Genome Mining Reveals Secondary Metabolites of Antarctic Bacterium *Streptomyces Albidoflavus* ANT_B131 Related to Antimicrobial and Antiproliferative Activities

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Abstract

The antimicrobial resistance requires the urgent mining of new antimicrobials. Actinobacteria, specially *Streptomyces* genus, are responsible for production of numerous clinical antibiotics and anticancer agents. The use of genome mining can reveal the genetic potential of a strain to produce natural products, however the potential may not be express under laboratory conditions. In the present study, the Antarctic bacterium was taxonomically affiliated as *Streptomyces albidoflavus*, ANT_B131. The crude extracts showed antimicrobial activity against fungi, Gram-positive and Gram-negative bacteria and antiproliferative activity against five human tumor cell lines. Whole genome sequencing reveals a 6.96 Mb genome size and the genome mining showed 24 BGCs, representing 13.3% of the genome. The use of three culture media and extraction methods reveal the expression and recovery of 20% of the BGCs. The natural products identified were surugamide A, surugamide D, desferrioxamine B + A1, desferrioxamine E, and ectoine. This study reveals the potential of *S. albidoflavus* ANT_B131 as a natural product producer. Yet, the diversity of culture media and extraction methods could enhance the BGCs expression and recovery of natural products, and could be a new strategy to intensify the BGC expression of natural products.

Introduction

In the past few years, Antarctica has been an important resource of new microbial natural products, to obtain antimicrobials, antiprotozoal, biosurfactants, enzymes, antitumor, antiviral, immunosuppressant, and antioxidant compounds¹⁻⁶. The extreme environmental conditions provide a unique metabolic response for microorganisms⁷. The growth of antimicrobial resistance became urgent the searching for new compounds capable of confronting resistant pathogenic microorganisms⁸. Also, cancer is one of the most complex diseases and acts in different organs in the human organism. The high level of mortality and the specificity of the treatment leads to the urgent need for novel antiproliferative compounds⁹. In this context, the phylum Actinobacteria, of which the *Streptomyces* genus belongs, are responsible for 90% of the production of antibiotics¹⁰ and novel natural products.

The usual methodologies for the discovery of natural products lead to a high rate of rediscovery of the same molecules. The increase in the microbial genome sequencing, metabolomic analyses, and synthetic biology have improved the search and discovery of novel natural products from microorganisms in environments that are still little explored^{11,12}. Furthermore, we aimed to perform the genome mining associated with a metabolomics approach of Antarctic *Streptomyces albidoflavus* ANT_B131, whose crude extracts showed antimicrobial and antiproliferative activity.

Material And Methods

Streptomyces isolation.

The tunicate, *Salp* sp., was collected in Punta Plaza, Admiralty Bay, King George Island, Antarctica during an expedition in the austral summer of 2010 by the Brazilian Antarctic Program team. The sample was ground in a homogenizer, serially diluted up (10^{-2} and 10^{-4}) and plated on Petri dishes with the culture media. The bacterium was isolated on M1 medium (peptone 2 g l⁻¹, yeast extract 4 g l⁻¹, starch 10 g l⁻¹, and agar 18 g l⁻¹; pH 7.0) prepared with artificial seawater, ASW, (KBr 0.1 g l⁻¹, NaCl 23.48 g l⁻¹, MgCl₂·6H₂O 10.61 g l⁻¹, CaCl₂·2H₂O 1.47 g l⁻¹, KCl 0.66 g l⁻¹, SrCl₂·6H₂O 0.04 g l⁻¹, Na₂SO₄ 3.92 g l⁻¹, NaHCO₃ 0.19 g l⁻¹, H₃BO₃ 0.03 g l⁻¹) at 15 °C and maintained by cryopreservation (-80 °C in 20% glycerol).

Phylogenetic analysis.

The genomic DNA was extracted¹³ and PCR amplified the 16S ribosomal RNA (Lane, 1991) and taken to sequencing in automated sequencer ABI 3500XL series (Applied Biosystems). The sequences were assembled^{14,15} in contigs of approximately 1100 bp and were subjected to comparison in the databases^{16,17}. The sequences retrieved from the databases were aligned in the CLUSTAL X¹⁸, edited and analyzed phylogenetically using MEGA X¹⁹. The evolutionary distance was calculated by Kimura model²⁰ and the construction of a phylogenetic trees based on the evolutionary distances were made by the neighbor-joining method²¹ with bootstrap values calculated from 1,000 replications²².

Crude extract production.

