Production of catechol siderophores by bacteria selected from feces of a healthy female adult through screening NRPS A domain

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Research article

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Abstract

Background: Catechol siderophores, which are widespread in the human gastrointestinal tract (GIT) and play important roles in maintaining iron-nutrition balance between microorganisms and host, are small molecules with a high affinity for ferric iron and are assembled by nonribosomal peptide synthetases (NRPS). In this study, we select strains encoding NRPS A domain gene from feces of a health female adult, predict its products type, and check it out in vitro .

Results: Firstly, eight bacteria were determined encoding NRPS A domain gene, and then two kind of catechol siderophores, Bacillibactin and Enterobactin, were predicted according to NRPS A domain substrate specificity. Secondly, Bacillibactin and Enterobactin were checked out in cultured medium aerobic incubated with selected strains of E. Coli, Bacillus sp. and B. Cereus. For the yield of catechol siderophores, B. cereus Gut 16 secreted highest yield of Bacillibactin and E. coli Gut 07 produce highest yield of Enterobactin.

Conclusion: By presence determination and activity prediction of NRPS A domain, two siderophores, Bacillibactin and Enterobactin, were checked out finally being secreted by selected strains cultured in a aerobic medium. Further research on the potential probiotic property is necessary to affirm the application in biological industry, as well as to elucidate their mechanism in human gut.

Keywords : Catechol siderophores, Enterobactin, Bacillibactin, NRPS A domain, Feces, Probiotics

Background

Siderophores are small organic molecules produced by microorganisms under iron-limiting conditions which enhance the uptake of iron to the microorganisms [1, 2]. More than 500 kinds of siderophore proteins have been discovered, and most of them are divided into three categories: catecholates, hydroxamaces, and carboxylates [3]. The iron-chelating ability of catechol siderophore is much higher than that of other iron-binding proteins secreted by vertebrate host cells [4].

Catechol siderophores belong to non-ribosomal peptides and are synthesized by NRPSs which is a class of giant enzymes with modular domains [5–8]. Further and more importantly substrate identification specificity reportedly exists in the NRPS adenine structure domain (NRPS A domain) can be used to predict the function and type of NRPS products [9, 10].

In recent years, more and more studies have found that siderophores have shown unique efficacy in the prevention and treatment of gastrointestinal diseases. Many microorganisms that produce siderophores are critical to the health of their hosts. These microorganisms show great adaptability in the low-iron environment of the GIT and can compete with pathogenic bacteria for iron to inhibit the growth of

pathogenic microorganisms [11]. Probiotic E. coli Nissle 1917 can compete with pathogenic bacteria in the gastrointestinal environment by secreting siderophores, thus inhibiting intestinal pathogenic bacteria [12]. Some microorganisms, such as Lactobacillus and Bifidobacterium, which can not synthesize siderophores by themselves, are co-cultured with iron-producing microorganisms in simulated gastrointestinal environment, in which siderophores-producing bacteria play a probiotic role as iron supplements to increase the number and growth rate of these strains significantly, thus maintaining the balance of the gastrointestinal flora in the GIT [13–15]. Studies have further shown that siderophores function in plant nutrition and protection, fish disease prevention and medical treatment [12, 16–20].

Most screened and isolated siderophores high-yielding strains originate from the ocean and plant rhizosphere soil, while the research on isolation and screening of siderophores high-yielding strains in human GIT from feces is less. In the present study, identified positive strains of NRPS A domain gene were screened from healthy adult feces. The categories and capacity producing siderophores were then analyzed to lay the foundation for application in future biological industry, also for further study on their mechanism of action in gastrointestinal nutrition and health.

Results

Positive strains containing the NRPS A domain gene

Twenty-one strains of facultative anaerobes from feces were isolated. Positive amplification products of NRPS A domain gene were found in all 21 bacteria. The amplification product size was 800 bp, consistent with the expected size of the target fragment (Fig. 1).

Species identification of strains containing the NRPS A domain

Comparative analysis of 16S rRNA gene sequence was performed, and the duplicated ones were excluded. Finally eight strains of bacteria remained, including Gut 01, Gut 07, Gut 12, Gut 13, Gut 20, Gut 03, Gut 14, and Gut 16. The sequencing results of the 16S rRNA gene sequence of eight strains were submitted to NCBI. The serial login number of GenBank was KU156682-KU156689 (Table 1) .

