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Short Report

Gene Deletion Leads to Improved Valinomycin Production by *Streptomyces* sp. CBMAI 2042

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The genus *Streptomyces* represents one of the largest producers of molecules with antibiotic activity. The whole genome sequencing of the endophytic microorganism *Streptomyces* sp. CBMAI 2042 revealed 35 gene clusters encoding for secondary metabolism including 3 non-ribosomal peptide synthetases (NRPS) and 7 NRPS-hybrids. Combining genome mining and cultivation profile analysis, the depsipeptide ionophore valinomycin was identified as one of the main metabolites produced by this strain. To better understand the metabolic machinery codified in CBMAI 2042 genome an adenylation domain from a hybrid NRPS cluster was deleted through a double crossing knockout experiment. Though the deletion was not plentiful to elucidate the encoded NRP metabolite related to the adenilation domain, an astounding increase of 10.5-fold in valinomycin production was observed in the mutant. These results suggest a metabolic flux redistribution of common substrates as an outcome of a gene target deletion.

Keywords: genome mining, gene target deletion, valinomycin

Introduction

The genus *Streptomyces* is a highly prolific producer of structural diverse natural products. These actinobacteria represent an outstanding source for leads that inspire and guide the drug discovery programs.[1,2] Among the diversity of biosynthesized metabolites, non-ribosomal peptides (NRPs) have a wide spectrum of biological activity including antibiotic (vancomycin),[3] immunosuppressant (cyclosporine A) and antineoplasics (enniatins).[4] Typically, complex peptides in bacteria are produced by non-ribosomal peptides synthetases (NRPS), a remarkable assembly-line multienzyme system able to extend and modify amino acid chain, in specific enzyme locus termed modules.[5] Many NRPS follow the canonical assembly line as described, but variations including different constructions of the modules, iterative mechanisms, as well as the incorporation of more unusual extender units are observed.

Despite the wide applicability, NRP discovery and exploitation are limited, as manageable amounts are necessary for subsequent biological and pharmacological studies. Reciprocally most of them are impractical to be obtained by chemical synthesis due to the complexity of their backbones. The possibility to interfere in biosynthetic pathways in a genetic level expanded the number of strategies available to study the megasynthetases enzymology responsible to outturn non-ribosomal peptides[6,7] enabling to improve efficiency of production and to increase fermentation titers. Correspondingly, the enhancement of natural hosts capable to overexpress a specific pathway is an attractive approach when a significant amount of purified material is needed to advance in clinical trials, for instance.[8] In addition, tools for gene deletions,[9,10] gene replacements, heterologous expression in appropriated hosts and insertion of stronger promoters[11,12] are among the most suitable methodologies of choice for genetic manipulation of the biosynthetic pathways.

Streptomyces sp. CBMAI 2042 is an endophytic isolated from *Citrus sinensis* branches. The whole genome sequencing of this actinobacteria evidenced a high biosynthetic potential uncovering third five gene clusters related to secondary metabolism. It was possible

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to identify suitable classes of enzyme assembly lines encrypted to produce polyketides (PKs) and NRPs. During our dereplication approach, valinomycin (Figure 1) was identified as one of the main metabolite produced by fermentation of this strain.

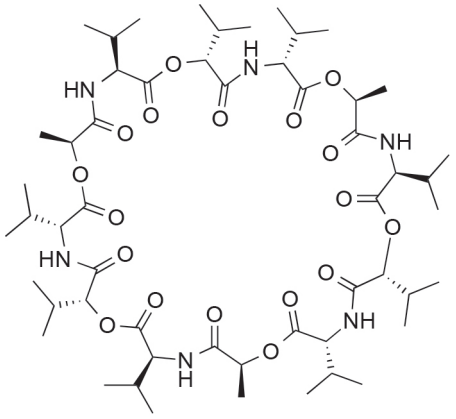

Figure 1. Chemical structure of valinomycin.

