A Novel Strategy for Heterologous Production of the Depsipeptide Ionophore Valinomycin

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Abstract: Streptomyces genome analyses have revealed an enormous abundance of gene clusters codifying enzymes from secondary metabolism inferring that their potential to produce metabolites is underestimated. Among the mostly useful natural products, those biosynthesized by nonribosomal peptides synthetases share both high molecular complexity and therapeutic activity. A gene cluster encoding the biosynthesis of valinomycin, a depsipeptide ionophore, was annotated from the whole genome sequencing of the endophytic strain Streptomyces sp. CBMAI 2042 isolated from Citrus ssp. In the present study, we describe the association of fermentation process, molecular networking and mass spectrometry imaging which revealed the production of the ionophore by the endophytic strain. Additionally, the heterologous expression of the entire valinomycin gene cluster in S. coelicolor M1146 host was successfully promoted, representing an alternative and unique platform for production of new valinomycin derivatives in future studies.

Keywords: valinomycin; ionophore; molecular networking; genome mining; heterologous expression.

1. Introduction

Genome analysis have helped to elect Streptomyces strains as one of the most prolific producers of secondary metabolites and a valuable source of natural products for drug discovery [1]. A pronounced progress in the search for new metabolites occurred mainly after the advances in genome sequencing and improvement of bioinformatics tools, revealing a massive abundance of biosynthetic gene clusters (BGC) codifying for enzymes involved in secondary metabolism that were not able to be accessed by laboratory culture conditions. The access to whole genome sequencing (WGS) is the first step of genome mining approach, that consists essentially in screening BGC based on genetic level by comparative analyses between databases and an available unexplored sequenced genome previously to in vitro/in vivo manipulations [2–4]. Methodologies leading to whole pathways heterologous reconstitution evolved markedly in recent years and are becoming the most efficient approaches to characterize and to study the enzymology of entire gene clusters of both known and unknown metabolites. The use of genomic tools therefore represents a promising avenue to isolate new molecules, elucidate biosynthetic pathways, study enzymes function and characterize biosynthetic intermediates.

During the last decades several biosynthetic pathways associated to polyketides (PKs) and nonribosomal peptides (NRPs) megasynthases were sequenced, cloned, and reconstituted by heterologous expression [5,6]. Such possibilities disclosed that it is possible both to improve the efficiency of fermentation processes and to generate “unnatural” natural products. Merging the development of vectors that accept entire gene clusters and heterologous expression in improved Streptomyces strains represents a genuine alternative to investigate unknown or cryptic biosynthetic
functions as well as to generate new derivatives through metabolic engineering and combinatorial biosynthesis [7–9].

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) [10]. Its production was already described in several Streptomyces strains around the world [11] and its biosynthetic gene cluster was well-defined during S. levis A9 [12] and S. tsusaiensis ATCC 15141 [10] studies. Two large NRPS genes vlm1 and vlm2 and other small functionally ORFs are involved in the biosynthesis of valinomycin. VLM1 and VLM2 are responsible for the incorporation of D-hydroxi-isovaleric acid, D-valine, L-lactic acid and L-valine. A thioesterase domain located in VLM2 promotes final cleavage and cyclization.

In 2014, Neubauer's research group [13] were able to clone and co-express VLM1 and VLM2 from S. tsusaiensis in an engineered E. coli strain (BJ01) and reconstituted the biosynthesis of valinomycin in heterologous host. After optimizing a fed-batch culture the VLM production [14] was increased 33-fold (10 mg L\(^{-1}\)) whereas the co-expression of a type II thioesterase (TEII) [15] 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 production [16]. Lee et al. [17] 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.

Herein we report the in vivo gene cluster expression in heterologous host of valinomycin identified in the endophytic strain Streptomyces sp. CBMAI 2042 isolated from Citrus ssp. [18]. Fermentation, molecular networking and mass spectrometry imaging initially pointed out the production of valinomycin by CBMAI 2042. Careful investigation of the WGS data confirmed the presence of the VLM gene cluster in the endophytic genome (Unpublished data). The whole BGC coding for the NRP was therefore cloned in ESAC (Escherichia coli-Streptomyces Artificial Chromosome) vector and transferred to S. coelicolor M1146 [19]. As a result, the production of valinomycin and montanastatin were reconstituted by the whole gene cluster expression in heterologous host.

