the vector. Collectively, this experiment strongly selected for double-crossover mutants of *C. violaceum* with part of the targeted *depD* replaced by the *FRT* cassette. Hundreds of exconjugants ap-

peared on a typical selection plate after incubation at 30°C for 2 days. The efficiency of conjugation and gene recombination was estimated to be in the range from 10^{-6} to 10^{-5} per cell.

Southern analysis (Fig. 3B) clearly showed that when genomic DNA of *C. violaceum* strains was digested with NruI (lanes 2 to 5), the wild-type strain showed two bands (1.7 and 5.8 kb; 0.46- and 0.49-kb DNA fragments ran off the gel during electrophoresis) that hybridized to the probe made from the 2.6-kb insert of pP4-G7. Considering that there are three internal NruI sites in the 2.6-kb insert of pP4-G7 and that one central NruI site was removed and two other sites were destroyed during the construction of pYC03-58a, insertion of the 1.8-kb *FRT* cassette via double-crossover DNA recombination was expected to result in a 9.3-kb (1.7 kb + 5.8 kb + 1.8 kb) hybridized band in the mutant genotype. Three of eight random exconjugants were proven in this experiment to have the correct genotype, and they were designated independent *depD*-inactivated mutant strains Cv58a, Cv58b, and Cv58c (collectively designated the Cv58a/b/c mutants). Similarly, when genomic DNA was digested with SalI (lanes 7 to 10), the size of a 2.7-kb hybridized band in the wild-type strain increased to 3.6 kb (2.7 kb − 0.49 kb − 0.46 kb + 1.8 kb) in the mutant strains, as expected. The 1.6-kb band in the wild-type strain remained unchanged in mutant strains because the DNA fragment is located outside the gene replacement region.

The same strategy was used to inactivate the P3-A6-SP6-associated gene (not named) and the P4-B4-SP6-associated *depD* gene (3′ part), to create mutant strains Cv56a/b/c and Cv57a/b/c, respectively, and their genotypes were verified by Southern analyses as well (data not shown).

During the course of method development, two other conjugation systems were also tested. One method used the methylation-deficient strain *E. coli* ET12567(pUZ8002) (25, 28) and the other used *E. coli* MT607(pRK600) (14) as donor strains to mobilize a conjugation construct (such as pYC03-58b) into *C. violaceum* cells. Both systems generated exconjugants, but they were at least 10-fold less efficient than the *E. coli* S17-1 strain-mediated conjugation between *E. coli* and *C. violaceum* cells (data not shown). In addition, it was noticed that, since the *FRT* cassette contains a functional *GFP* gene that encodes the green fluorescent protein (GFP), *E. coli* and *C. violaceum* colonies or cultures with the *FRT* cassette present on a replicable plasmid or integrated into the chromosome were distinguishable from the wild-type bacteria by a greenish color (data not shown). Therefore, bacterial exconjugants carrying the *FRT* cassette could be identified by direct observation or by a simple GFP assay. Furthermore, the marker genes (*aacC1* and *GFP* in the *FRT* cassette) integrated into the mutant chromosome could be excised precisely by a FLP recombinase encoded by the pFLP2 plasmid in the Flp-*FRT* system to create unmarked mutants (23). Unmarked mutants could be mutated at different loci sequentially to create multiple gene deletions or gene replacements. This feature could be very useful for future pathway engineering and combinatorial biosynthesis studies.

Confirmation of the necessity of the *depD* gene for FK228 biosynthesis in *C. violaceum* no. 968. The FK228 productivity of the wild-type and mutant (Cv56a/b/c, Cv57a/b/c, and Cv58a/b/c) strains of *C. violaceum* was examined by fermentation and LC-MS analysis. FK228 does not produce a characteristic UV spectrum because it lacks a chromophore, but its positive ion signals are strong and appeared near 20.8 min under the chromatographic conditions tested (Fig. 4). The calculated positive ion signal of FK228 is $[M + H]^+$ at *m/z* 541.2, and its ion adducts are $[M + Na]^+$ at *m/z* 563.2 and $[M + K]^+$ at *m/z* 580.2 for an authentic FK228 sample, but the actual observed signals were *m/z* 540.1, *m/z* 562.9, and *m/z* 578.7, respectively. The small mass differences between the calculated and observed values were likely due to inadequate instrument calibration. The samples from wild-type and Cv56a/b/c mutant strains yielded almost the same signals as the authentic FK228. However, no FK228 ion signal was detected in samples from Cv57a/b/c or Cv58a/b/c mutant strains. These results suggest that inactivation of *depD*, but not inactivation of the P3-A6-SP6-associated gene, completely abolished FK228 production, which confirmed the necessity of *depD* for FK228 biosynthesis in *C. violaceum* no. 968.