Starter culture (50 mL) was used to scale toward larger volume cultures (100 mL, 500 mL) of Nutrient Broth (NB, Oxoid™) medium with ASW, and incubated at 28 °C for 30 days under stationary conditions. The negative control was included in the production of crude extracts, and it represented the culture media without bacterial growth. After the incubation period, ethyl acetate was added to the liquid culture (1:1 ratio) and the medium and solvent were mixed in a homogenizer at 7,000 rpm for 6 min. The extracts were obtained by liquid-liquid extraction method and, the organic phases were concentrated under vacuum in a Rotary evaporator (Buchi® R-215) at 40 °C and 80 rpm. The crude extracts were stored at -20 °C until their use in assays.

Antimicrobial activity.

The antimicrobial activity of crude extract was tested to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) by microdilution assay according to the Clinical & Laboratory Standards Institute (CLSI)²³⁻²⁵. Crude extracts were solubilized in 2.5% of dimethyl sulfoxide and Muller-Hinton broth (BD Difco™) and assayed from 0.0078 to 2.0 mg.mL⁻¹. Bacterial extracts were tested against the strains of yeast *Candida albicans* ATCC 10231, Gram-positive and Gram-negative bacteria, that included virulent and pathogenic reference strains (Table 2). The Nalidixic acid was

included as positive control. The crude extracts were incubated without the microorganism inoculation to verify any type of contamination, the viability of the cells was confirmed during the test, and the medium was used as negative control. The test was performed in triplicate.

Antiproliferative activity.

The antiproliferative activity was performed in an in vitro assay²⁶ against a panel of human tumours glioblastoma (U251), mammalian adenocarcinoma (MCF-7), ovarian multi-drug resistant adenocarcinoma (NCI/ADR-RES), large cell carcinoma of lung (NCI-H460), adenocarcinoma of kidney (786-0), prostatic adenocarcinoma (PC-3), colon adenocarcinoma (HT-29), chronic myeloid leukemia (K-562) and non-tumour (HaCat, immortalized keratinocytes) cell lines. The cells grew in complete medium, RPMI 1640 supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin mixture (1,000 UI:1,000 $\mu\text{g}.\text{mL}^{-1}$), at 37 °C and 5% CO₂ in a humidified atmosphere. Human tumour and the human non-tumoural cell lines were provided by the Frederick Cancer Research & Development Center (National Cancer Institute, Frederick, MD, USA), and Dr. Ricardo Della Colleta (University of Campinas). The extract was solubilized in dimethyl sulfoxide (100 $\text{mg}.\text{mL}^{-1}$) followed by serial dilution in complete medium affording the final concentrations (0.25, 2.5, 25 and 250 $\mu\text{g}/\text{mL}$). Doxorubicin was diluted following the same protocol and used as a positive control at final concentrations of 0.025, 0.25, 2.5 and 25 $\mu\text{g}.\text{mL}^{-1}$. The complete medium was used as negative control. The GI₅₀ indicated the concentration ($\mu\text{g}.\text{mL}^{-1}$) to the crude extract reduce 50% of the cellular proliferation.

Whole genome sequencing.

Whole genome sequencing of the *Streptomyces* sp. ANT_B131 was performed to evaluate the encoded potential of secondary metabolites production related to biosynthetic gene clusters (BGCs) in this strain. The genomic DNA was performed with UltraClean Microbial DNA Extraction Kit (Mo Bio Laboratories, Carlsbad, USA). Paired-end libraries were prepared followed by 2 × 251 bp sequencing on Illumina HiSeq platform. The quality of the reads were determined using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and filtered with Trimmomatic 3.0²⁷. The assembled was performed with SeqMan NGen version 14.0 software (DNASTAR, Inc., WI, USA) and the quality evaluated by Quast 4.0²⁸. The contigs were realign using MAUVE²⁹ and *Streptomyces albidoflavus* J1074 genome (accession number PRJNA180996) was used as reference genome. The genome was annotated by NCBI Prokaryotic Genome Annotation Pipeline (PGAP)³⁰, and the Rapid Annotations using Subsystems Technology (RAST)³¹, PATRIC³², and the completeness was performed by BUSCO³³.

Multi-locus Sequence Analysis.

The phylogenetic analysis of the Multi-locus Sequence Analysis (MLSA) were performed using five house-keeping genes: *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit), *rpoB* (RNA polymerase beta subunit), *recA* (recombinase A), and *trpB* (tryptophan synthetase, beta subunit A) (See Supplementary Table 1), as recommended by Labeda et al. (2016). The *Streptomyces* sp. ANT_B131 genes were annotated by PATRIC³² and used in this study. The evolutionary history was inferred using the Neighbor-Joining method²¹. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)²². The evolutionary distances were computed using the Kimura 2-parameter method²⁰ and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 2525 positions in the final dataset. Evolutionary analyses were conducted in MEGA X¹⁹.

In silico DNA–DNA hybridization.

