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Identification and characterization of the spiruchostatin biosynthetic gene cluster enable yield improvement by overexpressing a transcriptional activator

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Abstract Spiruchostatins A and B are members of the FK228-family of natural products with potent histone deacetylase inhibitory activities and antineoplastic activities. However, their production in the wild-type strain of *Pseudomonas* sp. Q71576 is low. To improve the yield, the spiruchostatin biosynthetic gene cluster (*spi*) was first identified by rapid genome sequencing and characterized by genetic mutations. This *spi* gene cluster encodes a hybrid biosynthetic pathway similar to that encoded by the FK228 biosynthetic gene cluster (*dep*) in *Chromobacterium violaceum* No. 968. Each gene cluster contains a pathway regulatory gene (*spiR* vs. *depR*), but these two genes encode transcriptional activators of different classes. Overexpression of native *spiR* or heterologous *depR* in the wild-type strain of *Pseudomonas* sp. Q71576 resulted in 268 or 1,285 % increase of the combined titer of spiruchostatins A and B, respectively. RT-PCR analysis indicates that overexpression of heterologous *depR* upregulates the expression of native *spiR*.

Keywords Spiruchostatins · *Pseudomonas* sp. Q71576 · Biosynthesis · Genetic engineering · Yield improvement

Introduction

Spiruchostatins A and B were discovered as gene expression-enhancing agents and selective inhibitors of class I histone deacetylases (HDACs), while screening for activators of transforming growth factor-β (TGF-β) mediated signaling [14]. Spiruchostatins belong to a small family of natural products, which also includes FK228 (FR901228, romidepsin, marketed as Istodax) [18–20], FR901375 [1, 13] and thailandepsins [22–24]; all those compounds are produced by Gram-negative bacteria. Structurally spiruchostatins resemble FK228 in having a bicyclic depsipeptide scaffold and a signature disulfide linkage critical for prodrug stability and for bioactivities when reduced (Fig. 1). Close examination of the structure of spiruchostatins A and B reveals a likely sequence of building block polymerization starting with a derivative of L-cysteine, followed by two malonyl CoA units, a D-alanine unit, a D-cysteine unit, a derivative of L-valine (in spiruchostatin A) or L-isoleucine (in spiruchostatin B) unit, and finally another malonyl CoA unit. Biosynthesis of spiruchostatins in *Pseudomonas* sp. Q71576 is thus predicted to be catalyzed by a hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) pathway similar to that of FK228 [3, 15, 26].

As exemplified by FDA approval of FK228 for the treatment of cutaneous T-cell lymphoma and peripheral T-cell lymphoma [4, 21], other members of this FK228-family

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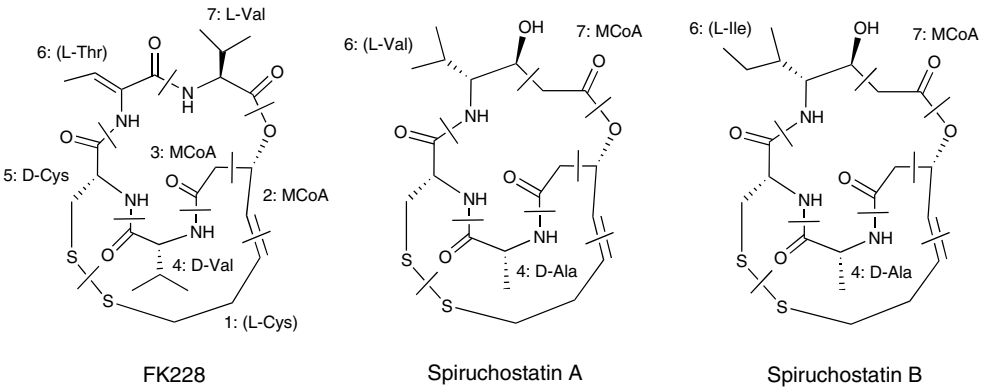

Fig. 1 The chemical structures of FK228 and spiruchostatins A and B. Each molecule is dissected into building blocks for easy comparison. In FK228 structure all building blocks are labeled; in spiruchostatin structures only building blocks that are different from those of FK228 are labeled. MCoA, malonyl coenzyme A. A bracketed amino acid name indicates a building block derived from that amino acid

of natural products have also drawn much attention due to their potent inhibitory activities of human HDACs and antineoplastic activities. For example, spiruchostatin A or B, either as a single agent or in combination with other drugs, has shown promising in vitro and/or in vivo efficacy in colon tumor xenograft model [17], leukemia cells [10], idiopathic pulmonary fibroblasts [6], endometrial carcinoma xenograft model [28], and renal cell carcinoma xenograft model [27].

