微生物学通报
Microbiology China
tongbao@im.ac.cn
http://journals.im.ac.cn/wswxtbcn

Jul. 20, 2021, 48(7): 2307−2317
DOI: 10.13344/j.microbiol.china.210193

研究报告

Bioinformatics-directed discovery of dehydrated linear trimer and dimer of 2,3-dihydroxybenzoyl-L-serine from *Streptomyces albofaciens* JCM 4342

WANG Lijun[1] FAN Keqiang[1] WANG Haiyan[1] HU Huitao[1,2] XIANG Lijun[1] AI Guomin[1] PAN Guohui[*1,2]

1 State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
2 University of Chinese Academy of Sciences, Beijing 100049, China

Abstract: [Background] As an essential element for bacterial growth, iron in its ferric form is almost insoluble in aqueous environments. Bacteria have evolved to produce various siderophores to facilitate iron uptake. For *Streptomyces*, the characteristic siderophores are desferrioxamines, while they can also produce other structurally different siderophores, such as ceolichelin, albomycin, enterobactin, and griseobactin. **[Objective]** We aimed to reveal the distribution and characteristic of siderophore biosynthetic gene clusters (BGCs), and to explore their product structures in streptomycetes. **[Methods]** We systematically investigated the distribution and conservation of siderophore BGCs in 308 annotated *Streptomyces* genomes using bioinformatics tools. Chromatographic and spectroscopic methods were utilized to isolate and characterize the enterobactin-related natural products. **[Results]** This enabled us to identify an orphan enterobactin BGC, which lacked genes encoding enzymes for the biosynthesis of 2,3-dihydroxybenzoic acid (2,3-DHB), together with a griseobactin BGC in *Streptomyces albofaciens* JCM 4342 and other strains. Four enterobactin-derived natural products, including linear trimer and dimer of 2,3-dihydroxybenzoyl-L-serine (2,3-DHBS), and their dehydrated products, were identified from *S. albofaciens* JCM 4342. **[Conclusion]** These results suggested an interesting synergistic biosynthetic mechanism executed by the two BGCs. The orphan enterobactin BGC encoding enzymes hijacked the 2,3-DHB, which was biosynthesized by the griseobactin BGC, to complete biosynthesis of the four aforementioned enterobactin-related natural products.

Keywords: enterobactin, griseobactin, siderophore, NRPS, *Streptomyces*

Foundation item: National Natural Science Foundation of China (32070067)
***Corresponding author:** Tel: 86-10-64806056; E-mail: panguohui@im.ac.cn
Received: 24-02-2021; **Accepted:** 15-04-2021; **Published online:** 19-05-2021
基金项目：国家自然科学基金(32070067)
*通信作者：Tel：010-64806056；E-mail：panguohui@im.ac.cn
收稿日期：2021-02-24；接受日期：2021-04-15；网络首发日期：2021-05-19

基于生物信息学分析从 *Streptomyces albofaciens* JCM 4342 中发现 2,3-二羟基苯甲酸酯-L-丝氨酸脱水三聚体和二聚体天然产物

王立军[1] 范可强[1] 王海燕[1] 胡会涛[1,2] 向丽军[1] 艾国民[1] 潘国辉[*1,2]

1 中国科学院微生物研究所 微生物资源前期开发国家重点实验室 北京 100101

2 中国科学院大学 北京 100049

摘 要:【背景】铁是细菌生长的基本元素，而三价铁在自然水环境中几乎无法溶解。细菌已经进化出产生各种铁载体的能力，以促进铁的吸收。对于链霉菌，其特有的铁载体是去铁胺，同时它们也可以产生其他结构的铁载体，如 Ceolichelin、白霉素、肠杆菌素(Enterobactin)和 Griseobactin。【目的】揭示链霉菌中铁载体生物合成基因簇(Biosynthetic Gene Clusters，BGCs)的分布特点和基因簇特征，并探索其所合成铁载体的化合物结构。【方法】利用生物信息学工具系统地分析 308 个具有全基因组序列信息的链霉菌中的铁载体生物合成基因簇，并用色谱和波谱方法分离和表征肠杆菌素相关天然产物。【结果】发现 *Streptomyces albofaciens* JCM 4342 和其他少数菌株同时含有一个缺少 2,3-二羟基苯甲酸(2,3-DHB)生物合成基因的孤立的肠杆菌素生物合成基因簇和另外一个推测可合成 Griseobactin 的基因簇。从 *S. albofaciens* JCM 4342 发酵液中鉴定出 4 个肠杆菌素衍生的天然产物，包括链状 2,3-二羟基苯甲酸酯-L-丝氨酸(2,3-DHBS)的三聚体和二聚体以及它们的脱水产物。【结论】 2 个基因簇间存在一种特别的协同生物合成机制。推测是 Griseobactin 基因簇负责合成 2,3-DHB，而孤立的肠杆菌素基因簇编码的生物合成酶可夺取该底物，进而完成上述 4 种肠杆菌素衍生天然产物的生物合成。

关键词: 肠杆菌素，Griseobactin，铁载体，非核糖体肽合成酶，链霉菌

1 Introduction

The insolubility of Fe^{3+} in aqueous environments severely limits the obtainment of this essential element for nearly all bacteria. To overcome iron starvation, bacteria generally produce and secrete low-molecule-weight siderophores to chelate Fe^{3+} with very high affinity[1]. To date, hundreds of siderophores with different structures have been discovered, and they contain key functional groups for Fe^{3+} solubilization, such as the commonly found catecholates, hydroxamates, and (α-hydroxy-) carboxylates[2]. The siderophore-iron complexes are transported into cells by the bacterial acquisition system (i.e., ABC transporters), and then Fe^{3+} is released via different mechanisms[3-6]. Siderophores showed various bioactivities, and played important roles in pharmaceutical development. Desferrioxamine and nocardichelins showed strong growth inhibitory activities toward tumor cells[7]. Desferrioxamine has been developed to a drug (trade name Desferal), which is administrated to patients suffering from iron overload[8-10]. Enterobactin could promote cancer cell apoptosis[11]. Interestingly, some natural product antibiotics, such as albomycin and ferrimycin A, consist of a siderophore part and a structural part with antibacterial activity. These antibiotics exhibited potent antibacterial activities, partially due to the presence of siderophore moieties that facilitated the cellular uptake of these antibiotics once the siderophore-iron complexes were formed[12-14]. Inspired by the chemical features of these natural products, a trojan horse strategy was developed by integrating two functional molecule parts into one structural scaffold, to form the so-called siderophore-antibiotic conjugates[1]. Many of these conjugates were synthesized and developed to fight membrane-associated antibiotic resistance[1,15-16].

