Complete genome sequence of the deep South China Sea-derived *Streptomyces niveus* SCSIO 3406, the producer of cytotoxic and antibacterial marfuraquinocins

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Abstract

*Streptomyces niveus* SCSIO 3406 was isolated from a sediment sample collected from South China Sea at a depth of 3536 m. Four new sesquiterpenoid naphthoquinones, marfur-aquinocins A-D, and two new geranylated phenazines, i.e. phenaziterpenes A and B, were isolated from the fermentation broth of the strain. Here, we present its genome sequence, which contains 7,990,492 bp with a G+C content of 70.46% and harbors 7088 protein-encoding genes. The genome sequence analysis revealed the presence of a 28,787 bp gene cluster encoding for 24 open reading frames including 1,3,6,8-tetrahydroxynaphthalene synthase and monoxygenase, seven phenazine biosynthesis proteins, two prenyltransferases and a squalene-hopene cyclase. These genes are known to be necessary for the biosynthesis of both marfuraquinocins and phenaziterpenes. Outside the gene cluster (and scattered around the genome), there are seven genes belonging to the methylerythritol phosphate pathway for the biosynthesis of the essential primary metabolite, isopentenyl diphosphate, as well as six geranyl diphosphate/farnesyl diphosphate synthase genes. The strain *S. niveus* SCSIO 3406 showed type I PKS, type III PKS and nonribosomal peptide synthetase cluster. The sequence will provide the genetic basis for better understanding of biosynthesis mechanism of the above mentioned six compounds and for the construction of improved strain for the industrial production of antimicrobial agents.

Introduction

Deep-sea *Streptomyces* are widely recognized as an emerging source of novel and bioactive secondary metabolites [1]. They have been phylogenetically classified in 13 groups (MAR1--MAR13) [2]. The MAR4 group is a rich source of polyketide-terpenoid secondary metabolites, such as marinone, azamerone, and napyradiomycins [3–5]. As a member of the MAR4 group, *Streptomyces* sp. CNQ-509 could produce two polyketide-terpenoids (naphterpin and...
debromomarinone), five new farnesyl-α-nitropyroles nitropyrolins A–E and O-prenylated phenazines marinophenazine A–B [6]. Here, S. niveus SCSIO 3406 was isolated from a South China Sea sediment sample collected at a depth of 3536 m. Four new sesquiterpenoid naphthoquinones, marfuraquinocins A–D (1–4) (Fig 1), which exhibited antibacterial activities against Staphylococcus aureus ATCC 29213 or methicillin-resistant Staphylococcus epidermidis (MRSE) were previously isolated from the strain SCSIO 3406 [7]. Additionally, two new gernatelylated phenazines, phenaziterpenes A and B (5–6) (Fig 1), were also isolated from this strain in spite of low production [7]. In order to gain insights about the genetic basis of the above six compounds and about the discovery of further new natural products, the genome of S. niveus SCSIO 3406 was sequenced.

Materials and methods

S. niveus SCSIO 3406 was cultivated in trypticase soy broth and grown for 2 days at 28˚C with 200-rpm aeration. High-molecular-weight DNA was prepared using standard genomic DNA isolation method [8].

Genomic DNA was then used to generate two libraries, one is paired-end (PE) library with insert sizes of 300~500 bp and the other is a SMRTbell™ template library with insert sizes of about 8~10 kb. The complete genome of S. niveus SCSIO 3406 was subsequently sequenced using a combination of PacBio RSII sequencing (Pacific Biosciences) and Illumina Hiseq 2500 technologies at Biozeron Biotech Co., LTD (Shanghai, China). Genome assembly was de novo performed with SOAPdenovo v2.04 [9] and Celera Assembler 8.0 [10]. Putative protein-coding sequences were predicted by Glimmer 3.02 [11]. Gene functional annotation was performed using BIASTP withNr, String, COG and KEGG databases. rRNA, tRNA were predicted using RNAmmer v1.2 and NCBI Prokaryotic Genome Annotation Pipeline respectively. Protein coding genes were analyzed for COG functional annotation using WebMGA server [12]. CRISPRFinder, freely accessible at http://crispr.i2bc.paris-saclay.fr is used to find clustered regularly interspaced short palindromic repeats (CRISPRs) in S. niveus SCSIO 3406 genome. Genes involved in secondary metabolic pathways were predicted using antiSMASH 2.0 (http://antismash.secondarymetabolites.org/) [13].