The *in silico* DNA–DNA hybridization (*isDDH*), local alignment by BLAST +, and difference in % G + C content of genomes was calculated using Genome-to-Genome Distance Calculator^{34,35} and the Average Nucleotide Identity based on BLAST + (ANIb), Average Nucleotide Identity based on Mummer (ANIm), and Tetra Correlation Search (TETRA) values were calculated using JSpeciesWS³⁶ between *S. albidoflavus* ANT_B131 genome and its phylogenetically closest species *S. albidoflavus* J1074 (accession number PRJNA180996) and *S. albidoflavus* SM254 (accession number PRJNA295319).

Genome Mining.

The genetic potential of *Streptomyces albidoflavus* ANT_B131 to produce secondary metabolites was analyzed using antiSMASH bacterial version 5³⁷ (default parameters including Cluster Blast and Cluster Pfam analysis), and the manual curation was performed using BLAST (*Basic Local Alignment Search Tool* – NCBI/NIH). The genomes of *S. albidoflavus* J1074 and *S. albidoflavus* SM254 were also evaluated by antiSMASH, following the same conditions, and the BGCs were compared between the three strains.

Cultivation of *Streptomyces albidoflavus* ANT_B131 in different culture media.

The *Streptomyces albidoflavus* ANT_B131 was cultivated in three types of culture media with the aim to support the expression of BGCs related to secondary metabolites. The media selected were Nutrient Broth (NB, Oxoid™), ISP2 broth (yeast extract 4 g.l⁻¹, malt extract 10 g.l⁻¹, dextrose 4 g.l⁻¹, pH 7,2) and GYE broth (glucose 15 g.l⁻¹, yeast extract 5 g.l⁻¹, CaCl₂·2H₂O 0.2 g.l⁻¹, pH 7,0), prepared with ASW. Starter cultures (15 mL) were used to scale toward larger volume cultures (100 mL) and incubated at 28 °C for

seven days under stationary conditions. The cultivation was performed in triplicate. Negative controls were conducted with the culture media without the bacterium growth.

Extraction of metabolites from different culture media.

Extracts were obtained by three different types of organic extractions. For each cultivation, we performed two aliquots of bacterial growth. In the first growth, we applied the liquid-liquid separation with ethyl acetate, as described above. In the second growth, we separated the cells, and extracted the intracellular metabolites with methanol. For this, the bacterial growth was centrifuged for 10 minutes (5,000 rpm) and the cells were separated from the culture media. 80 mL of methanol was added to the cells, carried to ultrasonic bath for 40 minutes, and the organic phase was collected. To the culture media were added XAD-2 adsorbent (Sigma-Aldrich), kept under shaking (150 rpm) for 3 hours, and methanol was used to recover the metabolites adsorbed in XAD-2. The crude extracts were dried in a Rotary evaporator (Buchi® R-215) under vacuum at 40 °C and 80 rpm. The crude extracts were stored at -20 °C until the use in the assays. The experiment was performed for all the culture media evaluated in triplicate. Negative controls were conducted with the culture media without the bacterium growth.

Mass Spectrometry analysis.

Crude extracts were diluted in methanol and analyzed by HPLC-ESI-MS/MS in Agilent 6550 series Q-TOF (G6550A) Dual AJS ESI. Parameters were set on positive mode, capillary voltage 3.5 kV, inlet capillary temperature of 290 °C. Method TIC (Total Ion Chromatogram) was selected for a general scan in 100–1700 m/z mass range. The injection volume of the samples was 2 μ L. Stationary phase: Agilent 1260 Infinity C18 (2.1 mm x 100 mm) column. Mobile phase: 0.1% formic acid (A) and acetonitrile (B). Eluent profile (A: B) 0–11 min, gradient from 2:98 up to 95:5; held for 5 min. Flow rate: 0.3 mL min⁻¹. MS/MS was performed by collision induced dissociation (CID with m/z range of 100–1500 and the collision energy of 30 V. Data was processed with MassHunter Qualitative Analysis B.07.00 software.

Molecular Networking Analysis.

ESI-MS/MS acquired data were subjected to msConvert³⁸ to convert the files to format .mzXML and were analyzed by molecular networking in Global Natural Products Social Molecular Networking (GNPS) server³⁹. A molecular network for *Streptomyces albidoflavus* ANT_B131 extracts was created using the online workflow (<https://ccms-ucsd.github.io/GNPSDocumentation/>) on the GNPS website (<http://gnps.ucsd.edu>). MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.5 and more than 5 matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most

similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.5 and at least 5 matched peaks³⁹. The resulting molecular networking is available at <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=bf96ebe6282048678b8da1b1affdf138>. The spectral networks were imported and visualized using Cytoscape 3.8.0 software (<https://cytoscape.org/>).

Results And Discussion

The phylogenetic analysis and the in silico DNA-DNA hybridization correlated the ANT_B131 to *Streptomyces albidoflavus*.