Bacterial siderophores are biosynthesized mainly

through two routes. One involves nonribosomal peptide synthetases (NRPSs), while the other is NRPS-independent and catalyzed by siderophore synthetases[17-19]. The NRPS-mediated assembly-line biosynthetic mechanism has been well studied[20]. One of the most representative NRPS-dependent siderophores is enterobactin, the cyclic trimer of 2,3-dihydroxybenzoyl-L-serine (2,3-DHBS)[21]. Enterobactin is mainly produced by bacteria of the family *Enterobacteriaceae*, and shows very high affinity for Fe^{3+} (i.e., a pFe^{3+} of 35.5 at pH 7.4)[22-23]. The biosynthetic process of enterobactin includes the formation and activation of 2,3-DHB, and the following condensation (between 2,3-DHB and Ser), elongation and cyclization steps catalyzed by the iterative one-module NRPS enzyme EntF[24]. In contrast to the "one-module" architecture of EntF, a group of "two-module" NRPS homologs are also identified in some siderophore biosynthetic gene clusters (BGCs), the products of which enrich the structural diversity of catecholate-type siderophores by having a spacer amino acid between 2,3-DHB and the ester core (e.g., griseobactin)[21,25-27].

The Gram-positive bacteria *Streptomyces* are well known for their complex life cycles and capability of producing various natural products with therapeutic applications[28]. They can also excrete different siderophores to increase access to iron. Desferrioxamines are the typical siderophores of streptomycetes, whereas some *Streptomyces* strains are also able to produce other siderophores, such as griseobactin, coelichelin, albomycin and enterobactin[21,29-33]. Among them, enterobactin [(DHB-^{L}Ser)$_3$] and griseobactin [(DHB-^{L}Arg-^{L}Thr)$_3$] belong to tricatecholate-type siderophores, which are biosynthesized by aforementioned NRPS pathways[34-35].

In this study, we analyzed the distribution of siderophore BGCs in 308 annotated *Streptomyces* genomes, and identified the orphan enterobactin BGCs, which lacked the biosynthetic enzymes of 2,3-DHB, in *S. albofaciens* JCM 4342 and other strains. We further identified four enterobactin-derived linear natural products from *S. albofaciens* JCM 4342. These results suggested an interesting functional cross-talk between the orphan enterobactin BGC and the griseobactin BGC in *S. albofaciens* JCM 4342.

2 Materials and Methods

2.1 Bioinformatics analyses of *Streptomyces* genomes

The siderophore BGCs in *Streptomyces* genomes were predicted using standalone BLASTp program[36] with the enzymes from the BGCs of desferrioxamine, coelichelin, enterobactin, griseobactin, and albomycin as query sequences[21,29-33]. Global protein sequence alignment was performed using EMBOSS 6.5.0[37]. The substrate specificities of adenylation domains were predicted by NRPSpredictor2 and NRPSsp web servers[25-26].

2.2 Strain cultivation and fermentation conditions

S. albofaciens JCM 4342 (genome accession number in GenBank: PDCM01000002.1) was purchased from CGMCC (No. 4.1655). This strain was cultivated on ISP2 agar plates for 3−4 days for sporulation. Then, fresh spores were collected and cultivated in TSB for 24 h to prepare seed culture. Next, the seed culture was inoculated (2%, *V*/*V*) to 50 mL of iron-deficient fermentation medium (*M*/*V*, 2.0% soluble starch, 1.0% glucose, 0.5% peptone, 0.5% yeast extract, 0.4% NaCl, 0.05% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2% $CaCO_3$, pH 7.0), and cultivated at 28 °C and 200 r/min for 5 days. On day 2, 1 g of XAD-16 resins was added to the fermentation culture.

2.3 HPLC, LC-MS, and LC-HRMS analyses of the products of *S. albofaciens* JCM 4342

Once the fermentation of *S. albofaciens* JCM 4342 (50 mL) was done, the XAD-16 resins were harvested, washed with distilled water, and allowed to air dry. Then, the resin was extracted with methanol (5 mL). After centrifugation (13 000 r/min, 1 min), 40 μL supernatant was analyzed by LC-MS. LC-MS (ESI source) experiments were performed on a triple-quadrupole LC-MS (Agilent 1260/6460) equipped with a diode array detector. An Agilent Zorbax SB-Aq column (3.5 μm, 2.1 mm×100 mm) and the following pump method were used: 0–2 min, 5% acetonitrile (0.1% formic acid); 2−27 min, a linear gradient to 80% acetonitrile (0.1% formic acid); 27−30 min, 95% acetonitrile (0.1% formic acid) at a flow rate of 0.25 mL/min. The UV absorptions, along with the molecular weights (MWs), suggested that one target compound might be enterobactin. To further

identify the products, the aforementioned methanol extracts were evaporated in vacuo, and then subjected to Sephadex LH-20 to afford ten fractions, all of which analyzed by analytical HPLC with a diode array detector. HPLC analyses were performed on an Agilent 1260 Infinity LC system equipped with an Agilent ZORBAX SB-C_{18} column (5 μm, 4.6 mm×250 mm). The following pump method was used: 0−5 min, 5% acetonitrile (0.1% formic acid); 5−40 min, a linear gradient to 80% acetonitrile (0.1% formic acid); 40−45 min, a linear gradient to 95% acetonitrile (0.1% formic acid); 45−50 min, 95% acetonitrile (0.1% formic acid) at a flow rate of 1 mL/min. The ninth fraction containing the target compounds was analyzed by LC-HRMS (high resolution electrospray ionization mass spectra), which was performed using an Agilent 1200 HPLC separation module and a qTOF-MS 6520 mass detector with a diode array detector. An Agilent SB-C_{18} column (1.8 μm, 4.6 mm×100 mm) and the following pump method were used: 0−5 min, 5% acetonitrile (0.1% formic acid); 5−30 min, a linear gradient to 60% acetonitrile (0.1% formic acid) at a flow rate of 1.0 mL/min.