Results and discussion

The complete genome of the strain S. niveus SCSIO 3406 consisted of a single linear chromosome of 7,990,492 bp with an average GC content of 70.46% without plasmids. Annotation revealed a total of 7,088 protein-coding genes, 6 rRNA operons with the order 5S-16S-23S, and 65 tRNA genes which could transfer all the twenty proteinic amino acids (Table 1, Fig 2).
the 7,088 protein-coding genes, 3345 (38.3%) were classified into different COG functional categories (Table 2, Fig 2), 4157 shared significant homology with genes from *S. niveus* NCIMB 11891, the producer of the gyrase inhibitor novobiocin [14]. Most of the genes in *S. niveus* SCSIO 3406 were associated with functions such as “transcription” (K), “amino acid transport and metabolism” (E), “carbohydrate transport and metabolism” (G), “Energy production and conversion” (C), and in particular, “Secondary metabolites biosynthesis, transport and catabolism” (Q) (Table 2).

Both marfuraquinocins A-D and phenaziterpenes A-B have a moiety of terpene. All terpenoids are synthesized from two precursors: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Two distinct biosynthetic pathways produce the essential primary metabolites IPP and DMAPP: the 2-C-methylerythritol 4-phosphate (MEP) pathway and the mevalonate pathway (MVA) [15]. IPP isomerase catalyzes the interconversion of IPP and DMAPP [16]. Based on the Nr annotation, no unigenes were identified as the genes of MVA pathway. However, seven genes scattered around the genome were identified as being involved in the MEP pathway: orf00197 coding for 1-deoxy-D-xylulose-5-phosphate synthase, orf01743 coding for 1-deoxy-D-xylulose-5-phosphate reductoisomerase, orf03854 coding for 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, orf04260 coding for 4-diphosphocytidyl-2C-methyl-D-erythritol kinase, orf03855 coding for 2-C-methyl-D-erythritol 2,4-cyclophosphate synthase, orf04113 coding for 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, and orf04123 coding for 4-hydroxy-3-methylbut-2-enyl diphosphate reductase. This implies that the isoprenoid moieties of marfuraquinocins A-D and phenaziterpenes A-B are derived from MEP.

We identified gene orf01170 coding for type III polyketide synthase and orf01166 for 1,3,6,8-tetrahydroxynaphthalene (THN) monooxygenase, responsible for the formation and modification of THN successively [17] to generate THN moiety of marfuraquinocins A-D. In many cases, the encoding genes responsible for the antibiotic biosynthesis are clustered in a continuous genomic DNA region, and usually in association with one or more genes that regulate their transcription and with resistance genes [18]. Therefore, the other biosynthetic genes for marfuraquinocins A-D are expected to exist in the vicinity of orf01170 and orf01166. By bioinformatic analysis of the genes located upstream and downstream of orf01170 and orf01166, a ~28.7 kb continuous DNA segment encoding for 24 open reading frames (ORFs) was predicted to contain the putative gene cluster of marfuraquinocins A-D. The function of the individual ORFs was deduced by BLAST analysis. The results are summarized in Table 3 and Fig 3. The identified DNA segment also included a squalene-hopene cyclase gene (orf01153), which is predicted to catalyze the cyclization of sesquiterpene to generate one of the parent skeletons of marfuraquinocins A-D. In addition, we found that, as shown in Fig 3 and Table 3, this continuous DNA segment also contained orthologues of most genes required for phenazine biosynthesis in two discontinuous loci (orf01155-01158 and orf01161-orf01163).
Complete genome sequence of *Streptomyces niveus* SCSIO 3406