Cloning, sequencing, and in silico analysis of the FK228 biosynthetic (*dep*) gene cluster. A series of overlapping cosmid clones were obtained by colony hybridization with digoxigenin-labeled insert DNA of pP4-G7 as a probe. Cosmid end sequencing indicated that, among these clones, cosmid 18 appears to contain the entire *dep* gene cluster; therefore, the nucleotide sequence of cosmid 18 was determined by the shotgun method, which revealed a 40,434-bp contig (Fig. 2A). Due to concern about the irregularity of the deduced protein domain organizations (see below for details), cosmid 2, which covers most but not all of the *dep* gene cluster, was also sequenced by a subcloning and primer walking strategy (Fig. 2A). A cosmid clone carrying a partial *dep* gene cluster was chosen for sequencing verification purposes because a partial gene cluster cloned from the gram-negative bacterium *C. violaceum* into another gram-negative bacterium, *E. coli*, should not result in acquired toxicity, minimizing possible gene deletion or recombination. The sequences of the overlapped region in cosmid 18 and cosmid 2 agreed perfectly, confirming the shotgun sequence quality and reliability.

The assembled contig contains 21 apparent genes or open reading frames (two partial sequences at the ends) (Table 3 and Fig. 2A). Bioinformatic analyses further predicted that the *dep* gene cluster consists of 14 genes, designated *depA* through *depN*, flanked by several housekeeping genes (*orf1* through *orf3* and *orf18* through *orf21*), although the exact boundaries of the *dep* gene cluster have not been experimentally verified yet. The flanking housekeeping genes have homology with genes in a single region of the *C. violaceum* ATCC 12472 genome (CV_3375 through CV_3386) (4). Interestingly, five ATCC 12472 genes (CV_3379 through CV_3383) are seemingly replaced by the *dep* gene cluster, suggesting that a lateral gene transfer event occurred (34). Further evidence that supports this notion comes from a G+C content analysis. The flanking housekeeping genes have an average G+C content of 62.9%, while the *dep* gene cluster has a G+C content of 69.0%. *C. violaceum* no. 968 could have acquired the *dep* gene cluster from an organism with a higher-G+C genome at the expense of a five-gene deletion of its own.

Cotranscription is common among related genes in bacteria. In the *dep* gene cluster and flanking regions, *orf1* through *orf3*, *orf18* through *orf21*, *depABCDEFGH*, and *depIJ* are very likely organized as operons, because genes within each putative