2.4 Isolation and characterization of enterobactin-related natural products

A 10 L fermentation was carried out with the above mentioned seed and fermentation medium. Similarly, the resins were harvested and extracted with methanol, and then the crude extracts were fractionated using Sephadex LH-20. Compounds **1** and **2** were obtained through HPLC purification. Semi-preparation was performed on a Shimadzu LC-20AT system equipped with a Silgreen C_{18} column (5 μm, 10 mm×250 mm). The following pump method was used: 0−5 min, 5% acetonitrile; 5−10 min, a linear gradient to 35% acetonitrile; 10−30 min, 35% acetonitrile at a flow rate of 3 mL/min. Finally compounds **1** (2.5 mg, 24.5 min) and **2** (2.0 mg, 18.2 min) were obtained.

All NMR spectra were conducted on a Bruker Avance III 500 MHz NMR spectrophotometer equipped with a cryoprobe. The compounds in this study were dissolved in the deuterated DMSO (Cambridge Isotope Laboratories Inc). Chemical shifts were reported in ppm, and the residual solvent signal was at 2.50 ppm.

Compound **1** (=dehydrated 2,3-DHBS trimer): yellow amorphous powder. HRESIMS *m*/*z* 670.151 8 $[M+H]^+$ (calcd for $C_{30}H_{28}N_3O_{15}$, 670.152 0, Δ= –0.4 ppm).

Compound **2** (=dehydrated-DHBS dimer): yellow amorphous powder. HRESIMS *m*/*z* 447.103 4 $[M+H]^+$ (calcd for $C_{20}H_{19}N_2O_{10}$, 447.104 0, Δ= –1.3 ppm).

Compound **3** (=linear 2,3-DHBS trimer): HRESIMS *m*/*z* 688.162 2 $[M+H]^+$ (calcd for $C_{30}H_{30}N_3O_{16}$, 688.162 6, Δ= –0.6 ppm).

Compound **4** (=linear 2,3-DHBS dimer): HRESIMS *m*/*z* 465.114 2 $[M+H]^+$ (calcd for $C_{20}H_{21}N_2O_{11}$, 465.114 5, Δ= –0.7 ppm).

3 Results and Discussion

3.1 Bioinformatic analyses of siderophore BGCs in *Streptomyces*

To have an overview of the distribution of siderophore BGCs in *Streptomyces*, we analyzed 308 annotated *Streptomyces* genomes available in NCBI Genome database (as of Dec, 2020) using the enzymes from desferrioxamine, coelichelin, griseobactin, and albomycin biosynthetic pathways[21,29-33], as query sequences. Overall, about 30% strains each contain more than one siderophore BGCs. The BGCs of desferrioxamines are widely distributed in *Streptomyces* (found in 83% analyzed genomes) (Figure 1A), further supporting that the desferrioxamine-type siderophores play a primary role in iron uptake for streptomycetes[13]. In addition, 73 BGCs of coelichelin and 21 BGCs of griseobactin were found, while only one BGC was identified to encode the biosynthesis of albomycin in *Streptomyces globisporus* strain TFH56 (Figure 1A). Most of these newly identified BGCs showed similar genetic organizations and high sequence identities (70%−100%) to previously reported siderophore BGCs in *Streptomyces*.

3.2 The genome of *Streptomyces albofaciens* JCM 4342 encodes two BGCs that potentially produce two groups of catecholate-type siderophores

Although previous studies reported the production of enterobactin in several *Streptomyces* strains including *S. tandae* Tü 901/8c and *Streptomyces* sp. Tü 6125[35], the corresponding BGCs of enterobactin have not been identified in *Streptomyces*. Using EntF from the enterobactin BGC

of *E. coli* as a query sequence, 18 BGCs were found to contain the EntF homologs (sequence identities ranging in 36%−40%) surrounded by enzymes responsible for 2,3-DHB biosynthesis[21], indicating that they could produce enterobactin-like siderophores (Figures 1A and 1B). Interestingly, four unusual BGCs each harboring an EntF homolog were observed in *Streptomyces albofaciens* JCM 4342 (ATCC 23873: PDCM01000002.1) and three *Streptomyces rimosus* strains (ATCC 10970: CP023688.1; WT5260: CP025551.1; R6-500: CP045803.1) (Figure 1C). However, they lacked the enzymes responsible for the biosynthesis of 2,3-DHB, and therefore, they were designed as orphan enterobactin BGCs. The orphan enterobactin BGC (*eno* cluster) in *S. albofaciens* JCM 4342 was annotated in detail, in which the one-module NRPS enzyme EnoE (with 38% sequence identity to EntF) was also predicted to activate L-Ser (Table 1)[38]. In addition, a 2,3-DHB carrier protein (EnoD), and the enzymes related to product hydrolysis (EnoF) and export (EnoABC) were identified (Figure 1C, Table 1).

