Interspecific interaction mediated crosstalk in the co-culture of Aspergillus sydowii and Bacillus subtilis produces novel benzoic acid derivates

Yu Sun
Dalian University of Technology
Xuan Shi
Dalian University of Technology
Liang-Yu He
Dalian University of Technology
Yan Xing
Dalian University of Technology
Qin-Feng Guo
Dalian University of Technology
Zhi-Long Xiu
Dalian University of Technology
yuesheng dong (yshdong@dlut.edu.cn)
Dalian University of Technology https://orcid.org/0000-0001-5010-6426

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Abstract

Interspecific interaction mediated crosstalk was regarded as an important feature among the microorganisms under natural conditions and also the key mechanism for the biosynthesis of newly induced compounds in the co-culture. However, there is still a lack of direct experimental evidence for this mechanism. In this study, the interaction between *A. sydowii* and *B. subtilis* in the co-culture was firstly verified by the morphological observation. Subsequently, through the strategy combining substrate feeding, stable isotope labeling and gene expression analysis, the crosstalk in the co-culture for the production of five benzoic acid derivatives (**N1**-**N4** and **N7**) were demonstrated: the secondary metabolites 10-deoxygerfelin of *A. sydowii* acted as an inducer to induce *B. subtilis* to produce benzoic acid, which was further converted into 3-OH-benzoic acid by *A. sydowii*. Subsequently, *A. sydowii* used 3-OH-benzoic acid as the substrate to synthesize the new compound **N2**, and then **N1**, **N3**, **N4** and **N7** were biosynthesized upon the up-regulation of hydrolase, hydroxylase, and acyltransferase during co-culture. The plate zone analysis suggested that the biosynthesis of the newly induced compounds **N1**-**N4** was mainly attributed to *A. sydowii*, and both *A. sydowii* and *B. subtilis* were indispensable for the biosynthesis of **N7**. The current study provides an important basis for a better understanding of the metabolites transfer and the biosynthetic pathways of the newly induced compounds in the co-culture, providing a new insight into the interaction-mediated crosstalk among microorganisms.

Introduction

Microorganisms have remarkable capacity of producing structurally unique secondary metabolites endowed with many biological activities [1]. Numerous secondary metabolites of microorganisms have been widely used for the treatment of human diseases [2]. However, the discovery of novel metabolites has been increasingly constrained by the excessive rediscovery of known metabolites. The mining of the full genome sequences of microorganisms demonstrated that there were many thousands of biosynthetic gene clusters within microbes encoding a series of unexplored secondary metabolites. The reason that the biosynthetic potential has not been fully discovered is that most of the microbial biosynthetic gene clusters remain silent and apparently have not been transcribed under standard culture conditions [3, 4]. To overcome this impasse, the microbial co-culture based on the interaction between microorganisms, which includes competition, symbiosis and allelopathy, has gained more attention and has been regarded as a useful approach to effectively induce the production of metabolites [5]. Using this strategy, Meng et al. demonstrated that the co-culture of *Penicillium bilaiae* MA-267 and *Penicillium chermesinum* EN-480 resulted in the accumulation of 2 new meroterpenoid derivatives and 3 known sesquiterpenoids [6]. Liu et al. indicated that the co-culture of *Phoma* sp. YUD17001 and *Armillaria* sp. generated 5 new secondary metabolites, including 2 phenolic compounds and 3 aliphatic ester derivatives [7].

Marine-derived *A. sydowii* can produce diverse structural classes of secondary metabolites which have been increasingly utilized in pharmaceuticals, food and chemicals, such as alkaloids, polyketides, terpenoids and sterols, and most of these metabolites exhibited a variety of bioactivities [8]. Moreover,

Bacillus subtilis is a well-known producer of bacterial agents and has been successfully used in agricultural biological control, which can produce a large number of metabolites with potential antibacterial and anti-insect activities [9]. In our previous study, the co-culture of *A. sydowii* and *B. subtilis* induced 25 newly biosynthesized metabolites [10]. Four of the newly biosynthesized metabolites were novel compounds and 5 compounds including one novel compound belong to benzoic acid derivates. However, the profiles of metabolites transfer, and how these newly induced benzoic acid derivates were biosynthesized symbiotically by the microorganisms were still unclear.

