New Insights into the Genetic Organization of the FK228 Biosynthetic Gene Cluster in *Chromobacterium violaceum* No. 968^{∇†}

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The biosynthetic gene cluster of FK228, an FDA-approved anticancer natural product, was identified and sequenced previously. The genetic organization of this gene cluster has now been delineated through systematic gene deletion and transcriptional analysis. As a result, the gene cluster is redefined to contain 12 genes: *depA* through *depJ*, *depM*, and a newly identified pathway regulatory gene, *depR*.

FK228 produced by *Chromobacterium violaceum* no. 968 is a potent histone deacetylase (HDAC) inhibitor recently approved under the commercial name Istodax by the FDA for the treatment of cutaneous T-cell lymphoma (9, 13). FK228 represents a small family of natural products that also includes spiruchostatins (12), FR901375 (11), and our newly discovered thailandepsins (Y.-Q. Cheng and C. Wang, U.S. patent application 61/235,253). All members of this family of natural products are produced by rare Gram-negative bacterial species, and each of them contains a signature disulfide bond that is known or presumed to mediate a novel mode of anticancer action in which a reduced thiol group “warhead” chelates a Zn²⁺ in the catalytic center of HDACs, thereby inhibiting the enzyme activities (2, 6).

We previously reported the cloning and preliminary characterization of the FK228 biosynthetic (*dep*) gene cluster in *C. violaceum* (2). Based upon limited genetic evidence and mostly bioinformatic analyses, we predicted 14 genes to be included in the *dep* gene cluster (*depA* through *depN*) (Fig. 1A). We also proposed a hybrid nonribosomal peptide synthetase-polyketide synthase pathway for FK228 biosynthesis. In this study, we redefined the genetic organization of the *dep* gene cluster; our studies also revealed an unexpected gene that regulates FK228 biosynthesis.

To precisely determine the upstream and downstream boundaries of the *dep* gene cluster, we employed a multiple cloning procedure (2, 7) and later a multiplex PCR method (4, 15) to have 13 genes or open reading frames systematically deleted (Table 1 and Fig. 1A; also see Materials and Methods and Table S2 in the supplemental material). *depG* could not be mutated despite multiple attempts, and *orf19* is a very small gene paired with *orf20*. Therefore, these two genes were mutated together, and no attempt was made to mutate *depB*, *depC*, or *depE*, because they are obviously cotranscribed with

depA (2) (also see below). In addition, mutants of *depD* and *depH* were created previously (2, 15). The genotype of each successful mutant was verified by PCR analysis (see Fig. S2 in the supplemental material), and the relative level of FK228 production by mutant strains was quantified by liquid chromatography-mass spectrometry (LC-MS) analysis (Fig. 1B; see also Fig. S3 in the supplemental material).

The upstream or left boundary of the *dep* gene cluster was determined to begin with *depM* according to the following observations (Fig. 1). First, the deletion of either *orf3*, *orf4* (previously annotated as *depK*), or *orf5* (previously annotated as *depL*) had no obvious effect on FK228 production, indicating that these three putative genes play no role in FK228 biosynthesis and thus are not part of the *dep* gene cluster. Second, deletion of *depM* or “*depN*” resulted in a 35% to 55% decrease in FK228 production, suggesting a positive involvement of these two “genes” in FK228 biosynthesis. The deduced product of *depM* is an aminotransferase presumably catalyzing the removal of an amine group from a cysteinyl-S-peptidyl carrier protein (PCP) intermediate in *trans* to form 4-mercapto-butanyl-S-PCP in the initiation module of the FK228 biosynthetic pathway (2). “*depN*,” originally annotated to encode a small hypothetical protein of unknown function (2), is now believed to be a pseudogene, because no *depN* transcript has ever been detected under the conditions in which other *dep* genes are expressed (see below for details). The stretch of DNA occupied by “*depN*” likely contains regulatory elements upstream of *depA*. The observed reduction of FK228 production by the *C. violaceum* Δ“*depN*” mutant might be an unintended result of the deletion of some of those regulatory elements of *depA* and its associated operon. Third, deletion of further downstream genes, *depA*, *depD*, or *depF*, resulted in complete loss of the FK228 production, confirming the critical biosynthetic roles of those genes as predicted in the originally proposed biosynthetic model (2). Fourth, *orf5* and *depM* share a 74-bp (A+T)-rich intergenic region but transcribe divergently, indicating that they are transcriptionally unrelated.