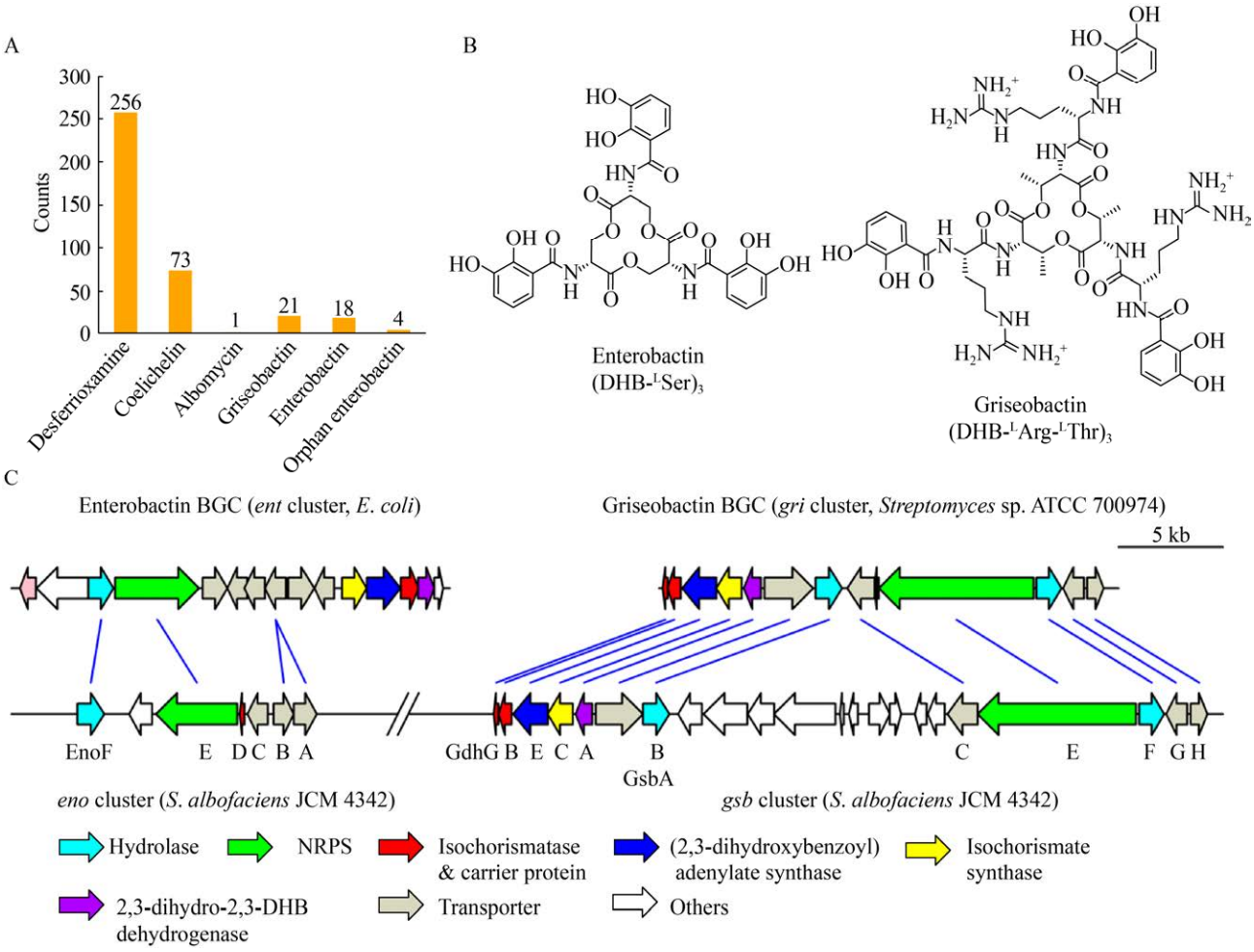

Figure 1 Bioinformatics analyses, chemical structures and biosynthetic gene clusters

图 1 生物信息学分析、化学结构式以及生物合成基因簇

Note: A: Distribution of different siderophore BGCs in 308 annotated *Streptomyces* genomes; B: Two catecholate-type siderophores enterobactin and griseobactin produced by streptomycetes; C: The *eno* and *gsb* clusters of *S. albofaciens* JCM 4342 in comparison to BGCs of enterobactin and griseobactin from *E. coli* and *Streptomyces* sp. ATCC 700974, respectively

注：A：不同铁载体生物合成基因簇在 308 个已注释链霉菌基因组中的分布；B：2 种由链霉菌产生的 Catecholate-Type 铁载体 Enterobactin 和 Griseobactin；C：*S. albofaciens* JCM 4342 基因组中 *eno* 和 *gsb* 生物合成基因簇与 Enterobactin (*E. coli*)和 Griseobactin (*Streptomyces* sp. ATCC 700974)生物合成基因簇的比较

Besides the orphan enterobactin BGC, each of these four strains also had another BGC proposed for the biosynthesis of griseobactin, in which the biosynthetic enzymes of 2,3-DHB were intact. The predicted griseobactin BGC (*gsb* cluster) in *S. albofaciens* JCM 4342 was annotated, and it highly resembled the griseobactin BGC from *Streptomyces* sp. ATCC 700974 (accession No. FN545130.1) (Figure 1C, Table 1)[21,33]. The two-module NRPS enzyme GsbE (with 72% sequence identity to GriE) was predicted to activate Arg and Thr as expected[33]. It was noteworthy that, in the middle of *gsb* cluster, there was an insertion of 10 CDSs without any sequence similarity to enzymes in known BGCs of triscatechol siderophores (Figure 1C). Based on these analyses, the *gsb* cluster very likely would produce griseobactin or its derivatives. Taken together, *S. albofaciens* JCM 4342 might be able to produce two groups of siderophores.

3.3 Discovery of dehydrated 2,3-DHBS trimer and dimer from *Streptomyces albofaciens* JCM 4342

Based on the above analyses, we were interested to know the siderophores that the strain JCM 4342 produced. The small scale fermentation of JCM 4342 was performed, and the crude extracts were fractionated before subjected to LC-MS analyses. The MS analyses revealed a product peak (compound **1**) with an *m*/*z* of 670.151 8 $[M+H]^+$ which seemingly accounted for the production of enterobactin (Figure 2A), whereas no griseobactin-like natural products were detected. To isolate compound **1**, a 10 L fermentation was carried out. After XAD-16 adsorption, LH-20 filtration (6 cm×19 cm, eluted with 70% acetonitrile in water) and HPLC purification, compound **1** as well as its congener **2** were isolated. Their structures were determined based on HRMS and 1D/2D-NMR spectra.