Table 1
Comparative analysis ofIdentification of selected strains by 16S rRNA gene sequenceing

	Accession No.	Closest relative and accession number	Similarity %
Gut 01	KU156682	Escherichia coli strain E191-4 (KJ477005.1)	99
Gut 07	KU156684	Escherichia coli O157:H7 strain WS4202 (CP012802.1)	99
Gut 12	KU156685	Escherichia coli strain E84-1 (KJ477001.1)	99
Gut 13	KU156686	Escherichia coli strain RCB273 (KT260485.1)	99
Gut 20	KU156689	Escherichia coli K-12 strain DHB4 (CP014270.1)	99
Gut 03	KU156683	Bacillus cereus strain BQAR-01d (FJ217203.1)	93
Gut 14	KU156687	Bacillus sp. B31(2008) (EU384285.1)	93
Gut 16	KU156688	Bacillus cereus strain S2-8 (CP009605.1)	99

Blast alignment of 16S rRNA gene sequences of eight culturable microorganisms from human digestive tract with 16S rRNA gene sequences were found in the GenBank database. If the similarity of the 16S rRNA gene sequence was > 99%, the microorganism was intrageneric; if the similarity was between 93% and 95%, the microorganism was extrageneric. Blast results showed that the 16S rRNA gene sequences of Gut 01, Gut 07, Gut 12, Gut 13, and Gut 20 were highly similar (99%) to those of E. coli strain E191-4 (KJ477005.1), E. coli O157:H7 strain WS4202 (CP012802.1), E. coli strain E84-1 (KJ477001.1), E. coli strain RCB273 (KT260485.1), and E. coli K-12 strain DHB4 (CP014270.1), respectively. The 16S rRNA sequences of Gut 03, Gut 14, and Gut 16 had the highest similarity with Bacillus cereus sp. Bqar-01d (FJ217203.1) (93%), Bacillus sp. B 31(2008) (EU384285.1) (93%), and B. cereus strain s2-8 (CP009605.1) (99%) respectively.

The 16S rRNA gene sequence of related strains with high similarity was downloaded and the phylogenetic trees of 16S rRNA genes were constructed with MEGA 5.2 software. Figure 2 shows that Gut 01, Gut 07, and Gut 13 were on the same branch, and Gut 12 and Gut 20 were on the other branch. They were homologous to different species of microorganisms of E. coli, and the similarity of 16S rRNA gene sequences between five strains and their homologous strains was 99%. Thus, these five strains may be new species or subspecies of E. coli. Gut 16 had homology with B. cereus strain s2-8 (CP009605.1) in the same branch and its 16S rRNA gene sequence similarity was 99%, so it may be a new species or subspecies of B. cereus. Gut 03 and Gut 14 had homology with Bacillus sp. B31(2008) (EU384285.1) and Bacillus sp. Bqar-01d (FJ217203.1) in the same branch, and the similarity of the 16S rRNA gene sequence with its homologous strain was 93%. Thus, Gut 03 and Gut 14 were new genera or subgenera of bacillaceae.

Product type of NRPS A domain

The amino acid sequences of the NRPS A domain of eight strains were submitted to NCBI. The serial login number of GenBank was KU156690-KU156697(Fig. 3 and Table 2).

The amino acid sequences of the NRPS A domain in Gut 01, Gut 03, Gut 07, Gut 12, Gut 13, and Gut 20 were in the same branch of the phylogenetic tree, which was homologous with the Ent F structure domain that participated in enterobactin synthesis. The similarity ranged from 97.7–99.2%. Therefore, in Gut 01, Gut 03, Gut 07, Gut 12, Gut 13, and Gut 20, the catechins synthesized by NRPS were enterobactin.The amino acid sequence of the NRPS A domain in Gut 14 and Gut 16 was the most homologous with the NRPS DHB F domain of Bacillus subtilis strain 168 (P45745), with similarity rates of 81.4% and 81.3%, respectively. In the evolutionary relationship, they were homologous. The DHB F domain is an important domain of Bacillaceae, which participates in bacillocin synthesis [21]. Thus, in Gut 14 and Gut 16, the catechin synthesized by NRPS was bacillibactin. Unlike Bacillus sp. Gut 14 which encode Bacillibactin of catechol siderophore, Bacillus sp. Gut 03 encode Enterobactin of catechol siderophore which were encoded by 5 E.coli bacteria in this study (Fig. 3 and Table 2).

Table 2
Similarity of amino acid sequences of NRPS A domain

	Accession No.	Closest NRPS relative and accession number	Similarity %	Original cell
E. coli Gut 01	KU156690	Enterobactin synthase component F (Ent F) (Q8XBV9)	97.7	Escherichia coli O157:H7
Bacillus sp. Gut 03	KU156691	Enterobactin synthase subunit F (Ent F) (P29698)	98.1	Shigella flexneri
E. coli Gut 07	KU156692	Enterobactin synthase component F (Ent F) (Q8XBV9)	98.9	Escherichia coli O157:H7
E. coli Gut 12	KU156693	Enterobactin synthase subunit F (Ent F) (P11454)	99.2	Escherichia coli
E. coli Gut 13	KU156694	Enterobactin synthase subunit F (Ent F) (P11454)	98.7	Escherichia coli
Bacillus sp. Gut 14	KU156695	Dimodular nonribosomal peptide synthase component F (DHB F) (P45745)	81.4	Bacillus subtilis strain 168
B. cereus Gut 16	KU156696	Dimodular nonribosomal peptide synthase component F (DHB F) (P45745)	81.3	Bacillus subtilis strain 168
E. coli Gut 20	KU156697	Enterobactin synthase subunit F (Ent F) (P29698)	97.9	Shigella flexneri