2. Results

As first step, in order to promote dereplication of the metabolites produced by the endophytic strain Streptomyces sp. CBMAI 2042 a bacterial molecular network was generated from direct infusion MS/MS analysis of extracts from two different growth conditions in A and GPMY Medium. The molecular networking permitted initially the dereplication of valinomycin (Figure 1) and a non-identified desferrichrome analog. Using ESI(+) as the ionization technique, valinomycin was detected as its ammoniated (m/z 1128.67), sodiated (m/z 1133.62) and potassiated molecules (m/z 1149.59) (Figure S1A). High accuracy MS/MS data acquired in an orbitrap mass spectrometer undoubtedly confirmed the production of the cyclic depsipeptide antibiotic via the structurally diagnostic fragment ions resulting from losses of CO (m/z 1083), Hiv-Val (m/z 884.52), Lac-Val (m/z 713.43), Hiv-Val (m/z 514.31), Lac-Val (m/z 343.22) and Hiv-Val (m/z 144.10) (Figure S1B).

Looking in depth the ESI(+) -MS/MS dereplication network we could identify the cyclooctadepsipeptide montanastatin (Figure 1), a valinomycin analogue, composed by doubled D-Val-L-Lac-L-Val-D-Hiv units and corresponding to cyclo-(D-Val–L-Lac–L-Val–D-Hiv). Montanastatin was previously described as a cell growth inhibitor produced by Streptomyces anulatus [20]. Its molecular formula was determined as C\(_{39}\)H\(_{56}\)O\(_{11}\) by HRESIMS (m/z [M+H]+ 741.4297 calcd 741.4280 error 2.26 ppm) and the molecule was also detected as its ammoniated (m/z 758.45), sodiated (m/z 763.41) and potassiated adduct (m/z 779.38) (Figure S2A). High accuracy MS/MS data of the most abundant ion (m/z 763.41) confirmed the production of the cyclic octapeptide via the structurally diagnostic fragment ions, related to valinomycin backbone, and resulting from neutral losses of CO (m/z 735), Lac-Val (m/z 592.26), Hiv-Val (m/z 321) and Lac-Val (m/z 150.04) (Figure S2B). Both valinomycin and montanastatin are supposed to share the same biosynthetic origin.
DESI(+)-MS imaging was also used to monitor VLM production during *Streptomyces* sp. CBMAI 2042 growth on agar plates as previously described [21]. When DESI-IMS was performed directly on the surface of *Streptomyces* sp. CBMAI 2042 culture on agar plate ranging from *m/z* 140 to 2000 (Figure S3A) the major ion detected was [VLM+K]+ of *m/z* 1149 and its isotopologues (Figure S3B). The spatial distribution of VLM over the medium and/or colony could therefore be easily imaged by DESI(+)-MS using the [VLM+K]+ “pixel”. As revealed in Figure S3, VLM is more abundant over the newest cells (border of colony) and, after 72 h it is highly spread over the media.

After indubitable identification of VLM production by the endophytic strain, the whole genome sequencing annotation of *Streptomyces* sp. CBMAI 2042 was cautiously analyzed. This endophytic strain from *Citrus* ssp. was isolated in Brazilian territory and sequenced using Illumina MiSeq Technology (Unpublished data). The complete genome consists in a linear chromosome with near to 8.2 Mb. A careful examination of the genome data revealed a modular NRPS gene cluster associated to the VLM production after protein BLAST. The antiSMASH platform (3.0.5) connected with the MIBiG repository (Minimal Information about a Biosynthetic Gene cluster) [22] revealed a similar operon type organization within VLM gene cluster of *S. tsusimaensis* ATCC 15141 described by Cheng [10] (genes 15, vlm1 and vlm2; Figure 2A) though a few outside genes differ between both strains (genes 22, 23 and 24; Figure 2B). All the functional assignments related to the VLM gene cluster were made by comparison within protein databases (final organization summarized in Table S2). The homologues ORFs 22, 23 and 24 corresponds to thioesterase, vlm1 and vlm2.