The downstream or right boundary of the *dep* gene cluster was determined to end with *depR* according to the following observations (Fig. 1). First, deletion of *depR* (previously annotated *orf18*) completely abolished FK228 production, and complementation of *depR* restored ca. 70% of the FK228 pro-

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FIG. 1. Redefined FK228 biosynthetic (*dep*) gene cluster and putative operon organization. (A) All redefined *dep* genes are shown by solid black bars, open reading frames (ORFs) beyond the *dep* gene cluster are shown by white bars with solid outlines, and a pseudogene ("*depN*") is shown by a white bar with a dotted outline. Previously annotated gene (*orf*) names that have been revised are in parentheses. Wavy lines with arrowheads indicate putative operons. (B) Relative levels of FK228 produced by the wild-type strain (CvWT), 14 gene deletion mutant strains, a complementation strain, and an overproduction strain of *Chromobacterium violaceum* no. 968, detected and quantified by LC-MS. Data are mean values of results from duplicate experiments, with error bars indicating standard deviation.

duction capacity, indicating a critical role of *depR* in FK228 biosynthesis. Second, deletion of *orf19-orf20* or *orf21* did not abolish FK228 production but resulted in a slight (16 to 20%) decrease of yield, presumably due to a reduced fitness of mutants. *orf19* to *orf21* are bacterial housekeeping genes encoding MinE, MinD, and MinC, respectively, which are components of the conserved bacterial Min system involved in cell division and septum formation (1, 5, 8). *orf19-orf20* or *orf21* mutants were found to be more sensitive to antibiotics and grew much slower than the wild-type strain, and these produced variable amounts of minicells. Third, deletion of other downstream genes, *depH*, *depI*, or *depJ*, resulted in various degrees of reduction of FK228 production, most likely due to the accessory roles of their deduced products involved in either FK228 biosynthesis or self-resistance (2, 15).

Furthermore, the role of *depR* as a pathway regulatory gene governing FK228 biosynthesis was ascertained with the following evidence. First, *depR* appears to encode an LysR-type transcriptional activator that contains an N-terminal helix-turn-helix DNA binding domain (pfam00126) and a C-terminal LysR-type sensor domain (pfam03466) (10); within this sensor domain there are two conserved catalytic cysteine residues (C199 and C208) that could form a disulfide bridge upon sensing oxidative stress (3). Second, deletion of *depR* abolished FK228 production, and complementation of the Δ *depR* mutant restored much of the FK228 production capacity (see above). In addition, an overproduction strain, *C. violaceum* wild type (WT)/pVP01-52b, containing an ectopic copy of *depR* on an expression vector, produced ca. 10% more FK228 than the wild type (Fig. 1B; see Fig. S3 in the supplemental material), suggesting that DepR is a rate-limiting factor in FK228 bio-

synthesis. This 10% increase of FK228 production also represents a moderate success of yield improvement through metabolic engineering. Third, semiquantitative reverse transcription (RT)-PCR experiments (Fig. 2) clearly indicate that the expression of most redefined *dep* genes, except for *depJ* and *depR* itself (mutation of *depR* was designed as such that a 520-bp internal part of *depR* was deleted, leaving a 260-bp 5' region of *depR* intact for RT-PCR detection), was drastically downregulated or turned off in the Δ *depR* mutant under a condition in which all genes were expressed in the WT strain. The expression of genes outside the redefined *dep* gene cluster was not affected by the *depR* deletion. Because *depR* was detected to cotranscribe with *orf19* to *orf21* and is the last gene in the operon (Fig. 1A; see below for details), *depR* thus does not self-regulate the operon where it resides. Considering that *orf19* to *orf21* are critical *min* genes for bacterial physiology, it is surprising to find that *depR* as part of a housekeeping gene operon turns out to be a gene regulating a secondary metabolite biosynthetic gene cluster. Perhaps there is an underlying link between bacterial septum formation and cell division, and between oxidative stress sensing and FK228 production, which is worth further investigation.

Finally, the operon organization of the *dep* gene cluster was assessed by overlap RT-PCR. As shown in Fig. 3, transcriptional coupling between two adjacent gene pairs, *depA* and *depB*, *depB* and *depC*, *depC* and *depD*, *depD* and *depE*, *depE* and *depF*, *depG* and *depH*, and *depI* and *depJ*, but not between *depM* and "*depN*," "*depN*" and *depA*, or *depH* and *depI*, could be detected by RT-PCR. Transcriptional coupling between gene pairs *orf21* and *orf20*, *orf20* and *orf19*, and *orf19* and *depR* was also detected. Therefore, the putative operon organization