Producation of catechol siderophores of selected Strains

The orange halo of 8 strains appeared on CAS solid plate, indicating that all 8 strains had siderphores formation (Fig. 4a). However, the CAS assay could not indicate whether catechol siderophores were secreted by bacteria in this study. By measuring the functional group DHB, all the eight selected strains secrete catechol siderophores more or less and their yield were significantly different ($P < 0.05$). The highest yield strain was B. cereus Gut 16 (94.75 ± 0.40 μM) which was the only one Bacllus cereus bacteria producing catechol siderophores other than two Bacillus sp. and five E. coli bacteria. in this study. For yield of Bacillibactin, B. cereus Gut 14 secrete less than B. cereus Gut 16. For yield of Enterobactin, E. coli Gut 07 and E. coli Gut 12 were the first and second on the list and secrete more than other 3 E.coli bacteria and Bacillus sp. Gut 03 (Fig. 4b).

Discussion

In the present study, detecting gene and predicting product of NRPS A domain positively from bacteria, which was selected in feces of a female health adult and cultured in a aerobic medium, was proved to be helpful to found out catechol siderophore from microbiome of human gastrointestinal habitat. Bacillibactin and Enterobactin, two kind of catechol siderophore, were checked out and measured in the supernatant of a succinic acid medium with eight catechol siderophore-producing bacteria inoculated in.

First of all, eight bacteria were determined encoding NRPS A domain gene. And two catechol siderophore, Bacillibactin and Enterobactin, were predicted according to NRPS A domain substrate specificity, which is consistent with the in vitro checking out of catechol siderophore from cultured medium with selected strains in this study. NRPS A domain sequence analysis was a useful way to screen and identify catechol siderophore and their chemical types of this secondary metabolites. Using this approach, one bacteria strain screened by the presence of conserved adenylation domains of NRPS genes and prediction of its antibiotic product, later identified as a Bacillus, was shown to strongly inhibit the growth of Staphylococcus aureus, S. epidermidis, and Enterococcus faecalis [22, 23]. In the present study, after two catechol siderophores were predicted according the presence of NRPS A domain, the yield of catechol siderophores (Bacillibactin or Enterobactin) were checked out in a cultured medium, as is a powerful proving about the catechol siderophore-finding approach of NRPS A domain catching.

A comparative analysis of 16S rRNA gene sequences and a phylogenetic tree showed that three strains of Bacillus and five strains of Escherichia secret catechol siderophores. NRPS A domain sequence analysis on all of the strains, except Bacillus sp. Gut 03, showed the same genus belonging, NRPS A domain sequence analysis on Bacillus sp. Gut 03 indicated that the siderophore procuct is Enterobactin from Shigella flexneri (P29698). Therefore, the amino acid sequence of the NRPS A domain can be used to predict the type of compound of secondary metabolites, but can not accurately reflect the evolutionary relationship between species.

In the present work, B. cereus Gut 16 with a high production of Bacillibactin and E. coli Gut 07 with a high production of Enterobactin were screened in vitro from feces of a health adult, indicating that these two strains play a critical role, good or bad, in human gut by siderophore pathway, also showing their

application prospects in biological technology. So future research on probiotic property of these selected strains is such a necessary and urgent issue because it looks like that whether a siderophore-producing strain is good or bad depends on its probiotic properties may have. Zawadzka et al. reported that the pathogenic role of Bacillus largely depended on the siderophores it secreted and the iron-transport protein on the membrane. So the siderophore was identified as one of the virulence factors [24]. On the contrary, Cursino et al. reported that E. coli strain H22 has several sets of iron-collection systems that have antagonistic effects on intestinal microorganism in vivo and in vitro. They also suggested that the E. coli strain H22 can be a potential probiotics for livestock and humans [25]. Some other researchers also reported that siderophores have beneficial effect in preventing and treating gastrointestinal diseases [26–29].

Conclusions

Two siderophores, Bacillibactin and Enterobactin, were family checked out by presence determination and activity prediction of NRPS A domain, were determinated to be secreted by selected strains cultured in a aerobic medium. Further research on the potential probiotic property is necessary to affirm the application in biological industry, as well as to elucidate the mechanism in human gut.