However, efforts to develop a promising natural product drug lead for clinical uses are often hampered by inadequate supply of material, and various approaches have been described for yield improvement, including classical strain mutagenesis, metabolic engineering, and fermentation optimization [2]. Spiruchostatins have the same supply issue, and the aim of this study was thus to improve the biosynthetic yield of spiruchostatins. To this end, we first identified and confirmed a gene cluster responsible for the biosynthesis of spiruchostatins in *Pseudomonas* sp. Q71576; we then individually overexpressed a native transcriptional activator and a heterologous transcriptional activator in two engineered bacterial strains. In both cases, the combined titer of spiruchostatins A and B in bacterial fermentation broth increased significantly.

Materials and methods

Bacterial strains, plasmids, and other general materials and methods

Bacterial strains and plasmids used in this study are listed in Table 1. Primers used for gene deletion and complementation, genotype detection and RT-PCR are listed in Table S1 and Table S2 in Electronic Supplementary Material. General DNA manipulations were performed according to standard methods [16] or manufacturer's protocols. *Pseudomonas* strains were grown in Super Optimal Broth (SOB) or Luria-Bertani (LB) Agar at 25–30 °C, with or without appropriate antibiotics. Chemicals or biochemicals were generally purchased from Thermo Fisher Scientific (Waltham, MA), and enzymes from New England BioLabs (Ipswich, MA), unless otherwise indicated.

Rapid genome sequencing and gene identification

Genomic DNA of the wild-type *Pseudomonas* sp. Q71576 strain (PsWT) was prepared from an overnight culture with an UltraClean Microbial DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA), and was submitted for single-end and paired-end shotgun sequencing on a G20 FLX Sequencer (454 Life Science, Branford, CT) at the Research Technology Support Facility of Michigan State University (East Lansing, MI). De novo assembly of sequence reads by instrument software was performed and resulted in a quality draft genome sequence of PsWT. Candidate genes and gene cluster were identified using FK228 gene cluster sequences as input to search the acquired draft genome sequence of PsWT.

Gene deletion and complementation

Gene deletion in PsWT and gene complementation in mutant strains of PsWT were performed generally according to our established protocols described previously [25]. In particular, a 907-bp DNA fragment containing the *spiR* ORF and its native ribosome-binding site was amplified by PCR, digested with XbaI/HindIII, and cloned into the same sites of a broad host-range expression vector pBMTL-3 to make the gene expression construct pBMTL3-*spiR*; the sequence fidelity of DNA insert was verified by re-sequencing. Gene expression construct pBMTL3-*depR* (pVP01-52b) was made and described previously [15]. pBMTL3-*depR* and pBMTL3-*spiR* were electroporated independently into *Pseudomonas* sp. strains with a Gene Pulser (Bio-Rad Laboratories, Hercules, CA). Cells were recovered into 1 ml of SOB, incubated at 30 °C for 1 h and then plated onto selection plates containing 160 µg/ml of

Table 1 Strains and Plasmids used in this study

Strains or plasmids	Description	Source or reference
E. coli strains		
DH5α	General cloning host	Lab stock
S17-1	Host strain for interspecies conjugation	Lab stock
Pseudomonas sp. strains		
Q71576	Wild-type strain (PsWT), spiruchostatin A and B producer, Ap^r	IPOD
PsWTΔ*orf–1*	Markerless mutant strain with *orf–1* gene deleted	This study
PsWTΔ*spiR*	Markerless mutant strain with *spiR* gene deleted	This study
PsWTΔ*spiA*	Markerless mutant strain with *spiA* gene deleted	This study
PsWTΔ*spiP*	Markerless mutant strain with *spiP* gene deleted	This study
PsWTΔ*orf* + *1*	Markerless mutant strain with *orf* + *1* gene deleted	This study
PsWT/vector	PsWT complemented with empty pBMTL-3 vector	This study
PsWT/*depR*	PsWT complemented with pBMTL3/*depR* vector	This study
PsWT/*spiR*	PsWT complemented with pBMTL3/*spiR* vector	This study
PsΔ*spiR*/*depR*	PsΔ*spiR* complemented with pBMTL3/*depR* vector	This study
PsΔ*spiR*/*spiR*	PsΔ*spiR* complemented with pBMTL3/*spiR* vector	This study
Plasmids/constructs		
pBMTL-3	Broad host-range gene expression vector; conjugative, Cm^r *mob pLac*	[12]
pBMTL3-Flp2	Vector encoding the site-specific DNA recombinase Flp that recognizes the *FRT* site; Ap^r *oriT* $sacB^+$	[24]
pEX18Tc	Gene deletion/replacement vector; conjugative, Tc^r *oriT* $sacB^+$	[9]
pPS858	Source of the *FRT* cassette; Ap^r Gm^r gfp^+	[9]
pVP04-21a	Gene deletion construct for *orf–1* based on pEX18Tc	This study
pVP04-21b	Gene deletion construct for *spiA* based on pEX18Tc	This study
pVP04-21c	Gene deletion construct for *spiR* based on pEX18Tc	This study
pVP04-21d	Gene deletion construct for *spiP* based on pEX18Tc	This study
pVP04-21e	Gene deletion construct for *orf* + *1* based on pEX18Tc	This study
pBMTL3-*depR*	Gene complementation construct with *depR* on pBMTL-3 vector	[15]
pBMTL3-*spiR*	Gene complementation construct with *spiR* on pBMTL-3 vector	This study