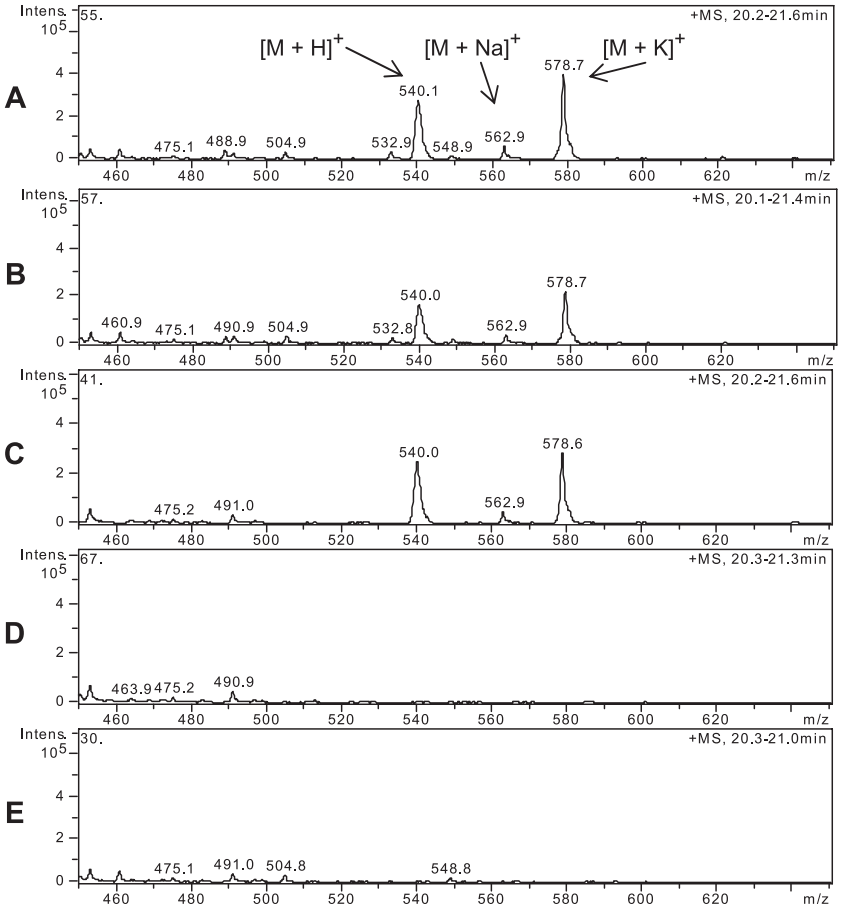

FIG. 4. Detection of FK228 positive ion signals by LC-MS. Samples were obtained from an authentic FK228 standard (A), wild-type *C. violaceum* (B), Cv56a/b/c mutants with the pP3-A6-associated gene inactivated (C), Cv57a/b/c mutants with the pP4-B4-associated *depD* gene (3′ part) inactivated (D), and Cv58a/b/c mutants with the pP4-G7-associated *depD* gene inactivated (E). For each mutation three mutants (a, b, and c) yielded identical results; therefore, only one data profile for each mutation is presented.

operon have overlapping stop and start codons. In contrast, the *depK*, *depL*, *depM*, and *depN* genes are separated by variable lengths of intergenic DNA. This analysis facilitated the prediction that *depJ* is the downstream boundary of the *dep* gene cluster because *orf18* through *orf21* are housekeeping genes in a single putative operon.

Model for FK228 biosynthesis by a hybrid NRPS-PKS-NRPS assembly line. Many natural products are often biosynthesized by modular NPRSs, PKSs, or hybrid NRPS-PKS or PKS-NRPS assembly lines in a colinearity model in which the substrate specificity and the number and order of modules dictate the chemical makeup of the products (for recent comprehensive reviews, see references 15, 16, and 22); meanwhile, variations from the canonical model, including colinearity violation, iterative polymerization (iteration), missing or misplaced domains, module skipping or stuttering, stand-alone domains, alternative chain termination, the presence of unique domains, or *trans*-acting enzymes, have all been documented in individual biosynthetic pathways (for recent comprehensive reviews, see references 16, 44, and 55). Based on extensive bioinformatic analyses of the domain and module organization of biosynthetic enzymes encoded by the *dep* gene cluster, a model for FK228 biosynthesis by a hybrid NRPS-PKS-NRPS assembly line is proposed (Fig. 2B), and this model should serve as a general guideline for future studies and experimental validation. The proposed pathway includes nine proteins (DepA, DepB, DepC, DepD, DepE, DepF, DepH, and DepM, as well as DepJ [not drawn in the model]) that constitute five NRPS modules, two PKS modules, and accessory activities; each module is responsible for the incorporation of one contributing building block.

Based on the model, FK228 biosynthesis starts with the activation of a Cys by the A domain in module 1 to form a cysteinyl-*S*-PCP intermediate. DepM (an aminotransferase) is proposed to act in *trans* to remove an amino group from the intermediate to form 4-mercaptobutanyl-*S*-PCP. Aminotransferase domains have been found to be an integral part of the PKSs in the biosynthesis of mycosubtilin (12) and iturin A (50), adding an amino group; no such domain, however, has been found to remove an amino group in a reverse reaction. The C domain in module 1 appears to be nonfunctional because of a lack of a critical catalytic motif, HHXXXDG; a nonfunctional C domain disconnects the possible chemical interaction between the upstream acyl coenzyme A ligase domain and the downstream A domain. Next, PKS modules 2 and 3 sequentially extend the growing chain with two C_2 units from MCoA.