Methods

Solution and medium

The simulated gastric juice (1000 mL; pH 1.8) contained the following: peptone, 8.3 g; glucose, 3.5 g; NaCl, 6.2 g; KCl, 2.2 g; $CaCl_2$, 0.22 g; $NaHCO_3$, 1.2 g; and pepsase, 3.0 g. The simulated intestinal juice (1000 mL; pH 7.5) contained the following: peptone, 8.3 g; glucose, 3.5 g; NaCl, 1.28 g; KCl, 0.239 g; $NaHCO_3$, 6.4 g; bile salt, 3.0 g; and pancreatic enzymes, 1.0 g). The solutions were passed through a 0.22 μm filtration membrane to remove bacteria.

The MRS liquid medium without iron (1000 mL; adjusted to pH 7.0–7.2 using 0.05 g of 8-hydroxyquinoline contained the following: peptone, 10.0 g; glucose, 10.0 g; yeast extract, 5.0 g; K_2HPO_4, 2.0 g; sodium acetate, 5.0 g; $C_6H_{14}N_2O_7$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and $MnSO_4 \cdot 5H_2O$, 0.05 g. The medium was extracted twice with isovolumetric chloroform, which was then discarded. The final solution was sterilized at 121 °C for 21 min. For solid MRS medium without iron, an extra 15.0 g of agar powder was added.

The CAS detection liquid (100 mL) contained the following: 0.0219 g of HDTMA and 4.3079 g of anhydrous piperazine in distilled water; 1.5 mL of 1 mM ferric chloride stock solution (1 mM $FeCl_3 \cdot 6H_2O$, 10 mM HCl), 7.5 mL of 2 mM CAS solution, and distilled water to 100 mL.

Isolation and purification of strains

A healthy female adult with no gastrointestine-related disease and who had not taken any medication for 2 weeks before sampling was selected (The participant provided informed consent for the research use of her samples). Feces was removed using cotton swab and was quickly collected in 10 mL of sterile saline and mixed. After centrifugation, 1 mL of supernatant was removed and 9 mL of simulated gastric juice was added. The liquid was cultured at 37 °C for 4 h, and then 40 mL of simulated intestinal juice was added. The liquid was incubated at 37 °C for 18 h for separation.

About 200 μL of liquid culture above was coated in different media (MRS, RCA, and BHI) at 37 °C for 4 h. Initial screening was performed according to the morphological characteristics of the colon. The screened colonies were inoculated in semisolid medium. After incubation, the purified strains were stored in glycerin at -20 °C.

Screening and identification of strains containing NRPS A domain gene

To screen positive strains containing NRPS A domain, the whole genome of the purified strains were extracted using a genome-wide extraction kit (Tiangen Biotech Beijing Co., Ltd.) and stored at -20 °C before use. By using CODEHOP software [30], primers were designed according to the NRPS gene conservative region (adenine structure domain, A domain). The primers were F2 and AR1 (Table 3). The PCR reaction system (25 ing to the NRPS gene conservative region (adenP (2.5 mmol/L), 2 μL, Taq polymerase (5 U/μL), 0.1 μL; 10 μmol/L upstream and downstream primer (10 μmol/L), 1 μL; template DNA, 1 μL; and ddH_2O, 17.4 μL. The reaction conditions were as follows: 94 °C, 5 min; 94 °C, 40 s, 55 °C 45 s, 72 °C 105 s, 35 cycles; 72 °C, 7 min. The amplified PCR products were evaluated by 1.2% (w/v) agarose gel electrophoresis. The target fragment size was about 800 bp.

To identify the NRPS A domain-positive strains, the 16S rRNA gene of NRPS A domain-positive strains were amplified using the universal primer 27F/1492R (Table 3). After purification, the products were connected with pmd19-T carrier (TaKaRa, Japan) and then imported into Escherichia coli DH5α (Sangon Biotech Shanghai Co., Ltd.) sensing cells. Positive clones were sequenced. The carrier sequence was removed from the sequencing results, and then the 16S rRNAs of the strains were analyzed with NCBI Blast. Their classification status was preliminarily determined. The 16S rRNA gene sequence with high similarity was downloaded as the reference sequence for phylogenetic analysis, and the comparison was conducted with Clustal W software. After the phylogenetic tree was constructed using MEGA 5.2 software (through the neighbor-joining method, setting up bootstrap to 1000 times) [31], the 16S rRNA gene sequence was submitted to the GenBank database.

Table 3
List of primers used for amplification of NRPS gene fragments and 16S rRNA gene sequences

Primers	Sequence (5'-3')
F2	5-GGCAAACCGAAAGGNGTNATGRT-3'
AR1	5'-GCTTCAATYTCRCMYARYTC-3'
27F	5'- AGAGTTTGATCCTGGCTCAG-3'
1492R	5'-TACGGYTACCTTGTTACGACTT-3'

Prediction of product type of NRPS A domain

The NRPS A domain gene of the positive strains were amplified. The product was purified and imported in E. coli DH5α (Sangon Biotech Shanghai Co., Ltd.) after being connected with pmd19-t vector (TaKaRa, Japan). The recovered plasmid from positive clones was sequenced. To verify if the NRPS A domains were sequenced correctly, the carrier sequence was removed from the sequencing results in advance. The Conserved Domain Database (CDD) software in NCBI was used for analysis.