Table 1 Predicted functions of ORFs in the two gene clusters
表 1 2 个基因簇中 ORF 的功能预测

ORF	Protein ID	Proposed function	Homolog (sequence identity)
eno cluster			
EnoF	KAA6212386.1	Esterase	GriB (43%)
EnoE	[a]	One-module NRPS	EntF (38%)
EnoD	KAA6215186.1	2,3-dihydroxybenzoate carrier protein	DhbG (63%)
EnoC	KAA6212388.1	ABC transporter	GriG (42%)
EnoB	KAA6215187.1	ABC transporter	FepD (38%)
EnoA	KAA6215188.1	ABC transporter	FepD (31%)
gsb cluster			
GdhG	KAA6215320.1	2,3-dihydroxybenzoate carrier protein	DhbG (77%)
GdhB	KAA6213416.1	Isochorismatase	DhbB (82%)
GdhE	KAA6213417.1	(2,3-dihydroxybenzoyl) adenylate synthetase	DhbE (79%)
GdhC	KAA6213418.1	Isochorismate synthase	DhbC (81%)
GdhA	KAA6213419.1	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	DhbA (73%)
GsbA	KAA6215335.1	ABC transporter	GriA (81%)
GsbB	KAA6213420.1	Esterase	GriB (73%)
GsbC	KAA6213431.1	MFS transporter	GriC (74%)
GsbE	KAA6213432.1	Two-module NRPS	GriE (72%)
GsbF	KAA6213433.1	Peptidase	GriF (77%)
GsbG	KAA6213434.1	ABC transporter	GriG (79%)
GsbH	KAA6213435.1	ABC transporter	GriH (83%)

Note: [a]: No protein ID was available in NCBI database

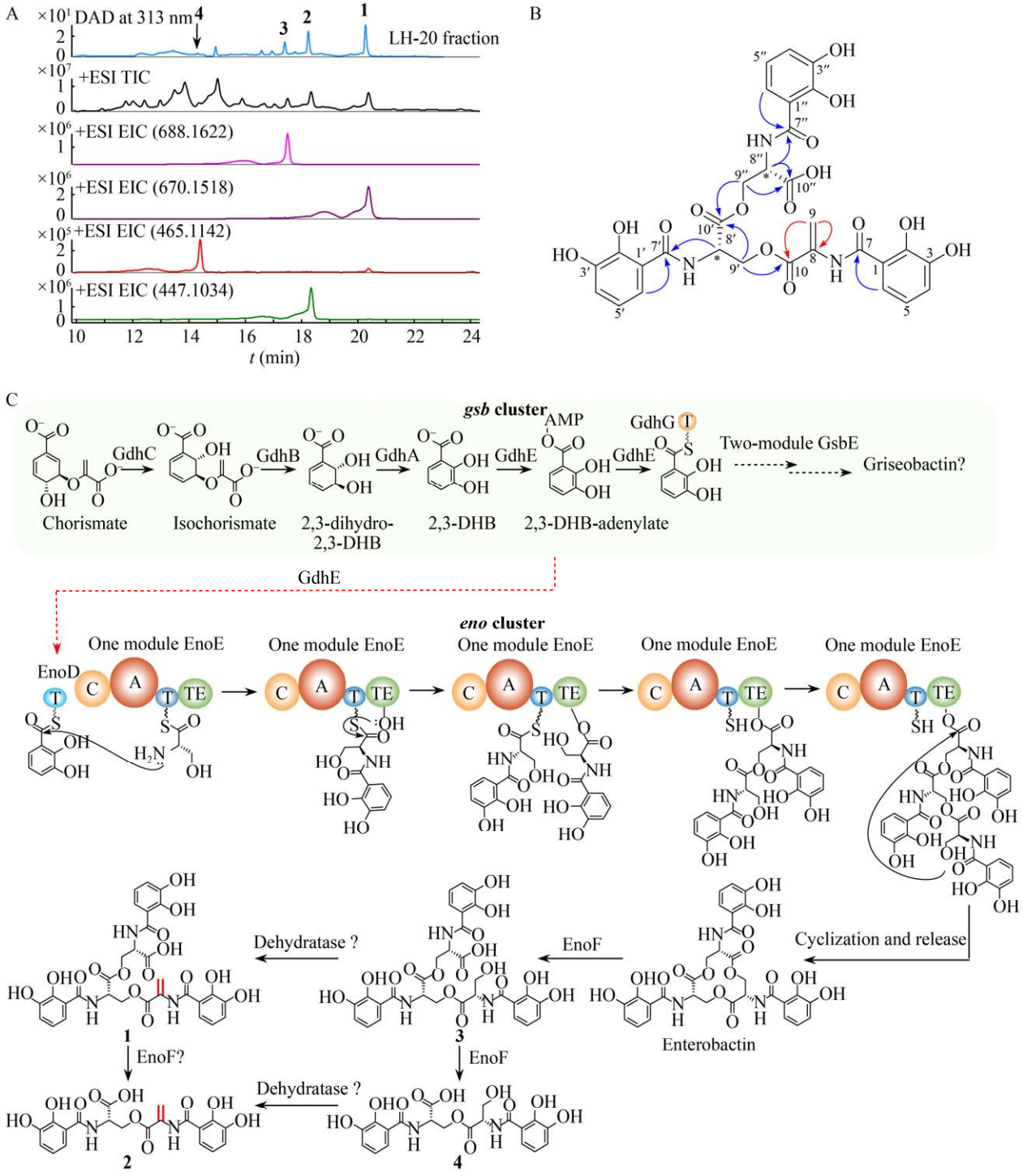

Figure 2 LC-MS data, structural elucidation and proposed biosynthetic pathway

图 2 液质数据、结构解析以及推测的生物合成途径

Note: A: LC-MS profile of the fractionated crude extracts of *S. albofaciens* JCM 4342; B: Key HMBC correlations of compound **1**; C: Proposed synergistic biosynthetic pathway of compounds **1**−**4**

注：A：*S. albofaciens* JCM 4342 粗分提取物的液质分析；B：化合物 **1** 的主要 HMBC 相关；C：推测的化合物 **1**−**4** 的协同生物合成途径