However, module 2 contains only a remnant nonfunctional AT domain that lacks essential motifs (e.g., GHSXG and A[FS]HS), and module 3 lacks an AT domain. The dehydratase (DH) domains in modules 2 and 3 also appear to be nonfunctional because of a lack of a conserved active site motif, HXXXGXXXXP. An unknown stand-alone AT-DH didomain protein (or, alternatively, discrete AT and DH proteins) is proposed to act in *trans* to compensate the modules in the PKS mode of biosynthesis. Furthermore, since no gene encoding a stand-alone AT-DH didomain is present in the *dep* gene cluster, it must exist in another region of the genome. Stand-alone AT domains or AT-X didomains (where X is any domain) have been identified in recent years in the biosynthetic pathways of natural products, including leinamycin (9), pederin (37), and many other compounds; a recent molecular cellular study of the bacillaene biosynthetic enzyme complex revealed an amazing interaction between a stand-alone AT-X didomain and the rest of a mega-PKS complex in *Bacillus subtilis* (48). In addition, DepF, an FadE2-like acyl coenzyme A dehydrogenase, has been proposed to act in *trans* on module 2 to generate a double bond on the β-hydroxyl-5-mercaptopentanoyl-*S*-ACP intermediate to form the β-5-mercaptopent-2-enoyl-*S*-ACP intermediate. If this is true, DepF would be functionally equivalent to an enoylreductase. KR domains in modules 2 and 3, although intact, are proposed to be inactive, probably due to a lack of proper interaction with the putative *trans*-acting AT-DH didomain. Modules 4, 5, and 6 extend the growing intermediate chain with activated D-Val, D-Cys, and 2,3-dehydro-2-aminobutanoic acid (2,3-dehydrothreonine) sequentially in the canonical model of the NRPS mode of biosynthesis. Module 7 is expected to incorporate a Val, but an A domain is completely missing in this module. It is proposed that the A domain in module 4, which specifies Val, acts in *trans* to aminoacylate the PCP domain in module 7. This phenomenon has been observed in the biosynthetic pathways of viomycin (49), yersiniabactin (19), and other compounds. Finally, terminal thioesterase (TE) on DepE should catalyze the formation of an ester linkage between a hydroxyl group originating from MCoA and a β-keto group from Val to form a 16-membered macrolactone ring, and a flavin adenine dinucleotide-dependent pyridine nucleotide-disulfide oxidoreductase encoded by *depH* is proposed to bring the free sulfhydryl groups from two Cys residues close to form an intramolecular disulfide bond. Disulfide bond formation hallmarks the formation of a 17-membered ring structure and brings the FK228 biosynthesis to completion. DepJ, a discrete type II TE, is not drawn into the model, and type II TEs are generally believed to have a proofreading function during chain elongation to ensure smooth biosynthesis by selectively removing misprimed thioesters or shunt intermediates (21). It is necessary to point out that, in the model described above, several unique features that include the *trans*-acting DepM, DepF, an unknown stand-alone AT-DH didomain, and a *trans*-acting A domain are highly speculative and require experimental validation.

Other genes in the *dep* gene cluster. There are two apparent resistance genes in the *dep* gene cluster. An esterase/lipase, encoded by *depI*, is proposed to hydrolyze the ester linkage and/or the disulfide bond in FK228 to prevent the accumulation of an excess concentration of FK228 in cells where FK228 may become toxic. A phosphotransferase, encoded by *depG*, is proposed to further mask and quench the hydrolyzed FK228 by adding a phosphate group to the freed hydroxyl and/or sulfhydroxyl group(s). Surprisingly, no gene encoding exportation machinery is found in the *dep* gene cluster. The *depL* gene encodes a typical transcriptional regulator that contains a helix-turn-helix motif, indicating its DNA-binding activity. The *depK* gene encodes a conserved functionally unknown protein. Finally, *depN* encodes a nonfunctional PCP remnant without a critical serine residue in a conserved motif, GX(HD)S, necessary for phosphopantetheinylation and covalent substrate aminoacylation.

In conclusion, FK228, a promising anticancer agent, is moving fast towards clinical uses. Its unique structural characteristics and mode of action warrant efforts to generate analogs that can be tested as additional and hopefully even better anticancer drug candidates. The adaptation of an efficient genetic system for *C. violaceum* no. 968, in combination with cloning of the *dep* gene cluster, provides an excellent platform for further investigations of the FK228 biosynthetic pathway and makes future genetic manipulations of the biosynthetic pathway for generating novel FK228 analogs by engineered biosynthesis possible. In addition, the Flp-*FRT* recombination system validated in this study could be applicable to other strains of *C. violaceum*.

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