Valinomycin (VLM) is a depsipeptide ionophore that plays several biological functions such as antibacterial, antiviral and anticancer. It is also a powerful therapeutic for the treatment of severe acute respiratory syndrome (SARS).[13] Its production was already described in several *Streptomyces* strains around the world[14] and its biosynthetic gene cluster was well-defined throughout *S. levoris* A9[15] and *S. tsusimaensis* ATCC 15141[13] studies. Two large NRPS genes *vlm1* and *vlm2* and other small functional (ORFs) are involved in valinomycin biosynthesis. VLM1 and VLM2 are responsible for incorporation of D-hydroxy-isovaleric acid, D-valine, L-lactic acid and L-valine. A thioesterase domain located in VLM2 promotes final cleavage and cyclization.

In 2014, Neubauer and co-workers[16] were able to clone and co-express VLM1 and VLM2 from *S. tsusimaensis* in an engineered *E. coli* strain (BJJ01) and reconstitute the biosynthesis of valinomycin in heterologous host. After optimizing a fed-batch culture VLM production[17] was increased 33-fold (10 mg L^{-1}) whereas the co-expression of a type II thioesterase (TEII)[18,19] allowed a 43-fold increase (13 mg L^{-1}), both compared to the initial batch culture (0.3 mg L^{-1}). This system was later used on fed-batch cultivation to develop a modeled scaled-up bioprocess for the antibiotic assembly. Lee *et al.*[20] also succeed to improve four-fold the VLM production in *Streptomyces* sp. M10 by redirecting the flux of a common precursor in bafilomycin and valinomycin biosynthesis by gene deletion experiments.

In this study we describe a 10.5-fold increase in valinomycin production by *Streptomyces* sp. CBMAI 2042 as a result of a target gene deletion. The deleted gene holds an adenylation domain of a hybrid NRPS-PKS (NRPS-polyketide synthase) cluster encoding to an unknown metabolite annotated from the whole genome sequencing and, apparently, unrelated to valinomycin gene cluster. Our experiments suggest a deep modification in the functional metabolism of CBMAI 2042 after punctual deletion, involving a redistribution of common precursors.

Experimental

Bacterial strain and culture conditions

Streptomyces sp. CBMAI 2042 was grown in TSBY liquid medium (3% tryptone soy broth, 10.3% sucrose, 0.5% yeast extract) at 30 °C and 250 rpm for preservation and genomic deoxyribonucleic acid (DNA) extraction. For valinomycin production, the seed medium was TSBY liquid medium and the expression liquid medium was GYM (1% malt extract, 0.4% yeast extract, 0.4% dextrose) or A liquid medium (0.4% peptone, 0.2% yeast extract, 0.4% meat extract, 0.2% soy-bean meal, 2.0% maltose and 1.0% potato dextrin). Fermentation was carried out after inoculating 10% of 2-day old seed culture in a 500 mL Erlenmeyer fitted with a metal spring containing 100 mL of GYM media, at 30 °C and 180 rpm for 7 days.

DNA manipulation

Chemicals were purchased from Sigma-Aldrich®, restriction endonuclease (*NdeI*) was purchased from Thermo Scientific®. Plasmid DNA was isolated from an overnight culture using Mini Prep kit (QIAGEN®) following the manufacturer's protocol. Polymerase chain reaction (PCR) amplification for plasmid construction was carried out using Phusion Flash High-Fidelity master mix from Thermo Scientific®. My RedTaq polymerase (Bioline®) was used for screening purposes. Genomic DNA was isolated using a protocol described and adapted from Sharma and Singh.[21]

Plasmid construction

The DNA fragments flanking the adenylation domain were amplified from *Streptomyces* sp. CBMAI 2042 genomic DNA by PCR using oligonucleotides pΔcal-F1 (5'-TGATCAAGGCGAATACTTCATATGCGCGTGGG CGAGCGAGCGCAGCTC-3') and pΔcal-R1 (5'-GGCCCGGTGTTGCGGGAGCCCTTCGCC GGTGCTCCG-3') to construct fragment 1 and pΔcal-F2

(5'-GCCGATCACCGCCGGAGCACCGGCGAAGGG CTCCCG-3') and pΔcal-R2 (5'-CCGCGCGGTCG ATCCCCGCATATGATGGACGGGTACGAGGAGG ATGTC-3') to construct fragment 2. For DNA assembly 1 μL of pYH7 vector previously digested with *NdeI* and 3 μL of each fragment were added in 13 μL of Gibson DNA assembly mix.[22] The reaction was carried out during 1 h at 50 °C, then 10 μL of the mix were used to transform chemically competent cells of *E. coli* DH10B. Around 30 recombinants were observed in lysogeny broth (LB)-agar plate supplemented with apramycin (25 μg mL^{-1}). At least 5 colonies were selected to extract plasmid and a double restriction assay using *NdeI* and *EcoRI* was used in a restriction analysis to confirm the correct construction of pBSΔcal (Figure S2, Supplementary Information (SI) section).