The *Streptomyces albidoflavus* ANT_B131 was isolated from *Salp* sp. collected in King George Island, Antarctica, during the summer of 2010. The phylogenetic identification using the sequencing of the 16S rRNA gene indicated that ANT_B131 belongs to the *Streptomyces* genera (See Supplementary Fig. 1), and was closest related to a clade with *Streptomyces albidoflavus* DSM 40455, *Streptomyces hydrogenans* NBRC 13475, *Streptomyces daghestanicus* NRRL B-5418, and *Streptomyces violascens* ISP 5183. According to the List of Prokaryotic names with Standing in Nomenclature (LPSN)⁴⁰ there are 660 species of *Streptomyces* with a validly published name. The 16S rRNA gene sequencing has an important role in the taxonomy of *Streptomyces*, however, it does not always have the power to delimit species⁴¹. To improve the taxonomic analysis, we choose to perform the Multilocus Sequence Analysis (MLSA) of house-keeping genes and the in silico DNA-DNA hybridization (isDDH). MLSA provide the improvement of taxonomic resolution among species within the family *Streptomycetaceae*⁴² and DDH is the gold standard for species delineation³⁴.

The studied *Streptomyces* strain had its whole genome sequenced (accession number JADIL1000000000) and, based on the genome annotation, five house-keeping genes were selected (see Supplementary Table 1) to the MLSA. The whole genome sequencing enables the comparison of the total genome of *Streptomyces* ANT_B131 with correlated strains, performing the isDDH. The DDH is a laborious technique, with which G + C content and melting profiles have been estimated, and could be done by HPLC and real-time PCR. After the advantage of genome sequence techniques, the G + C content analysis and the DDH between two bacterial genomes could be done in silico³⁴.

The whole genome was sequenced with Illumina and assembled with SeqMan NGen version 14.0 software, resulting in a total genome length of 6,968,465 bp in 49 contigs, with an N_{50} of 236,213 bp and a GC content of 73.2%. The genome was annotated using Prokka that identified 6,110 coding sequences (CDSs), PGAP identified 6,055 protein-coding genes, Patric identified 6,459 CDS and RAST identified

6,550 CDSs and 413 subsystems (Table 1). We provide a high-quality draft genome with 97.2% completeness, 0.6% duplicated and 0.3% fragmented.

Table 1 Genome annotation features of <i>Streptomyces albidoflavus</i> ANT_B131				
Feature	Prokka	PGAP	Patric	RAST
CDS	6,110	6,055	6,459	6,550
rRNA	7	16	65	16
tRNA	75	66	11	65
tmRNA	1	-	-	-
misc RNA	50	-	-	-
Subsystems	-	-	-	413

The MLSA included closest strains related to ANT_B131 and indicated that the Antarctic strain was closest related to *Streptomyces albidoflavus* NRRL B-1271 (Fig. 1). The average nucleotide identity based on BLAST + (ANib) and *in silico* DNA–DNA hybridization (*is*DDH) values were calculated between ANT_B131 and its phylogenetically closest species *S. albidoflavus* J1074 (ANib 97.02%; *is*DDH 79.60%; Difference in % G + C: 0.15) and *S. albidoflavus* SM254 (ANib 97.41; *is*DDH 79.50%; Difference in % G + C: 0.17) (see Supplementary Table 2). The ANI and *is*DDH were above the cutoff of > 95% and > = 70%, respectively, and the difference in %G + C was < 1.0, these results indicates that ANT_B131 belongs to the same species of the tested strains^{34,36}. The DDH values (%) are currently used as the gold standard for species delineation in bacteriology. The evaluation of the dDDH (digital DNA-DNA hybridization) enabled the delineation of the novel species *Micromonospora zhangzhouensis*, the producer of a diterpenoid with cytotoxic activity⁴³. In this study, attending to taxonomic criteria applied to the genome sequence, it was possible to correlate the bacterium ANT_B131 to the species *Streptomyces albidoflavus*.

In general, the genomic results are in agreement with the characteristics of *Streptomyces albidoflavus* genomes previously reported on NCBI. There are 46 genomes related to *Streptomyces albidoflavus* deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genome/genomes/32384>). The GC content ranging from 72.30 to 73.60% and the genome size ranging from 6.52 Mb to 7.45 Mb. The median total length of the genomes is 7,053,130 bp, the median protein count is 5,964 and the median GC% is 73.4.

Streptomyces albidoflavus ANT_B131 produces potential antimicrobials and antiproliferative.