The molecular formulas of **1** and **2** were assigned as $C_{30}H_{27}N_3O_{15}$ and $C_{20}H_{18}N_2O_{10}$ by HR-ESIMS, which showed $[M+H]^+$ ion peaks at *m*/*z* 670.151 8 (calcd 670.151 5, Δ= –0.4 ppm) and 447.1034 (calcd 447.103 4, Δ= –1.3 ppm), respectively (Figure 2A). The 1D-NMR data revealed that the planar structure of **1** consisted of six carbonyl carbons, three benzenes (nine are proton bearing and six are oxygenated), three methenes (in particular, one terminal double bond at δ_H 6.50/5.77 and δ_C 109.5) and two nitrogen-bearing methines at δ_H 4.97/4.80 (m) (Table 2). These data suggested that **1** was not enterobactin but a dehydrated product of linear 2,3-DHBS trimer (=N,N′,N″-tris(2,3-dihydroxybenzoyl)-O-seryl-O-seryl serine). The key HMBC correlations further supported the above structural elucidation (Figures 2B). Compared to **1**, 1D-NMR spectra of **2** also showed the α-aminoacrylyl residue signals (δ_H 6.54/5.81 and δ_C 109.4), but possessed additional proton signals corresponding to one more 2,3-DHBS monomer in the structure (Table 2). Based on bioinformatics analyses, the adenylation domain of EnoE was predicted to use L-serine as a substrate. EntF, the homolog of EnoE, also utilized L-serine as the precursor for the biosynthesis of enterobactin[38]. Taken together, the compounds **1** and **2** were determined as N,N′,N″-tris(2,3-dihydroxybenzoyl)-O-(α-aminoacrylyl)-O-L-seryl L-serine (dehydrated 2,3-DHBS trimer) and N,N′-bis(2,3-dihydroxybenzoyl)-O-(*α*-aminoacrylyl)-O-L-serine (dehydrated 2,3-DHBS dimer), respectively (Figure 2C). Further analysis of the LC-MS data of JCM 4342 revealed the other two products, which were proposed to be 2,3-DHBS trimer (**3**, MW=687.154 3) and 2,3-DHBS dimer (**4**, MW=464.106 3), respectively, whereas enterobactin was not detected (Figure 2C).

Table 2 ^{1}H and ^{13}C NMR data (δ in ppm, *J* in Hz) of compounds 1 and 2 in DMSO-*d6*
表 2 化合物 1 和 2 的 ^{1}H 和 ^{13}C 核磁数据(DMSO-*d6*)

No.	**1**[a]		**2**[b]		No.	**1**[a]	
	δ_H	δ_C	δ_H	δ_C		δ_H	δ_C
1		118.7		118.8	1″		115.4
2		145.8		145.6	2″		148.8[d]
3		146.2[c]		146.2	3″		146.1[c]
4	6.97, br. d (7.9)	118.8	6.98, br. d (7.9)	119.1	4″	6.93, br. d (7.9)	119.1[h]
5	6.75, t (7.9)	119.1	6.76, t (7.9)	119.2	5″	6.68, t (7.9)	119.0[h]
6	7.37, br. d (8.0)	120.2	7.38, br. d (8.0)	120.2	6″	7.31, br. d (7.9)	118.3
7		164.7		164.7	7″		169.1[e]
8		132.1		132.2	8″	4.80, m	51.6[f]
9	6.50, s	109.5	6.54, s	109.4	9″	4.65, dd (11.4, 5.1)	64.0[g]
	5.77, s		5.81, s			4.50, dd (11.2, 6.5)	
10		163.1		163.2	10″		170.4
1′		115.5		115.6			
2′		148.7[d]		148.6			
3′		146.2[c]		146.0			
4′	6.93, br. d (7.9)	119.1	6.95, br. d (7.9)	118.6			
5′	6.70, t (7.9)	115.5	6.72, t (7.9)	118.4			
6′	7.29, br. d (7.9)	118.3	7.35, br. d (8.0)	118.3			
7′		168.9[e]		168.9			
8′	4.97, m	51.5[f]	4.90, m	51.5			
9′	4.66, dd (11.2, 4.6)	64.1[g]	4.66, dd (11.2, 4.6)	64.4			
	4.55, dd (11.3, 7.2)		4.59, dd (11.3, 7.2)				
10′		168.6		170.3			
NH	9.05, d (7.3)		9.17, d (7.3)				
NH″	9.22, d (7.4)						
COOH	10.76, br. s		10.73, br. s				

Note: [a]: Assignments were mainly determined by analyses of HMBC spectra; [b]: Assignments were determined partially by comparing NMR data of **1** in our study and literature[39]; [c, d, e, f, g, h]: These signals are exchangeable

3.4 Proposed biosynthetic routes for enterobactin-related natural products

Apparently, based on structural comparisons, compounds **1**−**4** are enterobactin-related natural products. Except lacking the enzymes for 2,3-DHB biosynthesis, the *eno* cluster in JCM 4342 encodes adequate enzymes to catalyze the rest biosynthetic steps of enterobactin and its hydrolysis (Figure 2C). As the *ghd* cluster (proposed for griseobactin biosynthesis) retains the enzymes responsible for 2,3-DHB biosynthesis, we propose that the precursor 2,3-DHB biosynthesized by the *ghd* cluster might be hijacked and incorporated into the Eno pathway, leading to the formation of compounds **1**−**4** (Figure 2C). Specifically, 2,3-DHB was first biosynthesized by GdhABC, and then activated and loaded to the carrier protein EnoD by GhdE. Next, the iterative one-module NRPS enzyme EnoE catalyzed the elongation, and cyclization reactions to release the product enterobactin[24]. As compounds **3** and **4**[21,40] were previously shown to be the FeS-catalyzed hydrolysis products of enterobactin, EnoF (homolog of FeS) was herein predicted to carry out the same hydrolysis reactions to form **3** and **4**. Compounds **1** and **2** were proposed to be derived from **3** and **4**, respectively, through dehydrations catalyzed by unknown dehydratase(s). In addition, compound **2** might also be formed through hydrolysis of **1** by EnoF.

Compounds **1** and **2** were first reported as hydrolysis products of enterobactin in chemical reactions[41-43]. Both compounds were later detected as natural products in the crude extracts of *Streptomyces longisporous* JCM4261 and exhibited inhibition activity against hepatitis C virus protease, whereas no MS and NMR data were reported for structural elucidation[44]. Our study provided detailed chemical information, which enabled us to elucidate the structures of compounds **1** and **2**. Interestingly, we showed that the biosynthesis of compounds **1**−**4** was likely involving the functional cross-talk between *eno* and *gdh* clusters. As compounds **3** and **4** remained the ability of binding Fe^{3+} shown by a previous study[45], compounds **1** and **2** might also possess the iron chelating capability.