As a strategy to explore the biosynthetic machinery from *Streptomyces* sp. CBMAI 2042, an ESAC library was custom-made by Bio S&T Inc. (Montreal, Canada) and valinomycin BGC was screened using middle and flanking primers (Table S3). The ESAC clone identified as PAC4H was elected for further studies and intergeneric conjugation [23] from *E. coli* to heterologous host was confirmed by PCR analysis (Figure S4).

An isolated colony of the resulting exconjugants named M1146-PAC4H#5 was pre-inoculated in seed medium for 60 h and cultivated in A medium for 7 days. Afterwards ethyl acetate extracts were
obtained and production of valinomycin in heterologous host could be confirmed by LC-ESI-MS analysis and by tandem MS/MS spectrometry (Figure S5). Correspondingly, the production of montanastatin could be also confirmed along three additional structural unknown VLM analogues (peaks 1, 2, 3 and 5; Figure S6). The MS/MS of the unknown analogues revealed a 14 Da difference among the ions detected as ammoniated molecules suggesting the lacking of two (m/z 1100) and the one –CH₂ (m/z 1114) and one additional –CH₂ (m/z 1142) when compared to valinomycin (m/z 1128) – (Figures S7, S8, S9 and S10).

Conversely, looking back into *Streptomyces* sp. CBMAI 2042 metabolites profile from LC analysis allowed to undoubtedly confirm the production of the same analogues by the wild type strain (Figure S6). Through automated molecular networking analysis generated with the processed tandem mass spectrometry data it was possible to correlate the fragmentation pattern of the analogues to valinomycin-related compounds (Figure 3).

![Figure 3. Molecular networking clustering A) ions correlated with ammoniated valinomycin (m/z 1128.6); B) ions correlated with protonated montanastatin (m/z 741.4); C) Fully clustered depsipeptides ionophores ions from *Streptomyces* sp. CBMAI 2042.](image)

As next step, we monitored VLM production in wild type (WT) and recombinant *S. coelicolor* M1146-PAC4H#5 in small shake flasks cultivation and a 7 days time-course. The calibration curve linearity was evaluated in a range of 0.05-4.00 µg mL⁻¹ of standard solution of VLM showing a linear regression coefficients of 0.99 and equation equal \( y = 99360790.84x - 1328935.08 \) (Figure S11). Quantification of VLM was done by \( 10^4 \) dilution extracts analysis followed by linear regression equation interpolation (Table S4). These experiments display that wild type *Streptomyces* sp. CBMAI 2042 produces near to 0.5 mg L⁻¹ whereas in M1146-PAC4H#5 it was observed a 3-fold increase in VLM production with yields on average of 1.5 mg L⁻¹.

3. Discussion

Molecular networking is being applied as a dereplication methodology to identify known molecules and help to categorize related set of compounds that could share chemical similarities. Consequently, it is possible by comparing MS/MS fragmentation patterns to cluster families of structurally related natural products [24, 25]. By using molecular networking and ESI(+) as the ionization technique valinomycin and montanastatin were detected as metabolites produced by the endophytic strain *Streptomyces* sp. CBMAI 2042.

The imaging technique was crucial to afford information of the metabolite distribution in vivo as the distribution of VLM over the medium and/or colony is clearly visualized. The predominance of the [VLM+K⁺]⁺ ion in the DESI(+) spectra is in agreement with a well-known property of VLM, which functions as a selective potassium carrier within the cell membranes [26]. Hence, the in vivo analysis by DESI(+)−MS showed much greater abundance of [VLM+K⁺]⁺ as compared to others adducts plentifully detected by ESI(+)−MS directly from the extracts (Figure S3A). DESI(+)−MS was therefore invaluable to reveal the in vivo behavior of VLM production during *Streptomyces* sp. CBMAI 2042 growth.
Though the production of VLM is described in several *Streptomyces* strains [11] and its biosynthetic gene cluster precisely defined, some platforms are being explored in order to promote VLM production in heterologous strains in order to increase the titer and to get a better understanding of the enzymology targeting VLM analogues. As previously mentioned [13] the VLM production was reconstituted in *E. coli* and succeeded to improve the titer levels. However, the use of model strains, as *Streptomyces coelicolor* to promote heterologous expression of VLM whole gene cluster doesn’t have any precedent in the literature. *Streptomyces* is an effective platform to whole gene cluster expression and can be straightforwardly genetically modified (i.e. by punctual deletion and promoters’ insertion), representing an attractive organism to obtain VLM analogues.