Ap^r ampicillin resistance, *Cm^r* chloramphenicol resistance, *Gm^r* gentamicin resistance, *Tc^r* tetracycline resistance, *IPOD* International Patent Organism Depository, Tsukuba, Japan

chloramphenicol for growth overnight at 30 °C. Subsequently, six random colonies were inoculated in 4 ml of LB broth, grown overnight for the extraction of plasmid DNA for verification.

Fermentation and quantification

Fermentation of *Pseudomonas* sp. strains was carried out in triplicate in 50 ml of SOB containing 0.5 % (w/v) of HP-20 resin (Sigma-Aldrich) and 0.5 % (w/v) of XAD16 resin (Sigma-Aldrich) in 250-ml flasks at 25 °C with shaking at 200 rpm. Overnight grown seed culture was typically inoculated at a 1 % (v/v) ratio. In addition, 160 μg/ml of chloramphenicol and 1 mM of IPTG were added to the fermentation culture of engineered strains harboring a pBMTL-3 based expression construct. After 7 days, culture was centrifuged at 4,000×*g* for 30 min and the resulting pellet (resin and cell debris) was lyophilized to dryness. Dried pellet was eluted with 5 ml of ethyl acetate with agitation for 1 h at 30 °C. One milliliter of the extract was centrifuged at 17,000×*g* for 5 min, and 20 μl of the supernatant was injected into a liquid chromatography-mass spectrometer (LC-MSD Trap from Agilent Technologies, Santa Clara, CA) for the identification and quantification of spiruchostatins, similarly as the identification and quantification of FK228 described elsewhere [26].

Reverse transcriptase (RT)-PCR

For strains subjected to RT-PCR analysis, total RNA was extracted from a 12-h post-induction culture using an RNeasy Mini Kit (Qiagen, Valencia, CA). To remove DNA contamination, RNA samples were digested with DNase I for 1 h at 37 °C; the reactions were then stopped by adding EDTA to a final concentration of 0.5 mM and were denatured at 75 °C for 10 min. One hundred nanograms of RNA from each sample were added to each RT-PCR reaction performed with Qiagen OneStep RT-PCR kit. cDNA

synthesis was carried out at 50 °C for 30 min with OmniScript and SensiScript Reverse Transcriptases followed by an initial 15 min step at 95 °C to activate HostStarTaq DNA polymerase. Subsequently, PCR amplification for 25 cycles was performed as follows: 94 °C for 1 min, annealing at 56–64 °C for 1 min and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 10 min. For each batch of RT-PCR, a positive control reaction of 16S rRNA gene amplification from genomic DNA template and a negative control reaction to detect any DNA contamination by attempted amplification of the 16S rRNA gene without reverse transcription were included.

Nucleotide sequence accession numbers

The nucleotide sequences of the *spi* gene cluster, the 16S rRNA gene and a *Pseudomonas*-type carrier protein synthetase-encoding gene *pcpS* of *Pseudomonas* sp. Q71576 have been deposited in the GenBank database under accession numbers JQ045344, JQ045345 and JQ045346, respectively.