Organic crude extracts obtained by ethyl acetate solvent extraction showed antimicrobial and antiproliferative activities. The antimicrobial activity was observed against Gram-positive and Gram-negative pathogenic bacteria, and against the fungus *Candida albicans* (Table 2). The MIC ranged from

0.25 to 2.0 mg.mL⁻¹. Antimicrobial activity related to crude extracts of *Streptomyces* isolated from Antarctica is documented against Gram-positive and Gram-negative bacteria, but little information is available about the antifungal activity of those crude extracts^{2,3}.

The cytostatic antiproliferative activity of crude extracts were observed against cell lines: glioblastoma U251 (GI₅₀ 17.2 µL.ml⁻¹), human breast adenocarcinoma MCF-7 (GI₅₀ 195.3 µL.ml⁻¹), large cell carcinoma of lung NCI-H460 (GI₅₀ 0.35 µL.ml⁻¹), adenocarcinoma of kidney 786-0 (GI₅₀ 27.7 µL.ml⁻¹), and prostate PC-3 (GI₅₀ 24.8 µL.ml⁻¹) (Fig. 2).

Table 2

Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of crude extracts of *Streptomyces* sp. ANT_B_131.

Strain	Origin	MIC (mg/mL) / MBC (mg/mL)
Gram-positive strains		
<i>Bacillus subtilis</i> ATCC 6051	DM/UNICAMP	0,5 / 1,5
<i>Micrococcus luteus</i> ATCC 4698	DM/UNICAMP	0,25 / -
<i>Staphylococcus aureus</i> ATCC 6538	DM/UNICAMP	2,0 / -
Gram-negative strains		
<i>Neisseria meningitidis</i> Y USA (Clinical isolation)	INCQS/FIOCRUZ	2,0 / -
<i>Neisseria meningitidis</i> C 2135	INCQS/FIOCRUZ	2,0 / -
<i>Neisseria meningitidis</i> B4 (Clinical isolation)	INCQS/FIOCRUZ	2,0 / -
<i>Neisseria meningitidis</i> W135 ATCC35559	INCQS/FIOCRUZ	2,0 / -
Fungi strain		
<i>Candida albicans</i> ATCC10231	DM/UNICAMP	1,5 / 1,5
INCQS/FIOCRUZ: Special Laboratory of Bacteriology and Molecular Epidemiology, Department of Clinical, Toxicological and Bromatological Analysis, São Paulo State University (USP), Ribeirao Preto, SP, Brazil. DM/UNICAMP: Division of Microbiology, University of Campinas (UNICAMP), Paulínia, SP, Brazil. LABIOTEC: Biotechnology Laboratory, Faculty of Pharmaceutical Sciences, Campinas State University (UNICAMP), Campinas, SP, Brazil. ATCC: American Type Culture Collection.		

Genome mining uncovers the genomic potential for *Streptomyces albidoflavus* ANT_B131 to produce natural products.

The genome mining study was performed using antiSMASH database and the manual curation revealed 24 putative Biosynthetic Gene Clusters (BGCs). The BGC encoding polyketides type I (5 of 24) and type III

(1 of 24), non-ribosomal peptides (NRPS, 8 of 24), bacteriocins (2 of 24), terpenes (4 of 24), siderophores (2 of 24), hybrid compounds (4 of 24), ectoine, thiopeptide, lanthipeptide, and lassopeptide (Fig. 3, Table 3).

Despite the antiSMASH analysis, the manual curation validated the identification of three fragments of genes clusters related to surugamide A/D (cluster 13) and three related to candicidin (cluster 19). The surugamide A/D BGC had 82,441 bp and encodes a non-ribosomal peptide described as a antibacterial and antifungal compound⁴⁴. The candicidin BGC had 138,203 bp and the correspondent metabolite showed antimicrobial activity against *Candida albicans*⁴⁵.

The bioinformatic analysis by antiSMASH predicted terpenes with high BGC similarity: hopene (cluster 4, 100% of similarity), the volatile compound geosmin (cluster 8, 100% of similarity), the sesquiterpene antibiotic albaflavenone (cluster 9, 100% of similarity), the carotenoid Isorenieratene (cluster 17, 75% of similarity), naringenin (cluster 18, 100% of similarity). Albaflavenone and geosmin are responsible for the strong odor in streptomycete and are related to antimicrobial activity⁴⁶. Interestingly, antiSMASH identified the gene cluster of SGR PTMs (cluster 3, 100% of similarity) related to a polycyclic tetramate macrolactams first identified in *Streptomyces griseus*. The Desferrioxamine B/E (cluster 15) and Ectoine (cluster 16) BGCs showed 100% of gene similarity. The Desferrioxamine B is a Fe(III) chelator, and is associated to iron deliver to microbes⁴⁷. Ectoine provides protection against osmotic stress⁴⁸. The antimycin BGC (cluster 23, 80% of similarity) is a secondary metabolite of *Streptomyces* sp. with cytotoxic, antifungal, antibiotic, anticancer activities⁴⁹. The BGCs relate to kanamycin (cluster 8), thiopeptides fluostatins M-Q (cluster 10) and type I PKS Pacificanone A (cluster 20) showed less than 20% of similarity.