Several approaches for whole gene cluster reconstitution and heterologous host expression are prominent in the recent years either dependent or independent of library preparation. The ESAC libraries of *Streptomyces coelicolor* and *Planobispora rosea* were first reported by Sosio et al. [27] and Alduina et al. [28]. The phage P1-based artificial chromosome (PAC) libraries were exclusively fashioned for *Streptomyces* and *Actinomyces* using a vector derivative of pPAC-S1 [27] which contains an oriT site enabling the transfer of the resulting ESAC clones to *Streptomyces* by conjugation. Presumably the conception simplifies encoding biosynthetic gene cluster expression of innumerable secondary metabolites in heterologous host. Jones et al. [29] used this effective system to successfully promote conjugative transfer of a PAC clone containing the entire 83.5 kb sized FK506 gene cluster from *Streptomyces tsukubaensis* NRRL 18488 [30] to *S. coelicolor* [6, 19]. Luhavaya et al. [31] applied this strategy for site-specific modification of salinomycin biosynthetic pathway to obtain new analogues.

Based on these examples we explored the ESAC containing VLM whole BGC to obtain a recombinant *S. coelicolor* capable to produce the anticipated NRF. After confirmation of the entire gene cluster integration into the model strain chromosome, the exconjugants were cultivated and the production of valinomycin in heterologous host could be confirmed by LC-ESI-MS. These results endorse this recombinant system as an accessible model to genome encoded natural products. Furthermore, montanastatin could be confirmed as a metabolite associated with this same biosynthetic gene cluster. To the best of our knowledge these results have no precedent in the literature.

Moreover, three additional structural related VLM analogues could be associated to VLM biosynthetic gene cluster after automated molecular networking analysis generated with the processed tandem mass spectrometry data. As already mentioned, from networking results the ions fragments sharing common patterns in MS/MS can be clustered into families suggesting structural relationship. The losses of Lac-Val (m/z 713), Hiv-Val (m/z 514), Lac-Val (m/z 343) and Hiv-Val (m/z 144.10) are observed in all ESI(+)-MS/MS of the ammoniated valinomycin analogues (Figures S7, S8, S9 and S10). After data analysis adduct [VLM+NH4]+ (m/z 1128.6) was clustered within the ions m/z 1142.6, m/z 1100.5 and m/z 1114.6 indicating a deep correlation among these structures (Figure 3A). An association between [VLM+H]+ (m/z 1111.6) and montanastatin [VLM+H]+ (m/z 741.4) – (Figure 3B) was also observed. Though the structures associated with m/z 1142.6, m/z 1100.5 and m/z 1114.6 still unknown until these moment, only after isolating the whole VLM biosynthetic gene cluster and promote heterologous expression as described it was possible to elucidate that this BGC is responsible to biosynthesize analogues as stated in this work.

Although the VLM titer resulting from these experiments is still modest compared to the one achieved with the heterologous production in *E. coli*, the batch cultivation can be much improved, as *Streptomyces* is reliable machinery for antibiotic production. Additionally, this represents the first example of whole VLM gene cluster heterologous expression in *Streptomyces* and was directly reached without the need of extensive genomic handling. Unquestionably, in comparison with the methodologies already described, the recombinant *Streptomyces* carrying the whole enzymatic arsenal required to promote the biosynthesis of VLM complies an extremely versatile platform to achieve plausible analogues by genetic manipulation in future studies.

4. Materials and Methods

4.1. Organisms
The endophytic strain *Streptomyces* sp. CBMAI 2042 was isolated from *Citrus* spp. and is deposited (open access) at the Brazilian Collection of Environmental and Industrial Microorganisms, CBMAI. Other strains used in this study are described in Table S1.

4.2. Culture conditions

*Streptomyces* sp. CBMAI 2042 was grown in TSBY liquid medium (30 g L\(^{-1}\) tryptone soy broth, 103 g L\(^{-1}\) sucrose, 5 g L\(^{-1}\) yeast extract) for isolation of genomic DNA and PAC library construction, and on SFM solid medium (20 g L\(^{-1}\) D-mannitol, 20 g L\(^{-1}\) soya flour, 20 g L\(^{-1}\) agar, 10 mM MgCl\(_2\)) for conjugation and strain maintenance, with appropriate antibiotic selection (nalidixic acid 25 μg mL\(^{-1}\)).