Interspecific interaction mediated crosstalk was regarded as an important feature among the microorganisms under natural conditions, and in the co-culture, the crosstalk upon interspecific interaction, which included both the metabolites transfer among the microorganisms and the activation of silent biosynthetic gene clusters on demand by the compounds in a contrasting strain, was also common and often plays as an important role in the biosynthesis of newly induced metabolites [11, 12]. However, in the process of producing new compounds in co-culture systems, the research on the demonstration of interspecific interactions mediated crosstalk is still challenging currently. Only a few attempts have been reported. For instance, Xu et al. used ^{13}C-dynamic labeling techniques to reveal the association between 31 induced compounds and the corresponding producers in the co-culture of *T. versicolor* with *G. applanatum* [13]. However, the profiles of metabolites transfer and the biosynthetic pathways of newly produced metabolites have not been determined. To the best of our knowledge, there are no reports that reveal the interaction mediated crosstalk during the biosynthesis of the newly induced compounds symbiotically in the co-culture systems. Some experimental approaches have been applied to elucidate the biosynthetic pathways of the newly biosynthesized secondary metabolites in the single microorganism. For instance, the substrate feeding strategy, which was first developed to increase the yield of microbial metabolites, is an effective method to explore the biosynthesis pathways of microbial secondary metabolites [14, 15]. Supplementing amino acids and dipeptides to the culture of *S. lavendulae* led to the finding that the side chain of the saframycin Y3 was derived from the dipeptide units of alanylglycine [16]. These data demonstrated that the substrate feeding strategy had the advantages of high credibility, good operability and easy accessibility. In recent years, stable isotope labeling analysis has been widely used in qualitative and quantitative analysis in metabolomics, offering unique advantages by excluding false matrix positives and helping to discern unknown metabolites [17, 18]. For example, by coupling $^{13}C_6$-glucose labeling with off-line LC-NMR techniques, *de novo* fatty acid biosynthesis from glucose-derived acetyl-CoA was revealed as the main pathway of N-(1-oxooctyl)-tyrosine [19]. The stable isotope labeling combined with HRMS (SILMAS) was also used to identify the biotransformation products of gemfibrozil by *Arabidopsis thaliana* [20]. However, the application of these direct experiment approaches to elucidate the profile of interspecific interaction mediated crosstalk in the co-culture has not been reported.

In this study, to provide insight into microbial interaction and crosstalk in the co-culture of *A. sydowii* and *B. subtilis*, the influence of the interaction of the two microorganisms on their morphology was analyzed. The biosynthetic pathways and metabolite transfer profiles of newly induced benzoic acid derivates were

also elucidated by means of substrate feeding, stable isotope labeling, and gene expression analysis. The distribution of newly induced metabolites in different zones of the co-culture was also studied to further elucidate their biosynthetic process.

Methods

Strains and culture conditions

A. sydowii (CPCC 401353) and *B. subtilis* (CGMCC 13141) were activated in potato dextrose agar (PDA) medium (200 g potato/L, 20 g dextrose/L, and 15 g agar/L) for 3 days. Subsequently, each strain was suspended in 2 mL of sterile water. The co-culture was prepared by inoculating 4 μL bacterial suspension of the 2 different microbes on the opposite sides of bran dextrose agar (BDA) plates (100 g bran was boiled in 1 L deionized water for 14 min and filtered to obtain the filtrate, followed by supplementing 20 g glucose and 15 g agar into the solution). For the pure cultures, 4 μL of bacterial suspension was inoculated on the center of BDA plates. The plates were incubated at 28°C. After 12 days, the colony morphology was observed and the secondary metabolites were extracted from the culture medium with ethyl acetate (EtOAc).

Microscopic morphology observation

To observe the influence of mycelium and to investigate the interaction between *A. sydowii* and *B. subtilis* in the co-culture, the scanning electron microscopy (SEM, QUANTA 450, USA) was applied according to the methods described by Wang et al. [21]. The mycelium was collected on Day 12 of the co-culture and fixed with 2.5% glutaraldehyde overnight. The samples were then washed 3 times with 100 mM phosphate buffered saline (pH 7.4), and dehydrated by an alcohol gradient (30, 50, 70, 90, 100%) and tert-butyl alcohol. The dehydrated samples were subjected to critical point drying and palladium sputter coating and then applied to SEM. All the samples had 3 independent biological replicates.