Results

Draft genome sequencing of *Pseudomonas* sp. Q71576 (PsWT)

Shotgun sequencing of PsWT genome generated a total of 189,375,961 input bases, which were assembled into 165 contigs; among them 84 are large contigs with an average contig size of 77,009 bps. Those contigs were further aligned into 32 scaffolds with a total length of 6,486,271 bps. This draft bacterial genome was thus calculated to have 29.1-fold of sequence coverage, which is close to the desired 30-fold oversampling of raw sequence for the 454 pyrosequencing and *de novo* assembly technology platform [7]. Since the genome sizes of most *Pseudomonas* species fall between 6 to 7 Mb according to the GenBank data, this 6.48-Mb draft genome sequence of PsWT appears to be near complete. The quality of this draft genome sequence was further assessed by phylogenetic comparison of its full-length 16S rRNA gene sequence to those of 28 *Pseudomonas* strains and several outgroup bacterial strains, revealing a close taxonomical relatedness of this PsWT to *Pseudomonas* sp. UK4 and to *P. fluorescens* SBW25 (Fig. S1 in Electronic Supplementary Material).

Identification and confirmation of the spiruchostatin biosynthetic gene cluster

A Blastx search of the draft genome sequence of PsWT using our previously published protein sequences of the FK228 biosynthetic (*dep*) gene cluster (GenBank accession no. EF210776) [3] identified two scaffolds that carry a gene cluster containing genes homologous to most of the *dep*-genes. Three sequence gaps existing within a scaffold or between two scaffolds were closed by PCR amplification and sequencing, resulting in a 54,335-bp continuous DNA sequence that contains the target gene cluster designated *spi* for spiruchostatins (Fig. 2a; Table 2).

The boundaries of this *spi* gene cluster were determined by gene deletions (Fig. S2 in Electronic Supplementary Material) in conjunction with quantification of spiruchostatin titers in fermentation broth (Fig. 3a; Table 3; Fig. S3 in Electronic Supplementary Material). The upstream boundary of *spi* gene cluster was determined to start with *spiR* because deletion of *orf−1*, a putative gene upstream of *spiR*, had a negligible effect on the combined titer of spiruchostatins A and B, but deletion of *spiR* resulted in a 67 % reduction of spiruchostatin titer. Deletion of a key biosynthetic gene, *spiA*, completely abolished the spiruchostatin production. The downstream boundary of *spi* gene cluster was determined to end with *spiP* because deletion of *spiP* reduced the spiruchostatin titer by more than 80 %, while deletion of *orf+1*, a putative gene downstream of *spiP*, did not affect the combined titer of spiruchostatins. Although the observed relative abundance of spiruchostatins A/B varied somewhat in some of the mutants, we tended to believe that it was due to the variations of substrate assimilation by the spiruchostatin biosynthetic pathway.

Collectively, the *spi* gene cluster was defined to contain 14 genes (Fig. 2a; Table 2), among them, *spiA, spiDE1* and *spiE2* are NRPS genes, *spiB, spiC1* and *spiC2* are PKS genes, *spiF*, *spiH* and *spiJ* are genes encoding tailoring proteins, and *spiG* and *spiI* are putative resistance genes. *spiR* encodes an LysR-type transcriptional activator which appears to be dedicated to the biosynthesis spiruchostatins. *spiP* encodes a putative malonyl CoA acyltransferase which may be dedicated to the biosynthesis spiruchostatins.

The genes and their deduced proteins of this *spi* gene cluster exhibit a significant overall similarity to those of the *dep* gene cluster (Fig. 2a; Table 2). In particular, the deduced products of eight genes (*spiA*, *spiB*, *spiC1*, *spiF*, *spiG*, *spiH*, *spiI*, and *spiJ*) share 57 %/74 % or higher sequence identity/similarity with their respective counterparts from the FK228 biosynthetic pathway. The *dep* gene cluster does not contain a gene encoding the necessary phosphopantetheinyl transferase activity for post-translational modification of carrier proteins [11]; instead, a discrete *sfp* gene, physically detached from the *dep* gene cluster, encodes an Sfp-type phosphopantetheinyl transferase for the biosynthesis of FK228 [26]. Similarly, the *spi* gene cluster does not contain a phosphopantetheinyl transferase-encoding gene either; a search of the draft