For the prediction of product type of NRPS A domain, the nucleic acid sequence was translated into amino acid sequence by using the open-reading-frame finder software in NCBI, and amino acid sequence analysis was performed using the UniProtKB/Swiss-Prot online database. The amino acid sequence of the NRPS A domain with high similarity was downloaded as the reference sequence, and comparison was conducted with Clustal W software. The phylogenetic tree was constructed using MEGA 5.2 software through the neighbor-joining method, setting up bootstrap to 1000 times, and then the type of secondary metabolites of NRPS was predicted finally [32, 33]. The amino acid sequence of the NRPS A domain was submitted to the GenBank database.

Confirmation of producing catechol siderophores of selected Strain

Qualitative analysis of siderophores production

The detection of siderophores was determined using CAS method [34]. The solid medium for iron removal by MRS was prepared and sterilized at 121 °C for 21 minutes. After the medium was cooled, the strains were dotted on the surface of the medium and cultured at 37 °C for 48 hours. The reserve liquid of l0 mL l0um was cooled, the strains were dotted on the surface of the medium and cultured at 37 of NRPS was predictedson was conducted with Clustal W softsoft W softal W softor anaprepared.When the CAS solid detection solution is cooled to 60 °C, a layer of about l0 mL CAS solid detection solution is slowly poured onto the colonized MRS iron removal medium to cover the plate completely. After 15 minutes, if orange circles appeared around the colonies, it was proved that siderphoress were produced.

Determination of the yield of catechol siderophores

According to Rioux's experimental method, the yield of catechol siderophore was determined by measuring the functional group DHB of catechol siderophores [33]. The activated bacteria were inoculated in 10 mL of succinic acid medium with inoculum size as 10% (v/v) for shake culture (180 rpm) at 37 °C for 36 h and then centrifuged at 12,000 rpm for 30 min. The supernatant was collected and 5 mL of reaction system (sterile ultrapure water, 2.3 mL; 20% sulfuric acid solution, 0.2 mL; supernatant, 1 mL; 1% ammonium ferric citrate solution, 0.1 mL; 2 M ammonium fluoride solution, 0.4 mL; 1% phenanthroline solution, 0.4 mL; and 3 M hexamethylenetetramine, 0.6 mL) was added. After complete mixing and culturing in a water bath at 60 °C for 1 h, the absorbance value at 510 nm was measured at room temperature. Uninoculated succinic acid medium served as the blank. A modified group was set with sterile ultrapure water replacing phenanthroline. Each set of experiment was designed to be three parallels.

Data analysis

One-way ANOVA was performed using SPSS 19.0 software (α is 0.05). Duncan test was used to make multiple comparisons, and results were expressed as the mean and standard deviation.

Abbreviations

GIT: gastrointestinal tract; NRPS:nonribosomal peptide synthetases; CDD:Conserved Domain Database.

Declarations

• Ethics approval and consent to participate

The participant provided informed consent for the research use of her samples. The study was approved by the Ethics Committee of Shanxi Center for Disease Control and Prevention (Shanxi Health Committee, China), registered under code DCP-IDF-SX-12-112.

•Consent for publication

Not applicable.

•Availability of data and material

Raw data of 16S rRNA gene and amino acid sequences of NRPS A domain obtained from selected strains are accessible via NCBI Genbank database under accession number KU156682-KU156697.

•Competing interests

The authors declare that they have no competing interests.

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•Authors' contributions

XZ and HW conceived and designed research. QZ and MZ conducted experiments. YW contributed new reagents or analytical tools. HH analyzed data and wrote the manuscript. All authors read and approved the manuscript.