Valinomycin production was observed when *Streptomyces* sp. CBMAI 2042 was grown in seed medium (5 g L\(^{-1}\) peptone, 20 g L\(^{-1}\) soluble starch, 2 g L\(^{-1}\) soya flour, 4 g L\(^{-1}\) meat extract, 2 g L\(^{-1}\) yeast extract, 1 g L\(^{-1}\) CaCO\(_3\)) for 60 h, at 30 °C and 220 rpm, then 10% of seed culture was inoculated into A medium (4 g L\(^{-1}\) peptone, 4 g L\(^{-1}\) meat extract, 2 g L\(^{-1}\) yeast extract, 2 g L\(^{-1}\) soya flour, 20 g L\(^{-1}\) maltose, 10 g L\(^{-1}\) dextrin) and GPMY medium (20 g L\(^{-1}\) potato starch, 5 g L\(^{-1}\) yeast extract, 5 g L\(^{-1}\) malt extract, 20 mL L\(^{-1}\) glycerol) for 7 days at 30 °C and 250 rpm. For valinomycin heterologous production in *S. coelicolor* M1146 containing PAC DNA, 60h-old seed medium cultures were inoculated in A medium for 7 days at 30 °C and 250 rpm. *E. coli* DH10B containing PAC DNA was grown in LB (10 g L\(^{-1}\) tryptone, 5 g L\(^{-1}\) yeast extract, 10 g L\(^{-1}\) NaCl, 20 g L\(^{-1}\) agar) at 37°C with appropriate antibiotic selection (ampicillin 50 μg mL\(^{-1}\)). For conjugation purposes *E. coli* strains with or without PAC DNA were grown in 2TY medium (16 g L\(^{-1}\) tryptone, 10 g L\(^{-1}\) yeast extract, 5 g L\(^{-1}\) NaCl) with appropriate antibiotic selection (ampicillin 50 μg mL\(^{-1}\), chloramphenicol 25 μg mL\(^{-1}\), ampicillin 100 μg mL\(^{-1}\)).

4.3. LC-MS/MS analysis of metabolites

For analysis of *Streptomyces* sp. CBMAI 2042 metabolite profiles and *S. coelicolor* M1146 heterologous production, cells cultures were centrifuged (4,000 rpm, 10 min) and the supernatants were extracted three times with ethyl acetate (3:1, v/v). The solvent was evaporated, the residue was dissolved in methanol and the mixture was analyzed by LC-MS/MS. Liquid chromatography–mass spectrometry (LC-MS) was performed using an HPLC (Hewlett Packard, Agilent Technologies 1290 series) coupled to a Q-ToF iFunnel 6550 mass spectrometer fitted with an electrospray ionization (ESI) source and carried out on a Zorbax Extend C-18 1.8 μm column (2.1x50 mm, Agilent Technologies) column equilibrated with 90% A (H2O) and 10% B (CH3CN). The samples were eluted with a flow rate of 0.6 mL min\(^{-1}\) and the following linear gradient: 0-1 min 90% A/10% B; 1-10 min, 90% A/10% B to 30% A/70% B; 10-16 min 30% A/70% B to 0.1% A/99.9% B, 16-17 min 0.1% A/99.9% B to 90% A/10% B.

4.4. Nucleic acids accession numbers

Nucleotide sequences for *vlmTE*, *vlm1* and *vlm2* from *Streptomyces* sp. CBMAI 2042 VLM gene cluster are available at GenBank within the accession numbers MF668263, MF668264 and MF668265.