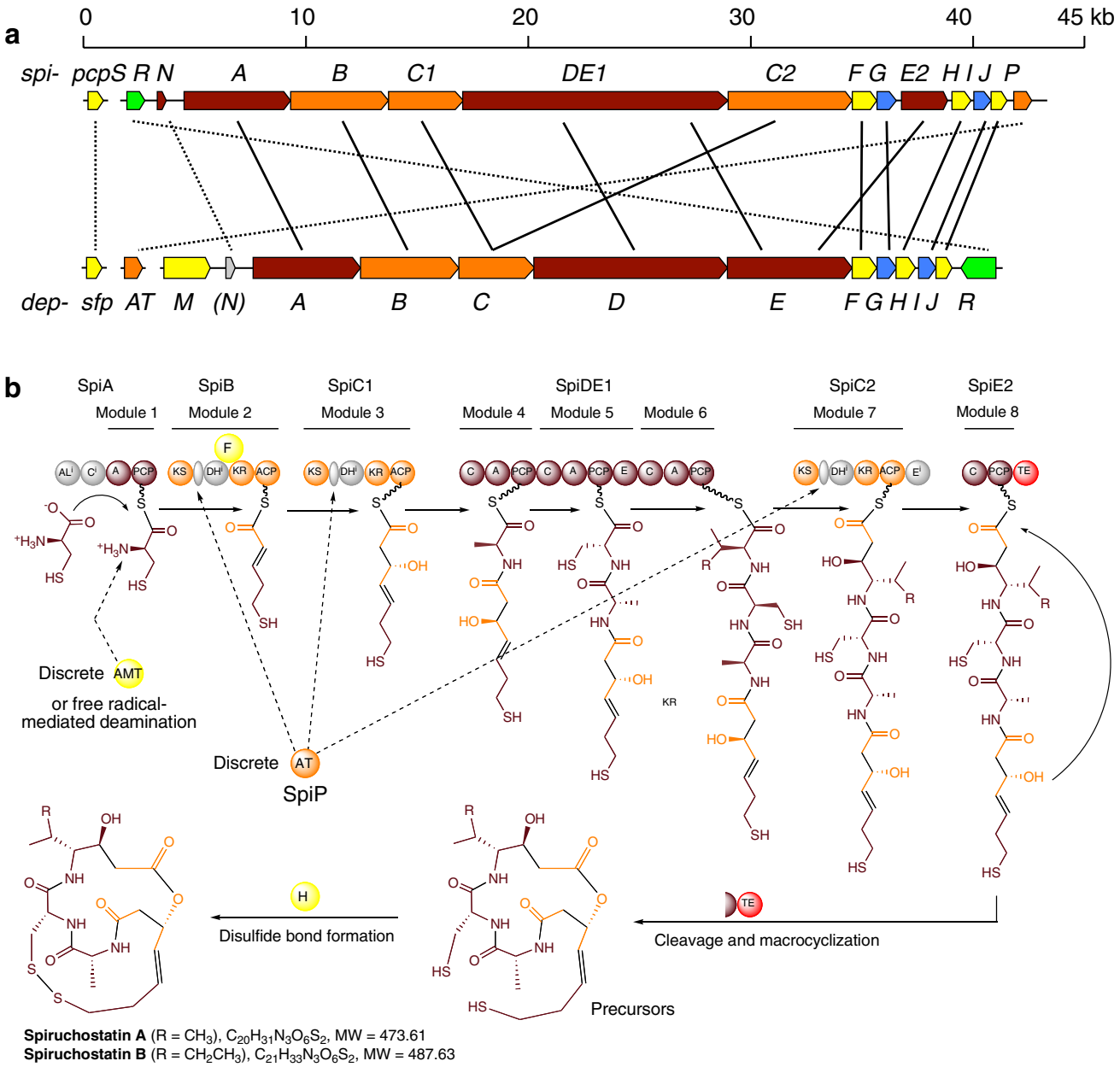

Fig. 2 Biosynthesis of spiruchostatins. **a** A comparative map of the spiruchostatin biosynthetic (*spi*) gene cluster and the FK228 biosynthetic (*dep*) gene cluster. Genes are color-coded in *dark red* for nonribosomal peptide synthetase genes, in *orange* for polyketide synthase genes, in *yellow* for genes of tailoring enzymes, in *blue* for resistance genes, and in *green* for regulatory genes. (*N*) in gray indicates a pseudogene *depN* previously defined [15]. A *solid line* connects two genes whose deduced products have a significant sequence homology and a similar functionality; a *dotted line* connects two genes whose deduced products may function similarly but do not share a sequence homology. **b** A proposed model for the biosynthesis of spiruchostatins A and B by a hybrid nonribosomal peptide synthetase-polyketide synthase pathway in *Pseudomonas* sp. Q71576

genome of PsWT identified a discrete candidate *pcpS* gene that encodes a sole *Pseudomonas*-type carrier protein synthetase (PCPS) [8] which may provide the missing phosphopantetheinyl transferase activity for the biosynthesis of spiruchostatins. Additional differences between the two parallel gene clusters or biosynthetic pathways were identified as follows: (1) unlike the FK228 biosynthetic pathway which employs a discrete acyltransferase encoded by either one of the two functionally overlapping housekeeping genes *fabD1* and *fabD2* located separately from the *dep* gene cluster [26], *spiP* appears to encode the acyltransferase activity necessary for *in trans* complementing the three "AT-less" PKS modules on SpiB, SpiC1 and SpiC2 proteins; (2) unlike *depR* which is located