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Figures

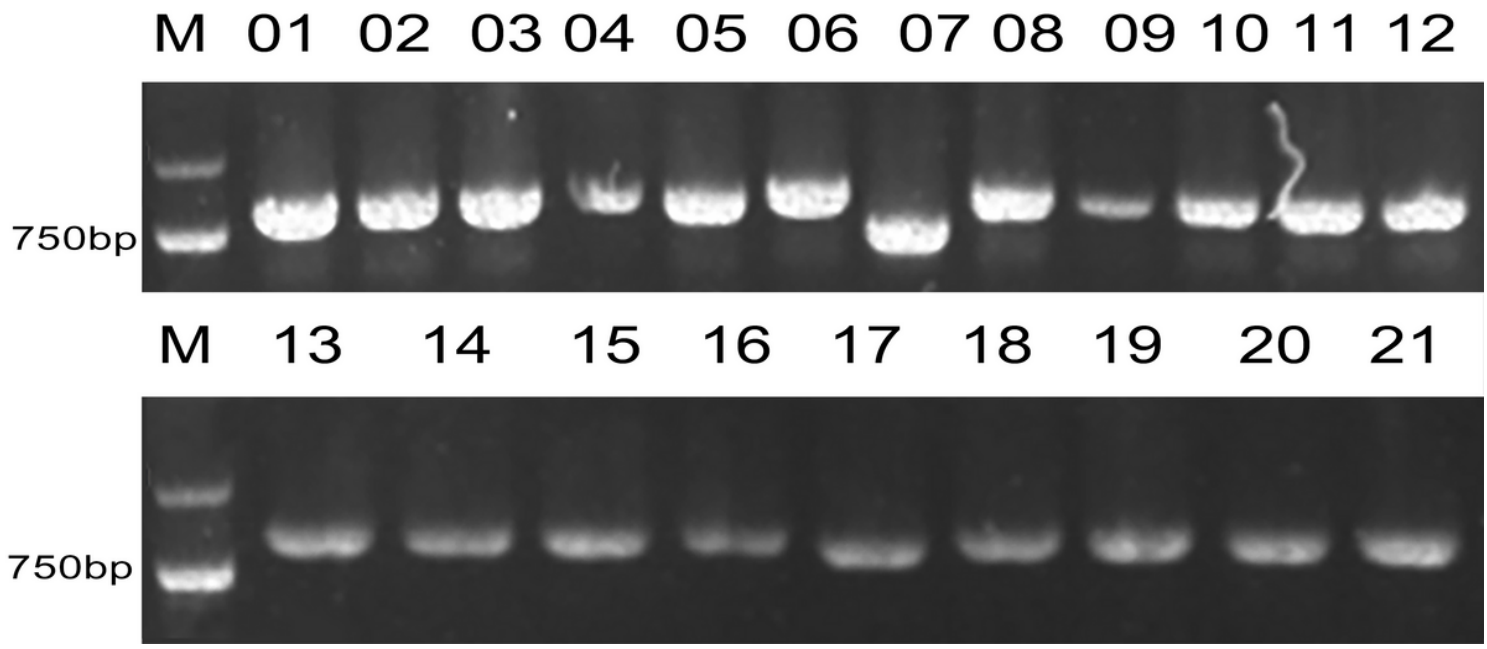

Figure 1

PCR amplification products of NRPS A domain gene. M: DNA ladder-2000 marker, 01–21: 21 amplification products of NRPS A domain gene.