**Supplementary Materials:** The following are available online at www.mdpi.com/link, Figure S1. A) ESI (+)-MS of the *Streptomyces* sp. CBMAI 2042 strain extract showing the detection of valinomycin as its ammoniated (m/z 1128), sodiated (m/z 1133) and potassiated molecule (m/z 1149). B) ESI(−)-MS/MS of the potassiated valinomycin; Figure S2. A) ESI (+)-MS of the *Streptomyces* sp. CBMAI 2042 strain extract showing the detection of montanastatin as its protonated as its protonated (m/z 741.42), ammoniated (m/z 758.45), sodiated (m/z 763.41) and potassiated molecule (m/z 779.38). B) ESI(−)-MS/MS of the sodiated montanastatin; Figure S3. A) DESI(+) IMS showing the spatial distribution of potassiated valinomycin over the *Streptomyces* sp. CBMAI 2042 culture and agar medium along bacterial growth and B) DESI(−)-MS data for the *Streptomyces* sp. CBMAI 2042 colony showing the detection of valinomycin as its ammoniated (m/z 1128), sodiated (m/z 1133) and potassiated molecule (m/z 1149); Figure S4. A) PCR photodocumentation (agarose 1.5%) using flanking (R and L) and middle (C) primers. M: 2-Log DNA Ladder (0.1-10.0 kb ~ NEB); (+) positive control: genomic DNA; Exconjugant #5 M1146/PAC4H (duplicates S1 and S2); The regions amplified by PCR correspond to 408 bp from the beginning (left – L) of the biosynthetic cluster spanning arm, 506 bp from the center (C) and 390 bp from the ending flanking arm; B) PCR photodocumentation confirming positive clones afforded by Bio S&T. Lane 4H: clone 5076-plate 11-4H; Lane
16L: clone 5076-plate 13-16L; Lane CK: positive control gDNA; M: 0.2; 0.4, 0.6 kb marker; Figure S5. Base peak ion chromatograms from culture extracts of recombinant valinomycin produced in S. coelicolor M1146 (back) and with an empty control plasmid (front). Peak 2 (m/z 1100.59), 3 (m/z 1114.61) and 5 (m/z 1142.63) corresponds to vlm analogues; peak 4 corresponds to valinomycin (m/z 1128.67); Figure S6. Q-TOF LC-MS of crude extract of A) S. coelicolor M1146 heterologous host containing PAC4H exconjugant #5; B) Streptomyces sp. CBMAI 2042 (wild type) – ISP2 medium. Peak 1: montanastatin m/z 758.45 [M+NH4]+; peak 2: vlm analogue m/z 1100.59 [M+NH4]+; peak 3: vlm analogue m/z 1114.61 [M+NH4]+; peak 4: valinomycin m/z 1128.67 [M+NH4]+; 5: vlm analogue m/z 1142.63 [M+NH4]+; Figure S7. A) ESI (+)-MS of the Streptomyces sp. CBMAI 2042 strain extract showing the detection of valinomycin analogue as its ammoniated molecule (m/z 1100.59) B) ESI(+)-MS/MS of the ammoniated valinomycin analogue; Figure S8. A) ESI (+)-MS of the Streptomyces sp. CBMAI 2042 strain extract showing the detection of valinomycin analogue as its ammoniated molecule (m/z 1114.61). B) ESI(+)-MS/MS of the ammoniated valinomycin analogue; Figure S9. A) ESI (+)-MS of the Streptomyces sp. CBMAI 2042 strain extract showing the detection of valinomycin as its ammoniated molecule (m/z 1128.66). B) ESI(+)-MS/MS of the ammoniated valinomycin; Figure S10. A) ESI (+)-MS of the Streptomyces sp. CBMAI 2042 strain extract showing the detection of valinomycin analogue as its ammoniated molecule (m/z 1142.63). B) ESI(+)-MS/MS of the ammoniated valinomycin analogue; Figure S11. Calibration curve plot for valinomycin; Table S1. Bacterial strains and plasmid used in this study; Table S2. Protein Homologues annotated from antiSMASH in vitro analysis in Valinomycin BGC; Table S3. Oligonucleotides used in PAC library screening; Table S4. Production of valinomycin in 7 days culture/ medium A.

Acknowledgments: The financial support for this study was provided by São Paulo Research Foundation – FAPESP (2014/50249-8; 2014/12727-5; 2010/51677-2). R.S. received a fellowship from São Paulo Research Foundation - FAPESP (2013/12598-8 and 2015/01013-4). C.F.F.A. received a fellowship from National Council for Scientific and Technological Development - CNPq (162191/2015-4) and B.S.P received a fellowship from National Council for Scientific and Technological Development - CNPq (140824/2017-0).

Author Contributions: The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

gene clusters from the environment: Piecing environmental DNA gene clusters back together with TAR. 


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