Table 2 Comparison of two homologous biosynthetic gene clusters and their associated discrete genes necessary for natural product biosynthesis

Spiruchostatin biosynthetic (*spi*) gene cluster		FK228 biosynthetic (*dep*) gene cluster [3, 15, 26]		Percentage identity/similarity between protein sequences	Confirmed or deduced protein function[b]
Gene[a]	Deduced protein[b]	*Gene*	Deduced protein		
spi_pcps (putative, discrete)[c]	Spi_PCPS	*dep_sfp* (discrete)[c]	Dep_Sfp	–	Phosphopantetheinyl transferase, PPTase
spiR	SpiR	–	–	–	LysR-type transcriptional regulator
–	–	*depM*	DepM	–	Aminotransferase
spiN	SpiN	–	–	–	Type II peptidyl carrier protein (PCP)
spiA	SpiA	*depA*	DepA	63 %/73 %	NRPS (1 module)
spiB	SpiB	*depB*	DepB	64 %/77 %	PKS (1 module)
spiC1	SpiC1	*depC*	DepC	64 %/75 %	PKS (1 module)
spiDE1	SpiDE1				
	First module	*depD*	DepD	62 %/74 %	NRPS (1 module)
	Second module	*depE*	DepE	38 %/53 %	NRPS (1 module)
spiC2	SpiC2	*depC*	DepC	31 %/44 %	PKS (1 module)
spiF	SpiF	*depF*	DepF	83 %/91 %	FadE2-like acyl-CoA dehydrogenase
spiG	SpiG	*depG*	DepG	64 %/76 %	Phosphotransferase
spiE2	SpiE2	*depE*	DepE	31 %/51 %	NRPS (partial module)
spiH	SpiH	*depH*	DepH	59 %/74 %	FAD-dependent disulfide oxidoreductase
spiI	SpiI	*depI*	DepI	66 %/76 %	Esterase/Lipase
spiJ	SpiJ	*depJ*	DepJ	57 %/76 %	Type II thioesterase
spiP	SpiP	*dep_fabD1*	Dep_FabD1	36 %/47 %	Acyltransferase, malonyl CoA-specific (AT)
		dep_fabD2 (both discrete)[c]	Dep_FabD2	30 %/46 %	
–	–	*depR*	DepR	–	OxyR-type transcriptional regulator

[a] Gene/protein names designated by the authors

[b] Standard abbreviations: *NRPS* nonribosomal peptide synthetase, *PKS* polyketide synthase

[c] Detached from the perspective gene cluster; –: not available

downstream of the *dep* gene cluster and encodes an OxyR-type transcriptional activator [15], *spiR* is located upstream of the *spi* gene cluster and encodes an LysR-type transcriptional activator; these two deduced regulatory proteins only share weak sequence homology (25 % identity/44 % similarity); (3) there is no *depM*-equivalent in the *spi* gene cluster; (4) there are two copies of *depC*-like gene in the *spi* gene cluster, the second copy is fused to DNA encoding a likely inactive epimerase (E) domain and is located after *spiDE1*; (5) a *depE*-like gene in the *spi* gene cluster is split into two parts, the first part is fused to the end of *spiD*, and the second part is transposed to a downstream location between *spiG* and *spiH*; (6) unlike the pseudogene "*depN*", the deduced protein of *spiN* appears to be a functional peptidyl carrier protein (PCP) containing a critical serine residue for phosphopantetheinylation, but the role of this PCP is yet to be defined.

Using the proposed FK228 biosynthetic pathway as a reference [3, 15, 26], we dissected the domain and module organization of six deduced NRPS- and PKS-type enzymes (SpiA, SpiB, SpiC1, SpiDE1, SpiC2 and SpiE2) encoded by the *spi* gene cluster and proposed a hybrid NRPS-PKS biosynthetic pathway model which also includes three accessory enzymes (SpiP, SpiF and SpiH) (Fig. 2b). This proposed pathway contains eight biosynthetic modules responsible for seven consecutive steps of building block polymerization that results in a full-length linear intermediate installed to a peptidyl carrier protein domain on the last module. A lactamization process catalyzed by the terminal thioesterase domain is predicted to release the linear intermediate and to form a macrolactam intermediate. Finally, an FAD-dependent oxidoreductase (SpiH) is predicted to catalyze a disulfide bond formation as the final step of the biosynthesis of spiruchostatins.