Non-coding RNA
- tRNA
- 5S RNA
- 16S RNA
- 23S RNA

COG Functional categories
- RNA processing and modification
- Chromatin structure and dynamics
- Energy production and conversion
- Cell cycle control, cell division, chromosome partitioning
- Amino acid transport and metabolism
- Nucleotide transport and metabolism
- Carbohydrate transport and metabolism
- Coenzyme transport and metabolism
- Lipid transport and metabolism
- Translation, ribosomal structure and biogenesis
- Transcription
- Replication, recombination and repair
- Cell wall/membrane/envelope biogenesis
- Cell motility
- Posttranslational modification, protein turnover, chaperones
- Inorganic ion transport and metabolism
- Secondary metabolites biosynthesis, transport and catabolism
- General function prediction only
- Function unknown
- Signal transduction mechanisms
- Intracellular trafficking, secretion, and vesicular transport
- Defense mechanisms
- Extracellular structures
- Nuclear structure
- Cytoskeleton
- No assigned COG
Although the orthologue of *phzF* necessary for phenazine biosynthesis is absent in this DNA segment, two *phzF* orthologues (*orf05123* and *orf05140*) were identified outside of the gene cluster.

Two prenyltransferase genes (*orf01154* and *orf01164*) were identified in this DNA segment. Prenyltransferases are a class of enzymes that transfer allylic prenyl groups to acceptor molecules [19]. Therefore, *orf01154* and *orf01164* were predicted to be responsible for the condensation reaction between the sesquiterpene moiety and the THN moiety to form the backbone of marfuraquinocins A-D and/or the condensation reaction between the monoterpane moiety and the phenazine moiety to form the backbone of phenaziterpenes A-B. Based on above all, we speculate that the continuous DNA segment of ~28.7 kb was involved in the biosynthetic pathway of both marfuraquinocins A-D and phenaziterpenes A-B.

Outside the gene cluster, we also identified gene *orf00932* encoding isopentenyl pyrophosphate isomerase (IPP isomerase). In particular, six geranyl diphosphate (GDP) synthase and farnesyl diphosphate (FDP) synthase genes (*orf00192*, *orf00915*, *orf01258*, *orf02165*, *orf04121* and *orf06133*) are located outside the ~28.7 kb gene cluster. GDP synthase and FDP synthase catalyze the addition of one and two molecules of IPP to DMAPP, yielding GDP and FDP, respectively [20]. Therefore, these six GDP/FDP synthases are proposed to be responsible for the formation of terpene core moieties of both marfuraquinocins A-D and phenaziterpenes A-B. It is obvious that the genes of the biosynthesis of marfuraquinocins A-D and phenaziterpenes A-B are not clustered at a single locus of the genome.

Table 2. COG functional categories of the complete genome sequences of *S. niveus SCSIO 3406*.

| COG code | Description                                      | Number of unigenes |
|----------|--------------------------------------------------|--------------------|
| B        | Chromatin structure and dynamics                 | 1                  |
| J        | Translation, ribosomal structure and biogenesis  | 129                |
| K        | Transcription                                    | 373                |
| L        | Replication, recombination and repair            | 129                |
| D        | Cell cycle control, cell division, chromosome partitioning | 17              |
| M        | Cell wall/membrane/envelope biogenesis           | 152                |
| O        | Posttranslational modification, protein turnover, chaperones | 86              |
| T        | Signal transduction mechanisms                   | 193                |
| U        | Intracellular trafficking, secretion, and vesicular transport | 20              |
| V        | Defense mechanisms                               | 86                 |
| C        | Energy production and conversion                 | 195                |
| E        | Amino acid transport and metabolism              | 349                |
| F        | Nucleotide transport and metabolism              | 56                 |
| G        | Carbohydrate transport and metabolism            | 345                |
| H        | Coenzyme transport and metabolism                | 108                |
| I        | Lipid transport and metabolism                   | 134                |
| P        | Inorganic ion transport and metabolism           | 186                |
| Q        | Secondary metabolites biosynthesis, transport and catabolism | 118             |
| R        | General function prediction only                 | 489                |
| S        | Function unknown                                 | 179                |