4 Conclusion

In this study, we systematically analyzed the distribution and conservation of siderophore BGCs in *Streptomyces*, from which the orphan enterobactin BGCs (lacking 2,3-DHB biosynthetic enzymes) were identified in several strains. *S. albofaciens* JCM 4342, which harbors an orphan enterobactin BGC (*eno* cluster) and a griseobactin BGC (*gdh* cluster), was found to be able to produce the enterobactin-derived linear compounds **3** and **4**, and their dehydrated products **1** and **2**. Based on bioinformatic analyses, the *gdh* cluster was proposed to biosynthesize the 2,3-DHB unit, and deliver it to the biosynthetic machinery encoded by the *eno* cluster, eventually leading to the formation of compounds **1**−**4**. These results suggested an interesting synergistic biosynthetic mechanism accomplished by two siderophore BGCs. As orphan enterobactin BGCs and griseobactin BGCs also coexist in *S. rimosus* strains, similar biosynthetic process might occur in these strains. In addition, the discovery and characterization of compounds **1** and **2** provides new substrates for the further structural diversification to generate novel enterobactin-related siderophores[21].

Acknowledgments

We thank Drs. WANG Wenzhao and REN Jinwei for the assistance in collection of LC-HRMS and NMR data, respectively.

REFERENCES

[1] Raines DJ, Sanderson TJ, Wilde EJ, Duhme-Klair AK. Siderophores in Reference Module in Chemistry, Molecular Sciences and Chemical Engineering[M]. Netherlands: Elsevier, 2015: 1-32

[2] Hider RC, Kong XL. Chemistry and biology of siderophores[J]. Natural Product Reports, 2010, 27(5): 637-657

[3] Garénaux A, Caza M, Dozois CM. The Ins and Outs of siderophore mediated iron uptake by extra-intestinal pathogenic *Escherichia coli*[J]. Veterinary Microbiology, 2011, 153(1/2): 89-98

[4] Krewulak KD,Vogel HJ. Structural biology of bacterial iron uptake[J]. Biochimica et Biophysica Acta (BBA) - Biomembranes, 2008, 1778(9): 1781-1804

[5] Mies KA, Wirgau JI, Crumbliss AL. Ternary complex formation facilitates a redox mechanism for iron release from a siderophore[J]. Biometals, 2006, 19(2): 115-126

[6] Köster W. ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12[J]. Research in Microbiology, 2001, 152(3): 291-301

[7] Schneider K, Rose I, Vikineswary S, Jones AL, Goodfellow M, Nicholson G, Beil W, Süssmuth RD, Fiedler HP.

Nocardichelins A and B, siderophores from *Nocardia* strain acta 3026[J]. Journal of Natural Products, 2007, 70(6): 932-935

[8] Sheth S. Iron chelation: an update[J]. Current Opinion in Hematology, 2014, 21(3): 179-185

[9] Kontoghiorghe CN, Kontoghiorghes GJ. Efficacy and safety of iron-chelation therapy with deferoxamine, deferiprone, and deferasirox for the treatment of iron-loaded patients with non-transfusion-dependent thalassemia syndromes[J]. Drug Design, Development and Therapy, 2016, 10: 465-481

[10] Brittenham GM, Griffith PM, Nienhuis AW, McLaren CE, Young NS, Tucker EE, Allen CJ, Farrell DE, Harris JW. Efficacy of deferoxamine in preventing complications of iron overload in patients with thalassemia major[J]. The New England Journal of Medicine, 1994, 331(9): 567-573

[11] Saha P, Yeoh BS, Xiao X, Golonka RM, Kumarasamy S, Vijay-Kumar M. Enterobactin, an iron chelating bacterial siderophore, arrests cancer cell proliferation[J]. Biochemical Pharmacology, 2019, 168: 71-81

[12] Braun V, Pramanik A, Gwinner T, Köberle M, Bohn E. Sideromycins: tools and antibiotics[J]. BioMetals, 2009, 22(1): 3-13

[13] Wang WF, Qiu ZQ, Tan HM, Cao LX. Siderophore production by actinobacteria[J]. Biometals, 2014, 27(4): 623-631

[14] Sheng MM, Jia HK, Zhang GY, Zeng LN, Zhang TT, Long YH, Lan J, Hu ZQ, Zeng Z, Wang B, et al. Siderophore production by rhizosphere biological control bacteria *Brevibacillus brevis* GZDF3 of *Pinellia ternata* and its antifungal effects on *Candida albicans*[J]. Journal of Microbiology and Biotechnology, 2020, 30(5): 689-699

[15] Schumann G, Möllmann U. Screening system for xenosiderophores as potential drug delivery agents in mycobacteria[J]. Antimicrobial Agents and Chemotherapy, 2001, 45(5): 1317-1322

[16] Kong HM, Cheng WY, Wei H, Yuan YL, Yang ZH, Zhang XJ. An overview of recent progress in siderophore-antibiotic conjugates[J]. European Journal of Medicinal Chemistry, 2019, 182: 111615

[17] Crosa JH, Walsh CT. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria[J]. Microbiology and Molecular Biology Reviews, 2002, 66(2): 223-249

[18] Huang TT, Lin SJ, Deng ZX. Recent advances in mechanism of siderophore biosynthesis in actinomycetes[J]. Microbiology China, 2011, 38(5): 765-773 (in Chinese)
黄婷婷, 林双君, 邓子新. 放线菌中铁载体生物合成机制研究进展[J]. 微生物学通报, 2011, 38(5): 765-773

[19] Challis GL. A widely distributed bacterial pathway for siderophore biosynthesis independent of nonribosomal peptide synthetases[J]. Chembiochem, 2005, 6(4): 601-611

[20] Fischbach MA, Walsh CT. Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms[J]. Chemical Reviews, 2006, 106(8): 3468-3496