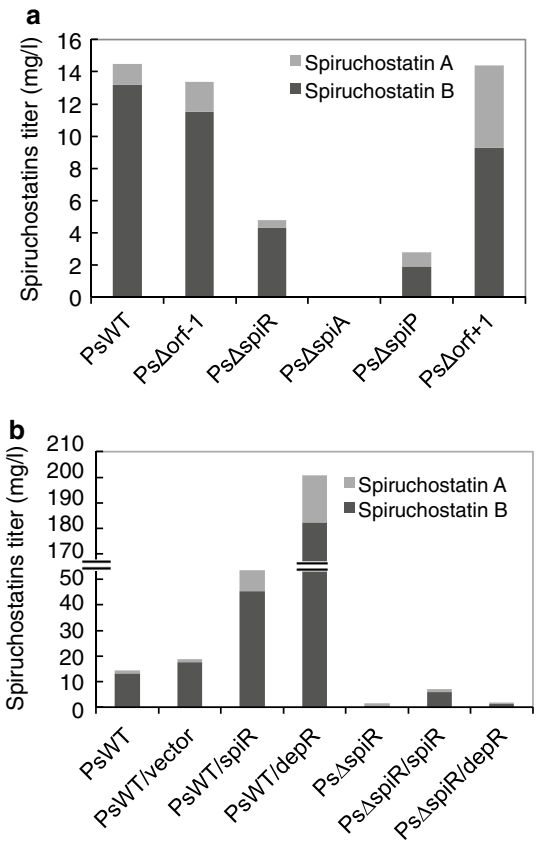

Fig. 3 Quantification of spiruchostatin titers in the wild-type and mutant strains of *Pseudomonas* sp. Q71576. **a** For the determination of spiruchostatin biosynthetic gene cluster boundaries. **b** Effects of gene overexpression. Titer results are the average value of triplicate experiments. Due to the overlaying of both spiruchostatin A titer and spiruchostatin B titer, standard deviations cannot be drawn into this figure but are presented in Table 3. Also see Fig. S3 in Electronic Supplementary Material for additional information

Table 3 Quantitative spiruchostatin titers for strains used in Fig. 3

Strain	Spiruchostatin A titer (mg/l) ± SD	Spiruchostatin B titer (mg/l) ± SD	Combined spiruchostatins A and B titer (mg/l)
Panel A			
PsWT	1.3 ± 0.49	13.2 ± 1.52	14.5
Ps*Δorf–1*	1.9 ± 0.13	11.5 ± 1.26	13.4
Ps*ΔspiR*	0.5 ± 0.11	4.3 ± 1.24	4.8
Ps*ΔspiA*	0	0	0
Ps*ΔspiP*	0.9 ± 0.14	1.9 ± 1.11	2.8
Ps*Δorf + 1*	5.1 ± 1.34	9.3 ± 0.35	14.4
Panel B			
PsWT	1.3 ± 0.58	13.2 ± 2.31	14.5
PsWT/vector	1.3 ± 0.57	17.6 ± 2.23	18.9
PsWT/*spiR*	8.0 ± 1.13	45.5 ± 3.23	53.5
PsWT/*depR*	18.5 ± 4.62	182.3 ± 13.1	200.8
Ps*ΔspiR*	0.9 ± 0.06	1.7 ± 0.06	1.6
Ps*ΔspiR/spiR*	1.3 ± 0.57	5.9 ± 0.58	7.2
Ps*ΔspiR/depR*	0.5 ± 0.06	1.3 ± 0.05	1.8

Spiruchostatin titer was determined by measuring peak area corresponding to spiruchostatin A and spiruchostatin B and then using a standard curve to generate approximate titer in mg/l. Results are the average value of triplicate experiments with standard deviation (SD). (Panel A) Spiruchostatin titers of strains determined in mutational analysis presented in Fig. 3a; (Panel B) Spiruchostatin titers of strains determined in complementation analysis presented in Fig. 3b

Titer improvement through gene overexpression and transcriptional regulation

PsWT produced c.a. 14.5 mg/l of combined spiruchostatins A and B in the fermentation broth under our laboratory conditions. Overexpression of *spiR* in PsWT resulted in a 268 % increase of the spiruchostatin production to a combined titer of 53.5 mg/l. Overexpression of *depR* from the FK228 biosynthetic gene cluster in PsWT resulted in a surprisingly 1,285 % increase of the spiruchostatin production to a combined titer of 200.8 mg/l (Fig. 3b).