Table 3
Biosynthetic gene clusters identified in the genome of *Streptomyces albidoflavus* ANT_B131.

Number	Cluster location	Cluster size (kb)	Type	Predicted product
1	Contig 79 (164714 to 210406)	45693	NRPS	Diisonitrile antibiotic SF2768
2	Contig 79 (229633 to 304621)	74989	NRPS / Terpene	Orphan
3	Contig 79 (423354 to 472764)	49411	NRPS / T1PKS	SGR PTMs
4	Contig 79 (503123 to 529681)	26559	Terpene	Hopene
5	Contig 79 (608808 to 619023)	10216	Bacteriocin	Orphan
6	Contig 42 (6276 to 17604)	11329	Bacteriocin	Orphan
7	Contig 66 (20876 to 35889)	15013	Siderophore	Orphan
8	Contig 104 (6303 to 28603)	22301	Terpene / Other	Geosmin/Kanamycin
9	Contig 52 (386568 to 407620)	21053	Terpene	Albaflavenone
10	Contig 2 (123277 to 155794)	32473	Thiopeptide	Fluostatins M-Q
11	Contig 10 (163019 to 224651)	48914	NRPS	Orphan
12	Contig 10 (263300 to 313589)	62236	NRPS	Orphan
13	Contig 80 (1 to 29890)	29890	NRPS	Surugamide A / Surugamide D
	Contig 33 (1 to 41377)	41377	NRPS	Surugamide A / Surugamide D
	Contig 102 (46186 to 77679)	30893	NRPS	Surugamide A / Surugamide D
14	Contig 71 (189212 to 233561)	44395	NRPS	Orphan
15	Contig 83 (59655 to 71475)	11820	Siderophore	Desferrioxamine B / Desferrioxamine E

Number	Cluster location	Cluster size (kb)	Type	Predicted product
16	Contig 81 (106501 to 116899)	10399	Ectoine	Ectoine
17	Contig 55 (39767 to 65616)	25849	Terpene	Isorenieratene
18	Contig 85 (1860 to 42957)	41097	T3PKS	Naringenin
19	Contig 85 (47893 to 101940)	54047	T1PKS	Candididin
	Contig 3 (1 to 22674)	22674	T1PKS	Candididin
	Contig 25 (11507 to 70522)	59015	T1PKS	Candididin
20	Contig 21 (1 to 31408)	31408	T1PKS	Pacificanone A
21	Contig 27 (1 to 5181)	5181	T1PKS	Orphan
22	Contig 40 (1 to 4749)	4749	T1PKS	Orphan
23	Contig 47 (1 to 74671)	74671	NRPS-T1PKS	Antimycin
24	Contig 22 (74379 to 96762)	22384	Lasso peptide	Orphan

In the antiSMASH analysis of the genomes of the two strains closest related to the *Streptomyces albidoflavus* ANT_B131, we found similarities in the BGCs number and type. The BGCs identification (Fig. 4) indicated that are common types of core biosynthetic genes for secondary metabolites production between the three strains, such as, the NRPS, PKS, Hybrid PKS-NRPS, Terpene and Siderophore. The metabolites hopene, geosmin, surugamide, desferrioxamine B, candididin, ectoine, isorenieratene and SGR-PTM were identified in all the genomes with similarity above 75%. Biosynthetic clusters related to RiPPs, such as lanthipeptides, thiopeptides and bacteriocins were also found. However, a blast analysis did not show similarity between the RiPPs BGCs of *Streptomyces albidoflavus* or even other bacteria. This result indicated that these orphan clusters (clusters 6, 10, 28, and 30) have a potential to be new molecules with unknown biological activities.

The three strains showed closest percentage of the genome attributed to biosynthetic genes involved in secondary metabolite production. *Streptomyces albidoflavus* ANT_B131 had 13.3% (925,376 bp) of the genome related to the secondary metabolism, *S. albidoflavus* SM254, genome size 7,170,505 bp, attributed 13.58% and *S. albidoflavus* J1074, genome size 6,841,649 bp, attributed 13.0% of the genome.

Five BGCs were expressed

After the genome sequencing and the BGC identification by antiSMASH, we performed an experiment to improve the expression of the biosynthetic gene clusters related to the secondary metabolites production. The Classical Molecular Networking analysis by GNPS enables the identification of compounds related to BGCs. GNPS provides the processes, analysis or identification of tandem mass (MS/MS) spectrometry data of a data set and compare the data to all publicly available data³⁹. To generate networks, Molecular Networking utilizes the MS/MS data. The nodes, in the network, represent molecules and edges represent spectral similarity between pairs of molecules¹².

