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Revelation and cloning of valinomycin synthetase genes in *Streptomyces lavendulae* ACR-DA1 and their expression analysis under different fermentation and elicitation conditions

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ABSTRACT

Streptomyces species are amongst the most exploited microorganisms due to their ability to produce a plethora of secondary metabolites with bioactive potential, including several well known drugs. They are endowed with immense unexplored potential and substantial efforts are required for their isolation as well as characterization for their bioactive potential. Unexplored niches and extreme environments are host to diverse microbial species. In this study, we report *Streptomyces lavendulae* ACR-DA1, isolated from extreme cold deserts of the North Western Himalayas, which produces a macrolactone antibiotic, valinomycin. Valinomycin is a K^+ ionophoric non-ribosomal cyclodepsipeptide with a broad range of bioactivities including antibacterial, antifungal, antiviral and cytotoxic/anticancer activities. Production of valinomycin by the strain *S. lavendulae* ACR-DA1 was studied under different fermentation conditions like fermentation medium, temperature and addition of biosynthetic precursors. Synthetic medium at 10 °C in the presence of precursors i.e. valine and pyruvate showed enhanced valinomycin production. In order to assess the impact of various elicitors, expression of the two genes viz. *vlm1* and *vlm2* that encode components of heterodimeric valinomycin synthetase, was analyzed using RT-PCR and correlated with quantity of valinomycin using LC–MS/MS. Annelid, bacterial and yeast elicitors increased valinomycin production whereas addition of fungal and plant elicitors down regulated the biosynthetic genes and reduced valinomycin production. This study is also the first report of valinomycin biosynthesis by *Streptomyces lavendulae*.

1. Introduction

Natural products, directly or indirectly are the major source of chemical scaffolds used as drugs in modern therapeutics. By virtue of enormous structural and functional diversity, low molecular weight and biological activity, the peptide secondary metabolites of microbial origin have established a niche in pharmaceuticals, agriculture and research sectors (Newman and Cragg, 2016). Biosynthetic mechanisms of these bioactive peptide metabolites employ complex ribosome independent megasynthetases, known as non-ribosomal peptide synthetases (NRPS). Genus *Streptomyces,* known for production of several bioactive compounds, are also prolific producers of non-ribosomal peptides (NRPs). Valinomycin is a relatively small crystalline cyclodepsipeptide NRP from natural neutral ionophores group with antibacterial, antifungal, anticancer, immunosuppressive and other activities (Cheng, 2006). It was first reported by Brockmann and Schmidt-Kastner (1955) from *Streptomyces fulvissimus*. Valinomycin is synthesized by a heterodimeric valinomycin synthetase (VlmSyn), components of which are encoded by *vlm1* and *vlm2* genes (Perkins et al., 1990). The expression levels of these core genes of valinomycin biosynthetic gene cluster may vary in different species (Matter et al., 2009). Many approaches are followed for optimizing expression of biosynthetic clusters encoding the desired bioactive peptides. Significant changes in the microbial metabolome are induced by the tradi-

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tional approach of manipulating the fermentation conditions such as medium composition, pH, and temperature (Marmann et al., 2014), known as "one strain many compounds" (OSMAC) approach and thus represents an effective platform for activating cryptic or poorly expressed biosynthetic pathways (Abdelmohsen et al., 2014a,b; Bode et al., 2002; Paranagama et al., 2007; Wei et al., 2010). An innovative strategy to generate biologically active metabolites is to challenge the microbial cells with external stimuli, called "elicitors" (Chiang et al., 2011; Muller and Wink, 2014). Sensitizing the producer strains with such elicitors results in a discrete microbial response that might lead to an altered metabolite profile such as the enhanced production of stress-response-related compounds (Romero et al., 2007) or in certain rare cases, even repression of the sought-after metabolites (Marmann et al., 2014). Inter-species interactions in the natural environment are well mimicked by biotic elicitors which may constitute live or dead cells supplemented into the culture media of an antibiotic producer thus eliciting complex metabolic and physiological responses. They can be used to produce novel metabolites and also to enhance the productivity of existing antibiotics as illustrated in the present work. Elicitation of gene clusters in fungi (Brakhage, 2013; Brakhage and Schroeckh, 2011; Chiang et al., 2009) as well as in myxobacteria (Krug et al., 2008; Wenzel and Müller, 2009) has now become a paradigm, but utility of this approach in activating metabolite gene clusters in actinomycetes is yet to be fully explored and exploited.

The present study focuses on a psychrotrophic strain *Streptomyces lavendulae* ACR-DA1, isolated from cold desert of the North Western Himalayas endowed with rich microbial biodiversity. The strain is capable of producing a small crystalline NRP macrolactone, Valinomycin, with broad spectra of bioactivities. Effect of various parameters such as production medium, temperature, precursor supplementation and elicitation were studied for optimal production of the NRP.

2. Material and methods

2.1 Reagents All materials and reagents were purchased from Sigma Aldrich (USA) and Thermo Fisher Scientific (USA). Molecular Biology reagents were purchased from New England Biology (USA). The plasmid pTZ57R/T (Thermo Fisher Scientific, USA) was used for cloning of *vlm1* and *vlm2* in *E. coli*. T4 DNA ligase and TaqDNA polymerase were obtained from Thermo Fisher Scientific, USA. *E. coli* strain DH5-α was used as host for cloning experiments. IPTG and X-gal were obtained from Sigma-Aldrich, USA.