Intergeneric conjugation

The resulting plasmid pBSΔcal was introduced in electrocompetent cells of *E. coli* ET12567/pUZ8002. The intergeneric conjugation with *Streptomyces* sp. CBMAI 2042 was performed based on methods described in the literature.[23] For selection of first crossover's exconjugants, apramycin and nalidixic acid were used at concentrations of 25 μg mL^{-1}. Exconjugants were grown in SFM-agar (2% soya flour, 2% D-mannitol, 2% bacteriological agar) at 30 °C without antibiotics during 16 h. Afterwards, the SFM plates were overlaid with a solution containing apramycin (25 μg mL^{-1}) and nalidixic acid (50 μL mL^{-1}) and left for 48 hours at 30 ºC. Colonies presenting apramycin resistance (potential mutants) were transferred to another SFM-agar plate without antibiotics (96 h, 30 ºC), to allow the second crossover event. To check the loss of apramycin resistance, potential mutants were transferred to SFM-agar plates supplemented with apramycin (25 μg mL^{-1}) and nalidixic acid (25 μg mL^{-1}). Colonies that did not appear in apramycin-SFM plates were cultivated in TSBY liquid medium and the genomic DNA was extracted for PCR analysis. Purified genomic DNA was used as a template with oligonucleotide pair scree_pΔcal_F (5'-CAGGAGCCACAGGAAACGCGGCAG-3') and scree_pΔcal_R (5'-CTGACCGTCACGCTGCGCGAA CTCC-3'). Detailed PCR characterization of the deleted mutant can be accessed on SI section (Figures S4 and S5).

LC-MS/MS analysis of metabolites

Liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis was performed using an ultra-performance liquid chromatography (UPLC, Agilent technologies 1200 series) coupled to a hybrid quadropole-time of flight Agilent Q-TOF mass spectrometer fitted with an electron spray ionization (ESI) source. The source condition gas was: gas temperature 290 °C, drying gas flow rate at 14 L min^{-1}; sheath gas temp of 300 °C; Vcap 300 V; Fragmentor at 150 V; Octa 750 V. The mass spectrometer worked in positive ion mode scanning mass from *m/z* 200 to 1500. The UPLC column used in this work was Extend C-18 1.8 μM (2.14 × 50 mm, Agilent) and the solvent gradient system of acetonitrile (solvent B) and water (solvent A) both containing 0.1% formic acid (v/v) was used. For quantification experiments the samples were eluted with a flow rate of 0.7 mL min^{-1} and the following linear gradient: 80% B; 0-3 min, 80% B to 90% B; 3-5 min, 90% B to 99.9% B, 5-7 min hold at 99.9%. For metabolic profile the linear gradient was: 5% B; 0-3 min, hold 5% B; 3-20 min 5% B to 70% B, 20-25 min 70% B to 99% B then 25-30 hold 99% B. All gradients finished with 3 min of equilibration time at initial gradient.

Results and Discussion

The *Streptomycetes* are among the most reliable sources of secondary metabolites bearing wide spectrum of biological activity. The biosynthetic potential of these genera was reinforced after more than 1500 *Streptomyces* whole genome sequences have been deposited in Genomes On Line Database (GOLD). It has been verified that symbiotic microorganisms as endophytic have been coevoluting with the plant host during many years and represent a generous source for new natural products with relevant pharmacological activity.[1]