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The major interest of *Streptomyces* is its potential to produce diverse secondary metabolites with biological activities. Here, analysis using antiSMASH showed 27 other gene clusters in the genome of *S. niveus* SCSIO 3406 (Table 4). Of these gene clusters, some have the really low similarity with the known clusters, revealing the potential of *S. niveus* SCSIO 3406 to produce novel natural products. In the putative T1pks-oligosaccharide gene cluster (Table 4, cluster_21), about 32% gene coding products showed similarity with the homologues of the known biosynthetic gene cluster of angucycline antibiotic, grincamycin, in *Streptomyces lusitanus* SCSIO LR32 [21]. We also identify an ectoine gene cluster (Table 4, cluster_23) which

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**Table 3. Deduced function of the open reading frames in Fig 3.**

| ORFs   | Amino acids | Proposed function                                                                 | Sequence similarity (protein accession number, origin, similarity/identity) |
|--------|-------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| orf01153 | 498         | squalene-hopene/tetraprenyl-beta-curcumene cyclase                                | SFG55119.1, *Streptomyces mirabilis*, 53/42                                |
| orf01154 | 305         | ABBA prenyltransferase                                                            | AFS18550.1, *Streptomyces tendae*, 77/60                                  |
| orf01155 | 657         | anthranilate synthase, 2-amino-2-desoxy-isochorismate synthase, PhzE              | CDH35391.1, *Streptomyces iakyrus*, 87/83                                  |
| orf01156 | 207         | isochorismatase, also known as 2, 3 dihydro-2, 3 dihydroxybenzoate synthase, PhzD | WP_03308464.1, *Streptomyces iakyrus*, 89/83                                |
| orf01157 | 390         | 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase, PhzC                     | CDH35389.1, *Streptomyces iakyrus*, 88/83                                  |
| orf01158 | 234         | phenazine biosynthesis protein A/B                                                | CDH35388.1, *Streptomyces iakyrus*, 94/88                                  |
| orf01159 | 704         | membrane protein                                                                  | CDH35387.1, *Streptomyces iakyrus*, 90/83                                  |
| orf01160 | 294         | RNA polymerase sigma factor                                                       | SER87811.1, *Streptomyces sp. Yr375*, 86/75                               |
| orf01161 | 168         | phenazine biosynthesis protein A/B                                                | CDH35386.1, *Streptomyces iakyrus*, 92/86                                  |
| orf01162 | 216         | FMN-dependent oxidase, PhzG                                                        | CDH35385.1, *Streptomyces iakyrus*, 89/84                                  |
| orf01163 | 408         | salicylate hydroxylase, PhzS                                                      | CDH35383.1, *Streptomyces iakyrus*, 85/79                                  |
| orf01164 | 301         | aromatic prenyltransferase                                                         | CDH35382.1, *Streptomyces iakyrus*, 84/70                                  |
| orf01165 | 68          | hypothetical protein                                                              | WP_062209276.1, *Streptomyces sp. NBRC 109706*, 78/65                     |
| orf01166 | 185         | 1,3,6,8-tetrahydroxynaphthalene monoxygenase                                       | SDJ65950.1, *Actinopolyspora mazalensis*, 84/74                            |
| orf01167 | 380         | pyridoxal phosphate-dependent aminotransferase                                    | WP_04956889.1, *Streptomyces sp. SBT349*, 93/87                            |
| orf01168 | 203         | NAD(P)H:quinone oxidoreductase                                                    | WP_079315493.1, *Microbispora sp. GKU 823*, 81/71                         |
| orf01169 | 332         | SAM-dependent methyltransferase                                                   | WP_073501777.1, *Streptomyces paucisporeus*, 85/76                        |
| orf01170 | 317         | type III polyketide synthase                                                      | SHN18252.1, *Streptomyces paucisporeus*, 90/83                            |
| orf01171 | 404         | putative transcriptional regulator                                                | CDH35377.1, *Streptomyces iakyrus*, 77/71                                  |
| orf01172 | 100         | hypothetical protein                                                              | WP_047018058.1, *Streptomyces sp. CNQ-509*, 74/57                         |
| orf01173 | 505         | FAD/FMN-containing dehydrogenase                                                  | SFF48976.1, *Streptomyces alni*, 67/53                                   |
| orf01174 | 520         | long-chain acyl-CoA synthetase                                                    | WP_047015640.1, *Streptomyces sp. CNQ-509*, 82/74                         |
| orf01175 | 509         | Puromycin resistance protein pur8                                                 | AKH84805.1, *Streptomyces sp. CNQ-509*, 80/70                             |
| orf01176 | 588         | FAD-dependent oxidoreductases                                                     | WP_073501787.1, *Streptomyces paucisporeus*, 87/79                       |