MS/MS spectra of *S. albidoflavus* ANT_B131 extracts were identified as a hit in GNPS database (see Supplementary Fig. 2–5) and four metabolites were putatively identified: surugamide A (**1**), surugamide D (**2**), desferrioxamine B + Al (**3**) and desferrioxamine E (**4**). Also, based on the MS/MS spectra and comparison with other databases (METLIN and MassBank of North America), the metabolite ectoine (**5**) was manually identified in *S. albidoflavus* extracts (see Supplementary Fig. 6). The identified compounds were detected only in the bacterial extracts and absent in the controls (culture media extracts) (see Supplementary Fig. 6–10), and all their structures are represented in Fig. 5. The MS data of the detected metabolites have mass errors below 5 ppm and are shown in Table 4.

Table 4
Genome annotation features of *Streptomyces albidoflavus* ANT_B131

Compound	Ion	Ion Formula	Calculated <i>m/z</i>	Experimental <i>m/z</i>	Error (ppm)
Surugamide A	[M + H] ⁺	C ₄₈ H ₈₂ N ₉ O ₈	912.6286	912.6294	0.9
Surugamide D	[M + H] ⁺	C ₄₇ H ₈₀ N ₉ O ₈	898.6129	898.6116	-1.4
Desferrioxamine B + Al	[M-2H + Al] ⁺	C ₂₅ H ₄₆ N ₆ O ₈ Al	585.3192	585.3202	1.7
Desferrioxamine E	[M + Na] ⁺	C ₂₇ H ₄₈ N ₆ O ₉ Na	623.3380	623.3404	3.8
Ectoine	[M + H] ⁺	C ₆ H ₁₁ N ₂ O ₂	143.0820	143.0818	-1.4

Ion [M + H]⁺ at *m/z* 912.6294 was identified as compound **1**. MS/MS spectrum of this ion yielded to fragments at *m/z* 261.1602, 298.2084, 374.2498, 539.3926, 652.4693 and 884.6316, typical fragments for the structure of **1** (see Supplementary Fig. 7E) and already reported in the literature⁵⁰. Also, surugamide **2** was identified as ion [M + H]⁺ at *m/z* 898.6116. Fragmentation of **2** was close to fragmentation of **1** due structure similarity (see Supplementary Fig. 8E) and yielded to fragments 261.1600, 374.2493, 638.4719 and 785.5196. Surugamide **1** was identified as intracellular and extracellular metabolite. Compound **1** was found in the mycelium and the media of two media, NB-ASW and GYE-ASW, when applied methanol as extract solvent (Fig. 6). However, compound **2** was identified only as intracellular metabolite of all culture media evaluated, also recovered by methanol extraction.

Surugamide **1** was reported with antifungal activity against *Saccharomyces cerevisiae* and was expressed in *Streptomyces albidoflavus* J1074⁴⁴, antimicrobial activity against *Staphylococcus aureus* (MIC of 10 μ M)⁵¹, and inhibit bovine cathepsin B (IC₅₀ of 21 μ M)⁵², a cysteine protease that implicated in invasion of metastatic tumor cells. Surugamide **2** showed the inhibitory activity against bovine cathepsin B (IC₅₀ of 18 μ M)⁵².

GNPS database identified ions [M-2H + Al]⁺ at m/z 585.3202 and [M + Na]⁺ at m/z 623.3404 as desferrioxamines B and E, respectively. MS/MS spectrum of **3** yielded to fragments at m/z 385.2015, 425.1973, 467.2067 and 568.2982, typical fragmentation pattern for the structure of desferrioxamine B complexed with Al(III) (see Supplementary Fig. 9E). Close fragmentation pattern was reported for desferrioxamine B complexed with Fe(III) (difference of 29 Da in the mass fragments due the difference of mass between Al and Fe)⁵³. For compound **4**, mass fragmentation yielded to fragments at m/z 423.2225 and 223.1101, this fragmentation pattern corresponds to the typical neutral losses of molecules of succinylcadaverine (200.1160 Da)⁵⁴. Compounds **3** and **4** were recovery using all types of extraction methods when GYE-ASW medium was used to bacterial growth. The compounds were also identified as intra and extracellular metabolite of ISP2-ASW medium, recovered by methanol extraction. Compound **3** was identified as in NB-ASW medium using methanol as extraction solvent (see Supplementary Fig. 7). Desferrioxamine B is an iron-chelating agent used clinically to treat pathological iron deposition in human and iron poisoning, as Al(III)⁵⁵. The antimicrobial activity of desferrioxamine B against *E. coli* is related to inhibition of bacterial motility⁵⁶. Desferrioxamine E or nocardamine belongs to a group of microbial siderophores and has weak antimicrobial activity against *E. faecium* and *B. subtilis* and showed inhibition of colony formation of melanoma cancer cells (IC₅₀ from 12 to 18 μ M)⁵⁷.