Streptomyces sp. CBMAI 2042 is a *Citrus sinensis* endophyte and was identified based on its 16S rDNA gene sequence. The interest in this strain relies in our previous observation on the ability to inhibit the growth of *Bacillus megaterium*, *Staphylococcus aureus* and *Candida albicans*[24] by diffusion test in agar plates. To better know its potential, this actinomycete was completely sequenced using ilumina technology (MiSeq) providing a linear chromosome with ca. 8 Mbp, which were divided in 4 contigs. Analysis through antiSMASH[25] platform highlighted 35 biosynthetic gene clusters (BGC) related to the secondary metabolism (Table S1, SI section). The presence of NRPS and PKS could be accented as previously implied.[24]

Based on the whole genome sequence information, *Streptomyces* sp. CBMAI 2042 was cultivated under several different nutritional conditions, however, A and GYM medium were elected for further fermentation experiments due to the most diverse profile of secondary

metabolites disclosed. Through LC-MS/MS analysis and dereplication valinomycin was identified as one of the main metabolites produced under culture cultivation. Using ESI(+) as ionization technique, valinomycin was detected as its protonated (*m/z* $[M + H]^+$ 1111.6399, calcd. 1111.6390, 0.8 ppm error), ammoniated (*m/z* $[M + NH_4]^+$ 1128.6693, calcd. 1128.6685, 3.4 ppm error), sodiated (*m/z* $[M + Na]^+$ 1133.6272, calcd. 1133.6209, 5.5 ppm error), and potassiated molecules (*m/z* $[M + K]^+$ 1149.5927, calcd .1149.5949, 2.3 ppm error) (Figure S1, SI section). High accuracy MS/MS data acquired in a QTOF mass spectrometer of the most abundant ion (*m/z* 1128.6693) undoubtedly confirmed the production of the cyclic depsipeptide antibiotic via the structurally diagnostic fragment ions resulting from losses of CO (*m/z* 1083.6451), Hiv-Val (*m/z* 884.5229), Lac-Val (*m/z* 713.4339), Hiv-Val (*m/z* 514.3104), Lac-Val (*m/z* 343.2215) and Hiv-Val (*m/z* 144.1035) (Table S2, SI section). The retention time (t_R 22.13 min) and MS data of standard VLM (Sigma-Aldrich®) also corroborate to undoubtedly identify the metabolite.

Depsipeptides scaffolds are typically biosynthesized by non-ribosomal peptide synthetases (NRPS). The genome of *Streptomyces* sp. CBMAI 2042 contains three NRPS related genes and five hybrid clusters codifying PKS-NRPS scaffolds. Among the gene clusters spotted in Table S1, SI section, only the BGC 30 shares important homology with the genes responsible to valinomycin biosynthesis. This BGC was fully analyzed and correlated with those reported for valinomycin production in *Streptomyces tsusimaensis*[13] and *S. levoris.*[15] antiSMASH output of the whole genome sequencing revealed two contiguous open reading frames, spanning 10341 bp to *vlm1* and 8031 bp to *vlm2*, both correlated with valinomycin biosynthesis. These ORFs encode two non-ribosomal peptide synthetase with very significant sequence identity to *vlm1* (84%) and *vlm2* (85%) from *S. tsusimaensis.*[13] Linked to *vlm1* there is a type II thioesterase believed to act as a proofreader during the release of misprimed thioesters from carriers proteins.[26] Transport related genes and other genes with no putative function on valinomycin biosynthesis were also observed (Figure 2).

By mining genome sequencing, the BGC 34 was predicted as a thiopeptide-NRPS-t1-PKS sharing 33% correspondence to a lactazole[27] framework. This BGC contains essential genes correlating to a thiopeptide natural product as a class I lantibiotic dehydratase from *Streptomyces lactacystinaeus* (48% lazB), a known lactazoles producer. Genes encoding for the enzymes homologs dehydrogenase (53% lazF), cyclodehydratase (64% lazE), hypotetical protein (42% lazD) and an own putative peptide leader (citA) were also predicted. An homolog for lazC was not observed. A lipopetide structure related NRPS-t1-PKS was annotated on the left arm of the gene cluster (Figure 3).