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The major interest of *Streptomyces* is its potential to produce diverse secondary metabolites with biological activities. Here, analysis using antiSMASH showed 27 other gene clusters in the genome of *S. niveus* SCSIO 3406 (Table 4). Of these gene clusters, some have the really low similarity with the known clusters, revealing the potential of *S. niveus* SCSIO 3406 to produce novel natural products. In the putative T1pks-oligosaccharide gene cluster (Table 4, cluster_21), about 32% gene coding products showed similarity with the homologues of the known biosynthetic gene cluster of angucycline antibiotic, grincamycin, in *Streptomyces lusitanus* SCSIO LR32 [21]. We also identify an ectoine gene cluster (Table 4, cluster_23) which
Table 4. Putative gene cluster coding for secondary metabolites in \(S. \text{niveus}\) SCSIO 3406 via antiSMASH.

| No. | Type                          | From (bp) | To (bp) | Most similar known biosynthesis cluster | Similarity |
|-----|-------------------------------|-----------|---------|-----------------------------------------|------------|
| 1   | NRPS-T1pks                    | 16717     | 104129  | Livipeptin                              | 100        |
| 2   | Other                         | 184608    | 228006  | _                                        | _          |
| 3   | NRPS-Linamarin                | 239107    | 354223  | Laspartomycin                           | 11         |
| 4   | Terpene-otherks-T1pks          | 425830    | 492314  | Carotenoid                              | 54         |
| 5   | T3pks                         | 515177    | 556304  | Herboxidiene                            | 2          |
| 6   | Terpene-otherks-lantipeptide   | 629756    | 732288  | Coelicichin                             | 100        |
| 7   | Terpene                       | 1046670   | 1073438 | Hopene                                  | 69         |
| 8   | terpene-Bacteriocin            | 1148109   | 1182287 | _                                        | _          |
| 9   | Terpene                       | 1439858   | 1461219 | _                                        | _          |
| 10  | Bacteriocin                   | 1563157   | 1574539 | _                                        | _          |
| 11  | Terpene                       | 1734097   | 1755776 | _                                        | _          |
| 12  | butyrolactone                 | 1763284   | 1774216 | _                                        | _          |
| 13  | Siderophore                   | 1883945   | 1898683 | _                                        | _          |
| 14  | Other                         | 2284811   | 2326703 | Stambomycin                              | 12         |
| 15  | Siderophore                   | 3740889   | 3756085 | Macrotetrolide                          | 33         |
| 16  | Terpene                       | 4086055   | 4109186 | _                                        | _          |
| 17  | Terpene                       | 4654606   | 4675796 | Mercchlorin                             | 7          |
| 18  | Bacteriocin                   | 4774818   | 4785109 | _                                        | _          |
| 19  | thiopetide-lantipeptide       | 4856826   | 4891992 | _                                        | _          |
| 20  | butyrolactone                 | 5081056   | 5092780 | _                                        | _          |
| 21  | T1pks-oligosaccharide          | 5780811   | 5848626 | Grincamycin                             | 32         |
| 22  | Nrps                          | 6228545   | 6274913 | _                                        | _          |
| 23  | Ectoine                       | 6339095   | 6349493 | Ectoine                                 | 75         |
| 24  | NRPS-T1pks                    | 6640764   | 6720100 | Streptomycin                            | 19         |
| 25  | otherks-Nrps                  | 6975232   | 7045695 | Naphthyridinomycin                      | 92         |
| 26  | T3pks                         | 7312845   | 7353951 | Alkylresorcinol                        | 100        |
| 27  | Melanin                       | 7672825   | 7683463 | Melanin                                 | 40         |