Ion [M + H]⁺ at m/z 143.0818 were identified as compound **5**, and yielded to fragments at m/z 101.0706 and 113.5968. MS/MS spectra of ectoine at METLIN and MassBank of North America databases showed close fragmentation pattern, with fragment at m/z 101.0709 as one of the main fragments for compound **5** (see Supplementary Fig. 6E). Compound **5** was identified as intracellular metabolite in methanolic extract of all culture media. Also, the compound could be identified in the NB-ASW culture medium as an extracellular metabolite recovered by methanol extraction (Fig. 6). Ectoine has an important role as osmoprotectant, helps to prevent whole cell damage and loss of viability⁵⁸. The crude extract of NB-ASW medium extracted using ethyl acetate was the extract used for the biological assays, antiproliferative and antimicrobial. However, the GNPS database did not correlate any secondary metabolite to this crude extract.

These results revealed that the use of different culture media enabled the condition to *Streptomyces albidoflavus* ANT_B131 express the BGCs related to the secondary metabolites. Also, the different extraction methods and solvents enable the recovery of these compounds. In this study, we could express 20% of the BGCs identified by antiSMASH (5 of 25). Studies conducted on *Streptomyces fildesensis* S013.3 and *Pseudoalteromonas luteoviolacea* showed the expression of zero and 10% of the BGCs identified, respectively^{2,59}. We showed that the diversity of culture media and extraction methods could

enhance the BGCs expression and recovery of natural products. This approach could be a new strategy to intensify the BGC expression of natural products.

Genome mining made possible the identification of BGCs related to RiPPs, such as bacteriocins, thiopeptide and lassopeptide. These clusters did not presented similarity with none BGC described in literature and Molecular Networking analysis did not show results for any class of RiPPs, but further work is required to detect their production in these organisms. The heterologous expression is a potential tool to discover these natural products.

Besides the efforts to get the compounds that showed similarities with biosynthetic gene clusters, we could not correlate any of the compounds identified in this study to the crude extract that showed antimicrobial and antiproliferative activity, the growth in NB-ASW media and extracted by ethyl acetate. Additional genome and biological assays should be done to confirm the presence and novelty of secondary metabolites for this condition and activity. Also, heterologous expression will be applicable, in future study, to get the orphan BGCs undefined associated to *S. albidoflavus* ANT_B131 genome.

Declarations

Competing Interest: No

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Figures

Figure 1

Multilocus Sequence Analysis (MLSA) of the genes sequence of *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit), *rpoB* (RNA polymerase beta subunit), *recA* (recombinase A), and *trpB* (tryptophan synthetase, beta subunit A) of the ANT B131 bacteria isolated from Antarctic and related microorganisms. Evolutionary distances were based on Kimura 2p model and tree reconstruction on the neighbor-joining method. Bootstrap values (1000 repetitions, showed as %) are listed. Scale bar represents 1 nucleotide substitution per 100 nucleotides position.

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Figure 2

Antiproliferative activity in tumor cell lines. (A) *Streptomyces* sp. ANT_B 131 crude extract; (B) Positive control doxorubicin.

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■ NRPS (8)	■ PKS (6)	■ Hybrid (4)	■ Lasso peptide (1)	■ Bacteriocin (2)
■ Ectoine (1)	■ Siderophore (2)	■ Terpene (4)	■ Thiopeptide (1)	

Figure 3

Secondary metabolite gene clusters predicted in *Streptomyces albidoflavus* ANT_B131 genome. From outside inward: DNA strands reverse and forward; contigs; BGCs; GC content. BGCs types are shown with different colors according to the legend.

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Figure 4

BGCs identified in *Streptomyces albidoflavus* strains genomes by antiSMASH. BGC count indicated by different colors.

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Figure 5

Structures of secondary metabolites detected in the extracts of *S. albidoflavus* ANT_B131. Surugamide A (1), surugamide D (2), desferrioxamine B + Al (3), desferrioxamine E (4) and ectoine (5).

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using methanol; E3; extraction intracellular metabolites by methanol. Precursor mass is shown above the node.

Figure 6

Streptomyces albidoflavus ANT_B131 Molecular Networking. Compounds 1 (surugamide A), 2 (surugamide D), 3 (desferrioxamine B + Al), 4 (desferrioxamine E), 5 (ectoine) and 6 (monensin) were identified in three different culture media (A) and three different types of crude extract extraction (B). E1;

extraction by liquid-liquid methanol with ethyl acetate; E2; extraction of metabolites in the culture media using methanol; E3; extraction intracellular metabolites by methanol. Precursor mass is shown above the node.

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