2.1. Microbial strain

Microorganism *Streptomyces lavendulae* ACR-DA1 was isolated from the soil of high altitude cold desert (at an elevation of about 10,000 feet above sea level, below sub-zero temperature) of the North Western Himalayas. Microorganism was isolated and maintained on DPB medium with composition (%): dextrin- 1.5, peptone- 0.5, yeast extract- 0.1, KH_2PO_4- 0.1, Na_2HPO_4- 0.06, $MgSO_4$- 0.05 and pH adjusted to 7.0. The psychrotrophic actinobacterium ACR-DA1 has been submitted to Sir R.N. Chopra Microbial Resource Centre, Jammu, India, with accession number **MRCJ 199**.

2.2. Molecular characterization of the strain ACR-DA1

Microbial strain ACR-DA1 was cultured on DPB broth medium at 30 °C for 4 days. Genomic DNA was extracted and PCR amplification of 16S rDNA gene was performed by using universal primers, B27F (AGAGTTTGATCMTGGCTCAG) and 1492R (CGGTTACCTTG TTACGACTT). Amplicon was sequenced and analyzed using the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov/) and a phylogenetic tree was constructed to ascertain the identity using MEGA 5.0 program (Saitou and Nei, 1987; Tamura et al., 2011). The nucleotide sequence obtained upon sequencing of the amplified PCR product of the 16S rRNA of the actinobacterium ACR-DA1 has been submitted to GenBank database with accession number **KX698040**.

Table 1
List of primers used in present study.

Primer	Sequence (5′ → 3′)	T_m(°C)	Use
vlm 1p1	GCATTCTCATGTCGCATCTG	54.1	amplification of *vlm1*
vlm 1p2	CAGCCTGATCCCGATCCT	56.6	amplification of *vlm1*
vlm 1p3	GCATTCTCATGTCGCATCTG	54.1	amplification of *vlm1*
vlm 1p4	TTGAGACCGTTCACGATCAG	54.6	amplification of *vlm1*
vlm 2p1	GTGCAGTTCTGCTCCGACTC	58.2	amplification of *vlm2*
vlm 2p2	GGATGTCGACGAAGGTCAC	55.4	amplification of *vlm2*
vlm 2p3	CACCGGTCTATCCGTCGTT	56.7	amplification of *vlm2*
vlm 2p4	GGATGTCGACGAAGGTCAC	55.4	amplification of *vlm2*
VLM1RTF	CTTCGCGAGTCCATCAAT	60.2	qPCR of VlmSyn; gene *vlm1*
VLM1RTR	CGAACTCCCGGTTGTAGA	60.0	qPCR of VlmSyn; gene *vlm1*
VLM2RTF	TCTTCGCCTCCTCGTAG	59.8	qPCR of VlmSyn; gene *vlm2*
VLM2RTR	GACTCATCATCCTCGACAC	59.3	qPCR of VlmSyn; gene *vlm2*
hrdBF	GAAGGTCATCGAGGTCCAGAG	62.1	housekeeping gene as control in qPCR
hrdBR	GTGGCGGAGCTTCGACATC	61.0	housekeeping gene as control in qPCR
M13F	GTAAAACGACGGCCAGT	52.6	amplification of insert in pTZ57R/T vector
M13R	CAGGAAACAGCTATGAC	47	amplification of insert in pTZ57R/T vector

2.3. Cloning of core fragment of vlm1 and vlm2 genes of VlmSyn

vlm1 and *vlm2* gene sequences available in NCBI GenBank database were used for designing primers from the conserved domains of the valinomycin synthetase of the producer species. For *vlm1* gene, primers vlm1p1 and vlm1p2 were used for initial amplification and the product of this primary PCR was used as a template for nested PCR using the primers vlm1p3 and vlm1p4. For *vlm2* gene, a similar strategy was employed, using primers vlm2p1 and vlm2p2 for primary PCR and primers vlm2p3 and vlm2p4 for nested PCR (Table 1). The amplification was carried out in total volume of 100 µl using Master Cycler® pro (Eppendorf, Germany). PCR program used for amplification was as follows: initial denaturation (5 min at 95 °C), followed by 35 cycles of denaturation (95 °C for 30 s), annealing (52 °C for 30 s) and primer extension (72 °C for 1 min), followed by final extension step for 10 min at 72 °C. The PCR amplified DNA fragments obtained from the nested PCRs were ligated into the pTZ57R/T InsTA cloning vector (Thermo Fisher Scientific, USA) and the ligated vectors were used to transform competent *E. coli* DH5α cells. Recombinants were identified by blue-white selection and colony PCR using the M13 vector specific primers. Plasmids were isolated from recombinants and amplified insert fragments were sequenced and the sequences were analyzed using NCBI-BLAST. The core sequences so obtained were used for designing real-time PCR primers (Table 1) using Light Cycler Probe Design software 2.0 (Hoffmann La Roche, Switzerland) for both the genes in order to carry out expression analyses. These primers were checked for single band amplification using conventional end point PCR.

2.4. Sequence analysis

The nucleotide sequences of *vlm1* and *vlm2* genes cloned from *S. lavendulae* ACR-DA1 were conceptually translated using ExPASy translate tool (http://web.expasy.org/cgi-bin/translate/dna) and the peptide sequences submitted to NCBI. Multiple sequence alignment was performed with related protein sequences of the two genes from