TABLE 1. Bacterial strains used in this study

Strain ^c	Description ^a	Source or reference
<i>Chromobacterium violaceum</i> strains		
968 (FERM BP-1968)	Wild-type strain, FK228 producing, Ap ^r Thio ^r	IPOD ^b (14)
$\Delta orf3::FRT$	Mutant strain with an internal part of <i>orf3</i> replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta orf3$	Mutant strain derived from the $\Delta orf3::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta orf4::FRT$	Mutant strain with an internal part of <i>orf4</i> (<i>depK</i>) replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta orf4$	Mutant strain derived from the $\Delta orf4::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta orf5::FRT$	Mutant strain with an internal part of <i>orf5</i> (<i>depL</i>) replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta orf5$	Mutant strain derived from the $\Delta orf5::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta depM::FRT$	Mutant strain with an internal part of <i>depM</i> replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta depM$	Mutant strain derived from the $\Delta depM::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta depN::FRT$	Mutant strain with an internal part of <i>depN</i> replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta "depN"$	Mutant strain derived from the $\Delta "depN"::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta depA::FRT$	Mutant strain with an internal part of <i>depA</i> replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta depA$	Mutant strain derived from the $\Delta depA::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta depD$	Mutant strain with an internal part of <i>depD</i> deleted	2
$\Delta depF::FRT$	Mutant strain with an internal part of <i>depF</i> replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta depF$	Mutant strain derived from the $\Delta depF::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta depH$	Mutant strain with an internal part of <i>depH</i> deleted	15
$\Delta depI::FRT$	Mutant strain with an internal part of <i>depI</i> replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta depI$	Mutant strain derived from the $\Delta depI::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta depJ::FRT$	Mutant strain with an internal part of <i>depJ</i> replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta depJ$	Mutant strain derived from the $\Delta depJ::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta depR::FRT$	Mutant strain with an internal part of <i>depR</i> (<i>orf18</i>) replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta depR$	Mutant strain derived from the $\Delta depR::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta orf19/20::FRT$	Mutant strain with most part of <i>orf19/orf20</i> (small genes manipulated together) replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta orf19/20$	Mutant strain derived from the $\Delta orf19/20::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta orf21::FRT$	Mutant strain with an internal part of <i>orf21</i> replaced with an <i>FRT</i> cassette (Gm ^r GFP ⁺) from pPS858	This study
$\Delta orf21$	Mutant strain derived from the $\Delta orf21::FRT$ mutant with the <i>FRT</i> cassette excised	This study
$\Delta depR/pVP01-52b$	The $\Delta depR$ mutant complemented with a <i>depR</i> (<i>orf18</i>) gene carried on broad-host-range expression vector pVP01-52b based on pBMTL-3	This study
WT/pVP01-52b	Wild-type strain harboring pVP01-52b, as an overproduction strain	This study
<i>Escherichia coli</i> strains		
DH5 α	General cloning host	Lab stock
S17-1	Host strain for interspecies conjugation	Lab stock

^a Ap^r, ampicillin resistance; GFP, green fluorescence protein; Gm^r, gentamicin resistance; Thio^r, thiostrepton resistance.

^b IPOD, International Patent Organism Depository, Tsukuba, Japan.

^c "*depN*" was previously annotated as a gene of unknown function. The current work revised it as a pseudogene that likely occupies part of the regulatory region of *depA* and its associated large operon.

of the *dep* gene cluster can be assigned as *depM* alone, *depABCDEF*, *depIJ*, and *depR* cotranscribed with *orf21*, *orf20*, and *orf19* (Fig. 1A). *depJ* appears to be able to transcribe from two alternative transcriptional start sites; one is located upstream of *depI* so that *depI* and *depJ* are cotranscribed, and this operon is under the control of *depR*; another one is located just upstream of *depJ* itself, independent of *depR* regulation.

In conclusion, the *dep* gene cluster is now redefined to contain 12 genes: *depA* through *depJ*, *depM*, and a newly identified pathway regulatory gene, *depR*. Those genes are organized into four putative operons, *depABCDEF*, *depIJ*, *depM*, and *depR* cotranscribed with *orf21*, *orf20*, and *orf19* in opposite orienta-

FIG. 2. Transcriptional regulation of *dep* genes by a newly identified pathway regulatory gene, *depR*. (A) Detection of individual gene expression by semiquantitative RT-PCR from an RNA sample of the wild-type strain. (B) Amplification of individual gene expression by semiquantitative RT-PCR from an RNA sample of the $\Delta depR$ mutant strain. 16S rRNA gene amplification was included as a control reaction.

FIG. 3. Detection of putative operon organization of the *dep* gene cluster and neighboring genes. (A) Detection of transcriptional coupling between two adjacent genes by overlap RT-PCR from an RNA sample of the wild-type strain. (B) Control reactions showing PCR amplification of an intergenic region of two adjacent genes from a genomic DNA sample of the wild-type strain. The unusually large amplicon of *depD-depE* was due to a special consideration in primer design to avoid two highly homologous intragenic regions toward the 3' end of *depD*.

tion. Precise determination of the gene organization of the *dep* gene cluster and identification of a pathway regulatory gene should facilitate complete elucidation of the mechanism by which FK228 is biosynthesized, production of FK228 in heterologous hosts, yield improvement of FK228 through metabolic engineering, and discovery and engineering of FK228 analogs as anticancer lead compounds.

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