To probe the molecular mechanism for this unexpected dramatic boost of spiruchostatin production, the same two overexpression constructs were also introduced into the *spiR* deletion mutant of *Pseudomonas* sp. (Ps*ΔspiR*). Complementation of Ps*ΔspiR* with *spiR* could restore 50 % production capacity of spiruchostatins, whereas *depR* alone failed to complement the loss of *spiR* in Ps*ΔspiR*.

Furthermore, semi-quantitative RT-PCR analysis of the expression of *depR*, *spiR*, *spiA* and *spiP* in PsWT, PsWT/vector control, PsWT/*depR* and Ps*ΔspiR/depR* showed that overexpression of *depR* in PsWT directly or indirectly upregulated the expression of *spiR*, *spiA* and *spiP* (Fig. 4); however, in the absence of *spiR* in Ps*ΔspiR*, overexpression of *depR* could not boost the expression of *spiA* or *spiP*. It was therefore deduced that the heterologous transcriptional activator DepR exerts its regulatory function through upregulating *spiR* expression to subsequently enhance the expression of other *spi*-genes.

Discussion

Spiruchostatins structurally resemble FK228 for having a bicyclic peptide scaffold, which bears a signature disulfide linkage critical for prodrug stability as well as for bioactivities when reduced. Spiruchostatins differ from FK228 for having three different building blocks (Fig. 1). Those overall structural similarity and yet minor differences are reflected well by the resemblance of the *spi* and *dep* biosynthetic gene clusters (Fig. 2a) and their deduced parallel biosynthetic pathways (Fig. 2b; see the deduced FK228 biosynthetic pathway in [3]).

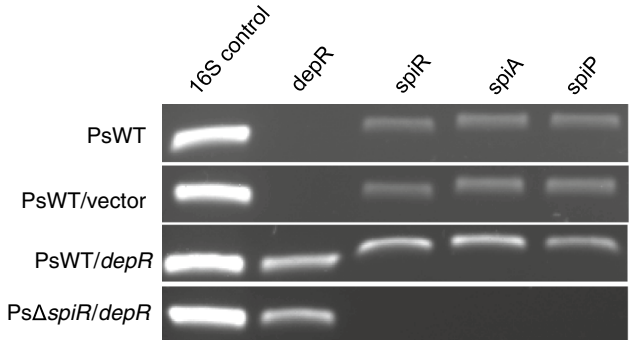

Fig. 4 RT-PCR analysis of the effect of *depR* overexpression on the transcription of *spiR*, *spiA* and *spiP* genes that represent the *spi* gene cluster. 16S rRNA gene amplification was included in Lane 1 of all panels as a positive control. Elevated transcript levels of *spiR*, *spiA* and *spiP* were seen in PsWT/*depR* whereas *spiA* or *spiP* transcript was not detected in PsΔ*spiR*/*depR* in conditions where they were expressed in PsWT at 12 h of growth

Spiruchostatins are produced by PsWT at a low titer of 14.5 mg/l, which hampered the development of spiruchostatins toward clinical applications. Possible reasons for the low yield include suboptimal level of gene expression or limited availability of biosynthetic precursors. Taking advantage of the biosynthetic gene cluster sequence obtained in this work, modulating the expression of the pathway regulatory gene *spiR* was first attempted and succeeded. Overexpression of *spiR* in PsWT resulted in a 268 % increase of the spiruchostatin production to a combined titer of 53.5 mg/l, even without a comprehensive optimization of the fermentation conditions. The biggest surprise came from the overexpression in PsWT of a heterologous regulatory gene *depR* from the FK228 biosynthetic gene cluster, which resulted in a lofty 1,285 % increase of the spiruchostatin production to a combined titer of 200.8 mg/l. This work demonstrated that the metabolic potential of natural product biosynthetic pathways could be exploited by overexpression of a native or heterologous transcriptional activator.

The deduced product of *depR* has a significant homology (87 % identity/93 % similarity) to OxyR protein of *Escherichia coli*, a pleiotropic regulator known to regulate a variety of cellular processes including oxidative stress response [5]. As determined by RT-PCR, overexpression of *depR* in PsWT increased the levels of expression of *spiR* and other *spi*-genes (Fig. 4), suggesting an artificial regulatory cascade where DepR activates the *spi* gene cluster through *spiR*. Thus, a plausible postulation for the DepR-induced dramatic increase in spiruchostatin titers may have been the binding of DepR to the regulatory elements of *spiR* which enhanced the *spiR* expression; elevated SpiR subsequently acts on other *spi*-genes to gear up the overall spiruchostatin biosynthetic machinery. Additional evidence supporting this postulation came from the observation that, in the absence of *spiR*, overexpression of *depR* failed to boost the production of spiruchostatins (Fig. 4).

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