With the intention to promote a metabolic profile differentiation a gene knockout was planned to generate a

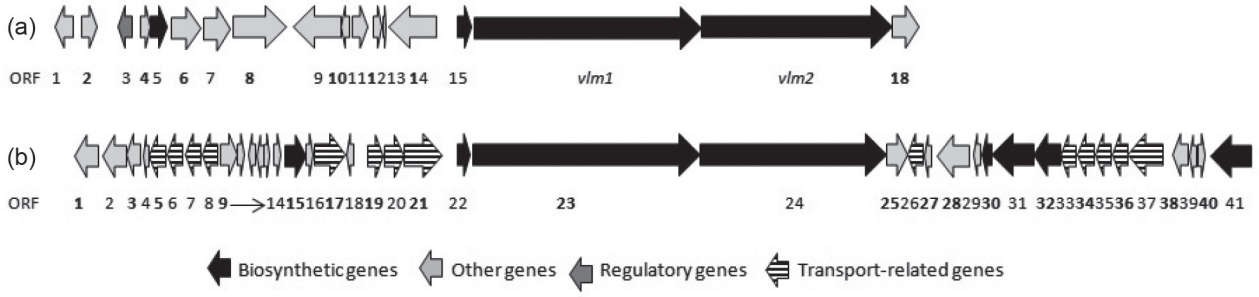

Figure 2. Homologues valinomycin biosynthetic gene clusters. (a) BGC from *Streptomyces tsusimaensis* ATCC 15141 (adapted from reference 13); (b) BGC from *Streptomyces* sp. CBMAI 2042 annotation. In black are the biosynthetic genes (23-*vlm1*; 24-*vlm2*), striped are transport-related genes and in grey are other/non-biosynthetic genes.

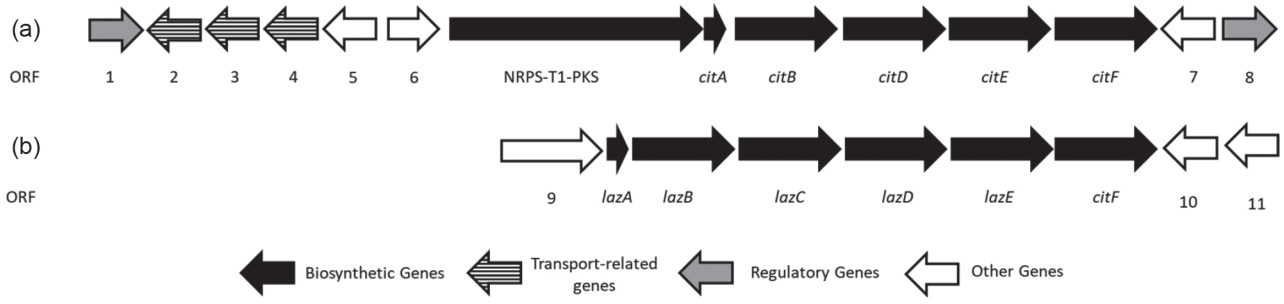

Figure 3. Comparison between (a) cluster 34 and (b) lactazole biosynthetic gene cluster from *Streptomyces lactacystinaeus*.

NRPS$^-$ mutant via double crossing over experiments. The elected gene encodes an NRPS-t1-PKS annotated in cluster 34. The expectation when designing this experiment was to get any insight about this gene cluster functionality as NRP producer or as a post-translational modifier during the thiopeptide biosynthesis. Remarkably, the main difference observed by comparison of the wild type and *cal*$^-$ mutant metabolic profile was the intensity of some peaks with a considerably enhancement in valinomycin production.

Exploring the outline that most gene clusters are substrate regulated, experiments involving knockouts of unique modules and domains responsible for recognition and modification of common building blocks could be a viable strategy to increase production of a target metabolite.[20] Meng *et al*.[28] succeeded to improve 24% the avermectin production in *Streptomyces avermitilis* by knocking out the gene *rpp* from a type III PKS specific to recruit malonyl-CoA subunits. The competing type I PKS responsible to biosynthesize the reduced polyketide uses this same precursor as building block. As cited before, Lee *et al.*[20] promoted the disruption of *bafV* gene responsible to provide isobutyric acid to the biosynthesis of bafilomycin in *Streptomyces* sp. M10. A null *bafV* mutant increased 1.5-fold the valinomycin production due to the redistribution of the common building block ketoisovaleric acid.