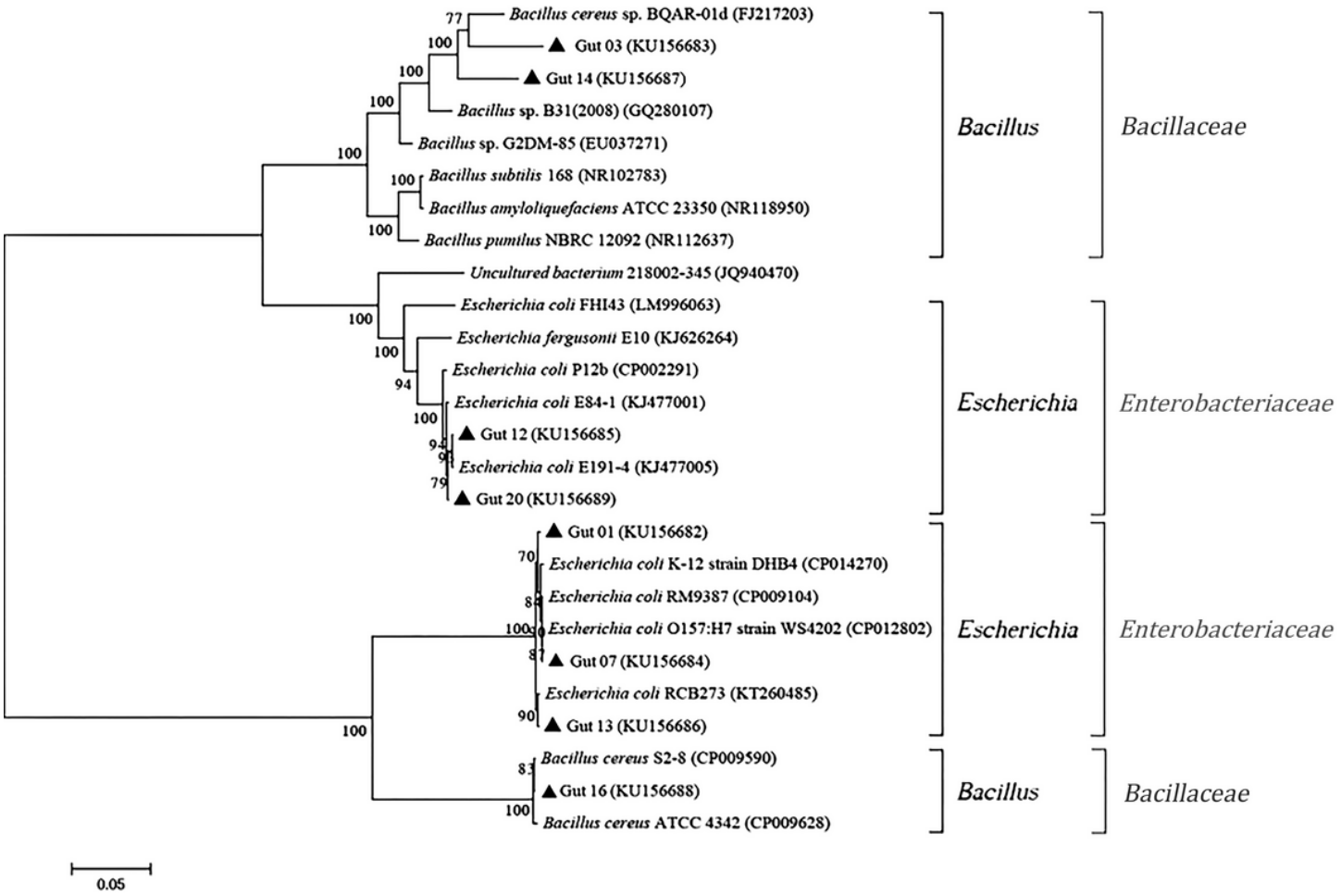

Figure 2

Phylogenetic tree based on the 16S rRNA gene sequences of positive strains. The figures at the branch nodes indicate the bootstrap values on the neighbor-joining analysis of 1000 replicates. Numbers in the brackets are the GenBank accession numbers. The ruler represents the degree of divergence between sequences, or the genetic distance. ▲ represent the tested strains.