[21] Reitz ZL, Sandy M, Butler A. Biosynthetic considerations of triscatechol siderophores framed on serine and threonine macrolactone scaffolds[J]. Metallomics, 2017, 9(7): 824-839

[22] Harris WR, Carrano CJ, Cooper SR, Sofen SR, Avdeef AE, McArdle JV, Raymond KN. Coordination chemistry of microbial iron transport compounds. 19. Stability constants and electrochemical behavior of ferric enterobactin and model complexes[J]. Journal of the American Chemical Society, 1979, 101(20): 6097-6104

[23] Raymond KN, Dertz EA, Kim SS. Enterobactin: an archetype for microbial iron transport[J]. Proceedings of the National Academy of Sciences of the United States of America, 2003, 100(7): 3584-3588

[24] Sattely ES, Fischbach MA, Walsh CT. Total biosynthesis: *in vitro* reconstitution of polyketide and nonribosomal peptide pathways[J]. Natural Product Reports, 2008, 25(4): 757-793

[25] Röttig M, Medema MH, Blin K, Weber T, Rausch C, Kohlbacher O. NRPSpredictor2 — a web server for predicting NRPS adenylation domain specificity[J]. Nucleic Acids Research, 2011, 39(suppl_2): W362-W367

[26] Prieto C, García-Estrada C, Lorenzana D, Martín JF. NRPSsp: non-ribosomal peptide synthase substrate predictor[J]. Bioinformatics, 2012, 28(3): 426-427

[27] Agüero-Chapin G, Pérez-Machado G, Sánchez-Rodríguez A, Santos MM, Antunes A. Alignment-free methods for the detection and specificity prediction of adenylation domains[M]. Nonribosomal Peptide and Polyketide Biosynthesis, 2016, 1401: 253272

[28] Van Der Meij A, Worsley SF, Hutchings MI, Van Wezel GP. Chemical ecology of antibiotic production by actinomycetes[J]. FEMS Microbiology Reviews, 2017, 41(3): 392-416

[29] Zeng Y, Kulkarni A, Yang ZY, Patil PB, Zhou W, Chi XL, Van Lanen S, Chen S. Biosynthesis of albomycin δ2 provides a template for assembling siderophore and aminoacyl-tRNA synthetase inhibitor conjugates[J]. ACS Chemical Biology, 2012, 7(9): 1565-1575

[30] Lautru S, Deeth RJ, Bailey LM, Challis GL. Discovery of a new peptide natural product by *Streptomyces coelicolor* genome mining[J]. Nature Chemical Biology, 2005, 1(5): 265-269

[31] Barona-Gómez F, Wong U, Giannakopulos AE, Derrick PJ, Challis GL. Identification of a cluster of genes that directs desferrioxamine biosynthesis in *Streptomyces coelicolor* M145[J]. Journal of the American Chemical Society, 2004, 126(50): 16282-16283

[32] Challis GL, Ravel J. Coelichelin, a new peptide siderophore encoded by the *Streptomyces coelicolor* genome: structure prediction from the sequence of its non-ribosomal peptide synthetase[J]. FEMS Microbiology Letters, 2000, 187(2): 111-114

[33] Patzer SI, Braun V. Gene cluster involved in the

biosynthesis of griseobactin, a catechol-peptide siderophore of *Streptomyces* sp. ATCC 700974[J]. Journal of Bacteriology, 2010, 192(2): 426-435

[34] Lee J, Postmaster A, Soon HP, Keast D, Carson KC. Siderophore production by actinomycetes isolates from two soil sites in Western Australia[J]. BioMetals, 2012, 25(2): 285-296

[35] Fiedler HP, Krastel P, Müller J, Gebhardt K, Zeeck A. Enterobactin: the characteristic catecholate siderophore of Enterobacteriaceae is produced by *Streptomyces* species[J]. FEMS Microbiology Letters, 2001, 196(2): 147-151

[36] Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. BLAST+: architecture and applications[J]. BMC Bioinformatics, 2009, 10: 421

[37] Rice P, Longden I, Bleasby A. EMBOSS: the European molecular biology open software suite[J]. Trends in Genetics, 2000, 16(6): 276-277

[38] Shaw-Reid CA, Kelleher NL, Losey HC, Gehring AM, Berg C, Walsh CT. Assembly line enzymology by multimodular nonribosomal peptide synthetases: the thioesterase domain of *E. coli* EntF catalyzes both elongation and cyclolactonization[J]. Chemistry & Biology, 1999, 6(6): 385-400

[39] Igarashi Y, Iida T, Fukuda T, Miyanaga S, Sakurai H, Saiki I, Miyanouchi K. Catechoserine, a new catecholate-type inhibitor of tumor cell invasion from *Streptomyces* sp.[J]. The Journal of Antibiotics, 2012, 65(4): 207-209

[40] Lin HN, Fischbach MA, Liu DR, Walsh CT. *In vitro* characterization of salmochelin and enterobactin trilactone hydrolases IroD, IroE, and Fes[J]. Journal of the American Chemical Society, 2005, 127(31): 11075-11084

[41] O'Brien IG, Gibson F. The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*[J]. Biochimica et Biophysica Acta, 1970, 215(2): 393-402

[42] O'Brien IG, Cox GB, Gibson F. Biologically active compounds containing 2,3-dihydroxybenzoic acid and serine formed by *Escherichia coli*[J]. Biochimica et Biophysica Acta, 1970, 201(3): 453-460

[43] Rogers HJ. Enterochelin complexes, pharmaceutical compositions containing them and a process for preparing them: Japan, EP0005346A1[P]. 1979.04.25. https://www.surechembl.org/document/EP-0005346-B1

[44] Tadashi Y, Yoichi O, Atsushi M, Hisafumiy S. Hepatitis C virus protease inhibitor: Japan, JP H10298151[P]. 1997.04.30. https://worldwide.espacenet.com/publicationDetails/biblio?FT=D&date=19981110&DB=EPODOC&locale=en_EP&CC=JP&NR=H10298151A&KC=A&ND=5

[45] Hantke K. Dihydroxybenzoylserine: a siderophore for *E. coli*[J]. FEMS Microbiology Letters, 1990, 55(1/2): 5-8