Neubauer's VLM synthetases studies identified L-Val as precursor for both the L- and D-form of valine in valinomycin. A deamination reaction to the corresponding keto acids and subsequent reduction produces the hydroxy acid precursors constituents, D-α-hydroxyisovaleric acid (D-Hiv) and L-lactate (L-Lac) from the α-amino acids. These results also demonstrate that VLM1 and VLM2 are highly specific for α-ketoisovaleric acid (Kiv) and pyruvate (Pyr) incorporation.[16]

The NRPS-t1-PKS gene is predicted to compose three units of condensation domain, one of them specific to insert subunits from the pyruvate pathway in the construction of lipopeptides also called *cal* domain.[29] Consulting the MIBiG repository prediction we have found homology to an NRPS gene associated to cystomanamides (CtmA) lipopeptides (37%) from *Cystobacter fuscus* Mcy9118[30] and a thiazoline related compound, micacocidin (MicC), from the bacterium *Ralstonia solanacearum* (31%).[31] The similarity among these primary sequences within valinomycin main proteins were compared using multiple sequence alignment (Muscle)[32] (Figure 4). All incorporated subunits encoded by those sequences rely on pyruvate pathway essential to afford precursors involved in valinomycin biosynthesis.

Based on these results we proceed to perform valinomycin quantification in the mutant (*NRPS*$^-$) and wild type. Both strains were grown during 2 days in TSBY liquid medium at 30 °C and 250 rpm, after that an aliquot of 1 mL of each flask were transferred to GYM and A liquid medium which were maintained for 7 days. Cells were harvested by centrifugation at 4000 rpm, during 10 min at 25 °C. Aliquots (100 mL) of supernatant were extracted with same volume (1:1) of ethyl acetate and evaporated, then dissolved in 1 mL of acetonitrile/water. From those stock solutions, samples were prepared in triplicate by 100 times dilution and quantified in LC-MS/MS (Q-TOF 6500 iFunnel series).

The calibration curve linearity was evaluated in a range of 0.05-4.00 μg mL^{-1} of VLM standard solution purchased from Sigma-Aldrich. The curve showed a linear regression coefficient of 0.99 (Figure S3, SI section). Quantification of VLM was done by 10^3 dilution extracts analysis followed by linear regression equation interpolation (Table S1, SI section). The wild type *Streptomyces* sp. CBMAI 2042

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VLM1          EPDGAAVIFFTSGSTGAPKGAVQTHGAILARE-----AGVMQSDGGGRDVQLNWMPLEHA
CtmA          RPEDIALIQFSSGSTGEPKGVIVTHRAAIVNAQ----DMLATLRVTGKDVFLGWMPLTHD
MicC          TPESIAFLQYTSGSTGSPKGVVNRHGALLRNLQFLGRLTRPQDRAPEDTAVASWLPLFHD
NRPS-34       GPDDVAFVQFSSGSTGAPKGVEVTHRGVLANLE----QIREASALNADDVLVSWMPYFHD
VLM2          DPESPALILFTSGSTGTPKGVVQSHANIVHKQQ----AAVQHSAYAADDVFLNWLAIEHV
                *:. *.: ::***** ***.   *   : .                .  .*:.  *

VLM1          ELDLEFIGRVDHQVKVRGYRVELSEIELAVRSLEYVRDVAIEARTPEGGTLRLVAFYA-A
CtmA          DGRLEYLGRGDGQVKLRGHRIELGEIESALLKHERIRATTVVLHASPEGQRALVAYVVGD
MicC          DGTLEFLGRRDFQVKIAGNRIELGEIESALLRHAGLRDAVVDAIGPARGNKRLAAWVVPK
NRPS-34       DGTLEFLGRLDDQVKVRGNRVEPGEIEAVLQARADIAQAVVLLQNG-----RLVAFVTPR
VLM2          DGQLHYHGRRDGQVKVRGHRVELNAVRSALCSLAGVKDAFVVARPGAAG--ALTAHVA-A
              :  * : ** * ***: * *:* . :  .:     :  . :          *.*  .
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Figure 4. Comparison among the conserved adenylation domain motifs responsible to biosynthesize valinomycin (VLM1 and VLM2), cystomanamides (CtmA), micacocidin (MicC) and the investigated NRPS-34 gene (NRPS-34).