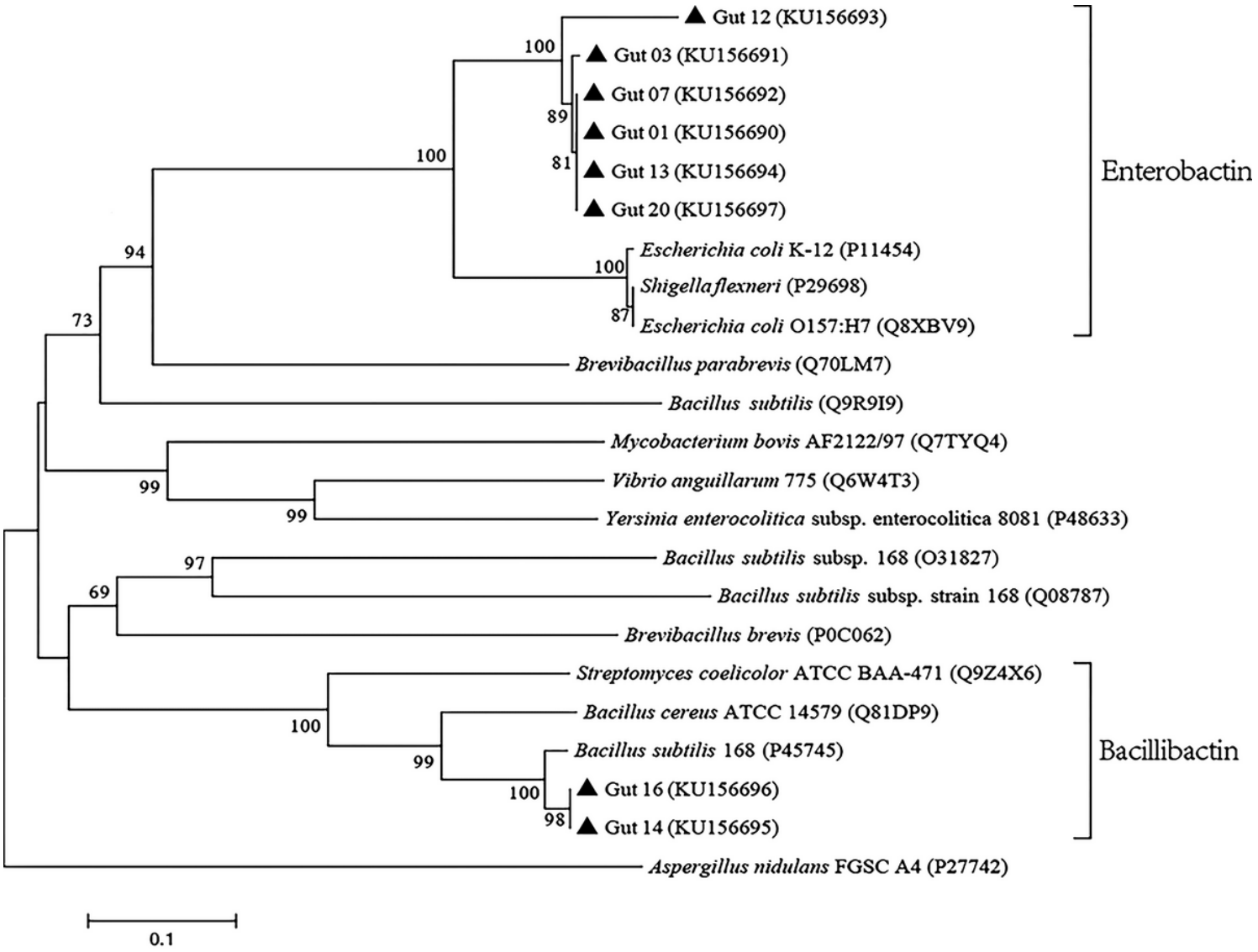

Figure 3

Phylogenetic tree based on the NRPS A domain amino acid sequences of isolated strains. The figures at the branch nodes indicate the bootstrap values on the neighbor-joining analysis of 1000 replicates. Numbers in brackets are the accession numbers of the NRPS A domain amino acid sequences in the UniProt-KB/Swiss-Prot database. The ruler represents the degree of divergence between sequences, or the genetic distance. ▲ strains isolated from human GIT in this study. Predicted chemical classes are indicated on the right.

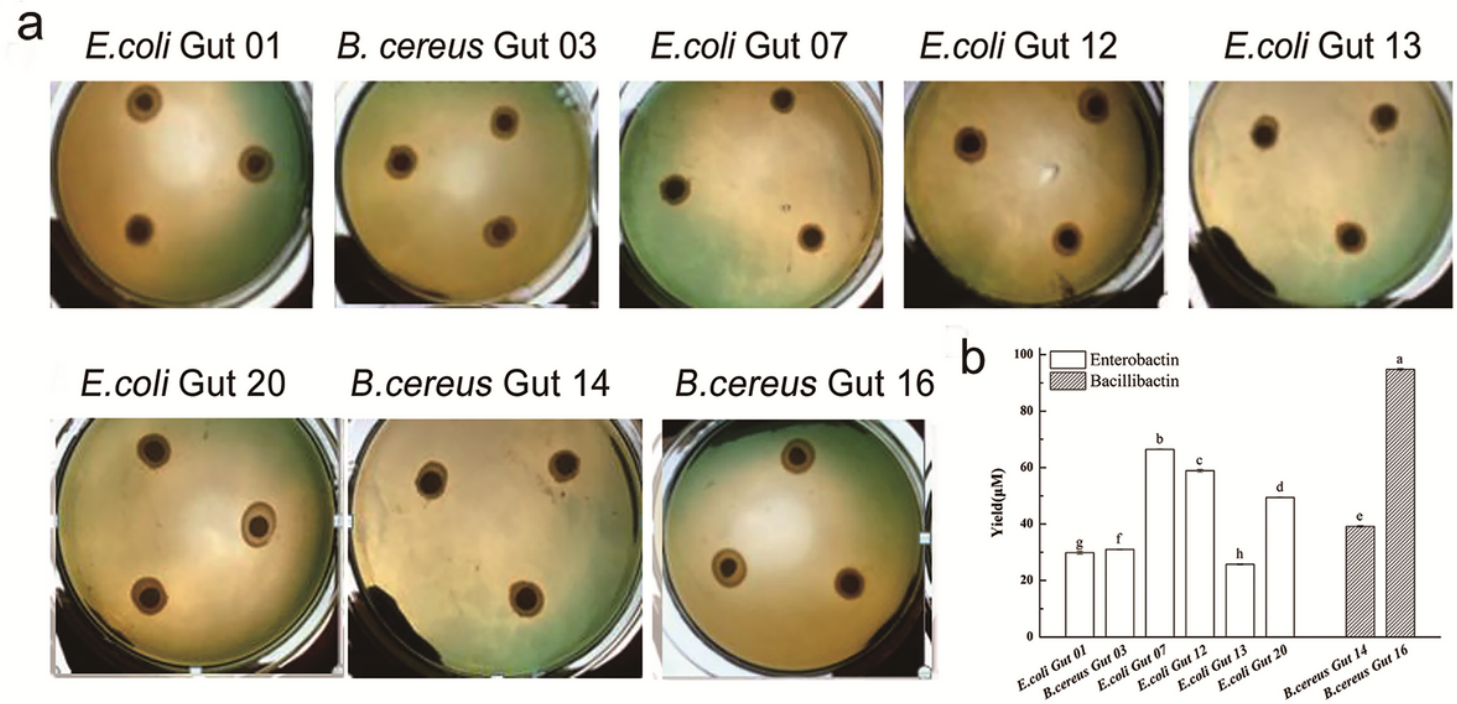

Figure 4

a:Siderphores formation of eight strains. b:The yield of catechol siderophore secreted by selected strains. a-h Superscripts on the column indicate the significant difference (P□0.05).