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consists of hydroxylase (Orf05563), L-ectoine synthase (Orf05564), diaminobutyrate-2-oxo-glutarate transaminase (Orf05565) and L-2,4-diaminobutyric acid acetyltransferase (Orf05566) in \(S. \text{niveus}\) SCSIO 3406; these gene coding products shows 75% similarity with the homologues of the known ectione biosynthetic cluster (BGC0000853_c1). As one kind of compatible solute, ectoine can be used for protecting enzymes, membranes and whole cells against stresses [22].

CRISPR (Clustered regularly interspaced short palindromic repeat) acronym was proposed by Jansen et al. [23]. CRISPR was observed first in 1987 in \(E. \text{coli}\) [24] and were subsequently reported in a wide range of prokaryotic genomes. CRISPR associated proteins (Cas) use the CRISPR spacers to recognize and cut foreign genetic elements [25]. Therefore, the CRISPR/Cas system is a prokaryotic immune system [26]. Here, 22 CRISPRs candidates spreading over \(S. \text{niveus}\) SCSIO 3406 genome, including 6 confirmed CRISPRs and 16 questionable CRISPRs [27], were identified in \(S. \text{niveus}\) SCSIO 3406 genome via CRISPRFinder, well above the average level in \(S. \text{fradiae}\) whose genome sequences have been published (Table 5). The CRISPR sequence contains 168 spacer sequences in size from 19 to 99 bp. The number of spacers in each locus varies from 1 to 78. Only two of the spacer sequences (5’-gcgcgccgacggagcggggtgagcgcgcagg-3’ and 5’-gtcctcgcggccggtc-3’), namely, protospacers match any sequences in the public sequence databases. The rest of the spacers remain the CRISPR “dark matter”. We also
identified ten genes (orf03151, orf05316, orf06507-orf06514) coding for CRISPR-associated proteins. CRISPR loci together with cas (CRISPR-associated) genes form the powerful immune system for *S. niveus* SCSIO 3406.

Interestingly, several antibiotic resistance genes were identified in *S. niveus* SCSIO 3406 genome. The gene orf04161 encodes penicillin amidase catalyzing the hydrolysis of benzylpenicillin [28], that efficiently accounts for the fact *S. niveus* SCSIO 3406 can survive on plates which contained 100μg/ml penicillin; the gene orf00505 encodes erythromycin esterase hydrolyzing the lactone ring of the 14 membered macrolides erythromycin and oleandomycin [29]; the gene orf02495 encodes virginiamycin B lyase inactivating the type B streptogramin antibiotics by linearizing the lactone ring at the ester linkage [30].

In summary, we have completely sequenced the genome of the deep South China Sea-derived *S. niveus* SCSIO 3406. By bioinformatic analysis, we have identified a biosynthetic gene cluster for both the marfuraquinocins A-D and phenaziterpenes A-B. The identified gene cluster provides important genetic basis for better understanding of biosynthesis mechanism of the marfuraquinocins A-D and phenaziterpenes A-B, as well as the construction of improved strain for the industrial production. Notably, 27 other gene clusters were also predicted in the genome of *S. niveus* SCSIO 3406. More importantly, some of these clusters have the really low similarity with the known clusters, strongly suggesting the potential of *S. niveus* SCSIO 3406 to produce diversity of novel natural products. This sequence information paves the way for the genome mining of *S. niveus* SCSIO 3406 for the novel natural product discovery.

**Author Contributions**

**Conceptualization:** Jianhua Ju, Qinglian Li.

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Funding acquisition: Qinghua Zhu, Yongxiang Song, Qinglian Li.

Project administration: Jianhua Ju.

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Supervision: Jianhua Ju.

Writing – original draft: Qinghua Zhu, Qinglian Li.

Writing – review & editing: Qing He, Qinglian Li.

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