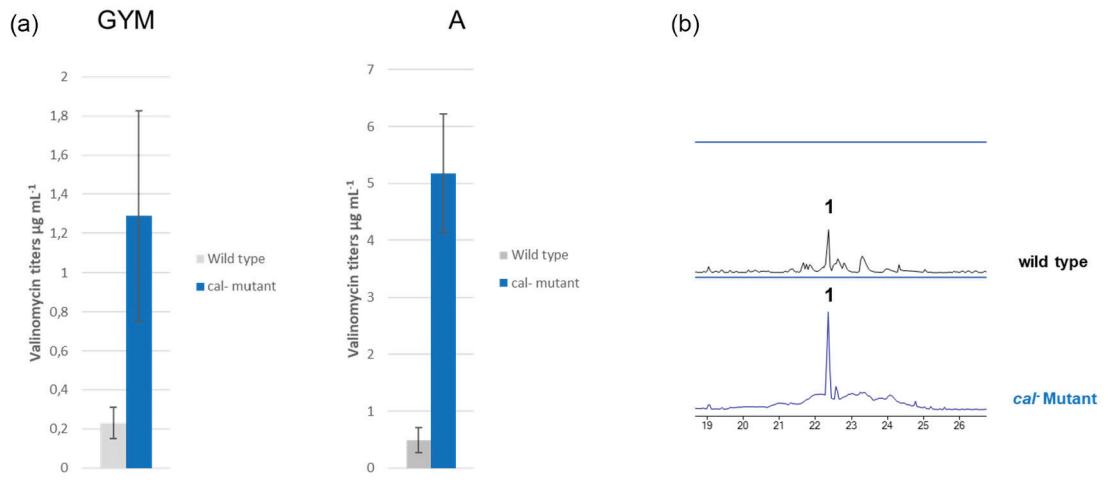

Figure 5. (a) Valinomycin increment production by *cal*⁻ gene disruption in *Streptomyces* sp. CBMAI 2042 in GYM and A liquid medium; (b) comparative total ion chromatogram between wild type and *NRPS*⁻ mutant concerning valinomycin production.

produces near to 0.231 ± 0.087 mg L^{-1}, whereas *cal*⁻ mutant yields near to 1.290 ± 0.538 mg L^{-1} representing an increasing titer about 5.58-fold. Changing to A liquid medium we could obtain 0.491 ± 0.221 mg L^{-1} for the wild type, whereas mutant produces 5.171 ± 1.048 mg L^{-1}, an increased production of 10.5-fold.

Quantification experiments demonstrated an increased production of valinomycin titers by 10.5-fold in A liquid medium and 5.58-fold in GYM liquid medium. For each 1000 mL of GYM liquid medium analyzed we could expect 1.29 mg of valinomycin in *cal*⁻ mutant, whereas in the wild type we could observe only 0.23 mg of valinomycin (Tables S3 and S4, SI section). The most productive medium liquid A resulted in 5.17 mg of valinomycin in *cal*⁻ mutant, while 0.49 mg was quantified in the wild type (Figure 5). Apparently, A liquid medium is richer in nutrients compared to the GYM (DNA manipulation subsection), favoring incremental production of the depsipeptide.

Conclusions

Genome sequencing of the endophytic *Streptomyces* sp. CBMAI 2042 identified at least 35 gene clusters encoding to secondary metabolism. Dereplication methodologies revealed that *Streptomyces* sp. CBMAI 2042 is a promising producer of cyclodepsipeptides scaffolds. Analyzing those extracts it was possible to identify the production of valinomycin.

To get a better understanding about the metabolic functionality of adenylation domain involved in biosynthesis of a hybrid NRPS gene cluster, a knockout experiment was performed and resulted in overexpression of valinomycin titers about 5.58-fold in GYM medium, whereas in A medium the increment was about 10.5-fold. Those sets of results revealed a deep modification in substrate efflux that can be a powerful tool to increase titers of related secondary metabolites in *Streptomyces* species.

Supplementary Information

Supplementary information (LC, LC-MS and MS/MS data) is available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

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