Extraction of metabolites and HPLC analysis

The thalli of *A. sydowii* and *B. subtilis* were obtained respectively for pure cultures. The thalli of the confrontation zone was obtained for co-culture, and transferred into 100 mL glass flasks using the same method as introduced in our previous article [10]. The thalli were extract for 3 times with 70 mL EtOAc to obtain the extract solutions, and the extract was then filtered through filter paper. A total of 70 mL of crude EtOAc extract was collected and evaporated to dryness by a rotary evaporator (120 rpm at 38°C). Dried extracts were re-dissolved in 1 mL of methanol and were centrifuged at 1600 g for 10 min. To study the biosynthetic pathways of the compounds, the extraction solvents were aqueous, petroleum ether, EtOAc and ethanol. All extracts were analyzed by a Waters HPLC system equipped with a 2998 detector and a 1525 pump. Routine detection wavelengths were at 235, 254, 280, and 340 nm. According to our previous method, twenty (20) μL of the samples was injected to a Shimadzu TC-C_{18} column (10×250 mm, 5 μm), and the following gradient was used (mobile phase A: 0.2% CH_3COOH in H_2O, mobile phase B:

acetonitrile): 0-30 min (20-80% B), 30-35 min (80-100% B), 35-40 min (100% B) at 37°C with a flow rate of 1 mL/min. All the samples had 3 independent biological replicates.

LTQ Orbitrap XL mass spectrometry analysis

The mass spectrometry analysis was performed with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) at a flow rate of 0.6 mL/min with an electrospray interface (ESI), using the same method described in our previous article [10]. In separate runs, the m/z range was set as 50-1200 Da in centroid mode with a scan rate of 1.5 spectra/s. The detection was achieved in both positive and negative ion modes, and the ESI conditions were as follows: the spray voltage was 4.2 kV, the heater temperature was 320°C, the capillary temperature was 300°C, dry low was 10/min and the sheath gas pressure was 35 arbs. Each full MS scan was followed by data-dependent MS/MS on the 3 most intense peaks using stepped collision-induced dissociation (35% normalized collision energy, isolation width of 2 Da, activation Q at 0.250). Samples of 20 μL were injected into a Shimadzu TC-C_{18} column (10×250 mm, 5 μm) with the following mobile phase: (A) 0.1% acetic acid (v/v) in water; (B) 0.1% acetic acid (v/v) in acetonitrile. The analysis was performed with a gradient of 20–80% B for 35 min, followed by a gradient of 80–100% B for 5 min, and an isocratic step of 100% B for 5 min, which was followed by a re-equilibration of 5 min with 20% B. The total run time was 50 min. The temperature was maintained at 30°C. The solvent (MeOH) and the samples of the pure cultures were injected to mass spectrometer under the same conditions as the controls. All the samples had 3 independent biological replicates.

The substrate feeding experiment

To explore the factors inducing the production of new secondary metabolite in the microbial co-culture, the EtOAc extract, aqueous extract, petroleum ether extract and ethanol extract of the confrontational microbe on one BDA plates were separated uniformly on BDA plates, respectively. Subsequently, one single microorganism cultured with the extract of confrontational microbe was inoculated and cultured for 12 days. For the co-culture and pure cultures supplemented with benzoic acid or 3-OH-benzoic acid, 100 μL of 20 mg/mL benzoic acid or 20 mg/mL 3-OH-benzoic acid dissolved in sterile ethanol was supplemented to the 20 mL BDA medium before the inoculation of the microbes. The identification of compounds by MS-DIAL and MS-FINDER was based on the approach provided in our previous article [10].

Stable isotope labeling combined with LC-HRMS

In order to investigate the relationship between newly produced compounds and benzoic acid, the stable isotope labeled benzoic acid was supplemented into the co-culture. A mixture solution of benzoic acid and benzoic acid-d_5 (2:1) was prepared in sterile ethanol. The aim of the addition of benzoic acid-d_5 was to create a unique companion twin-ion pair of benzoic acid-d_5 and to retain the deuterated label metabolites. A total of 100 μL the mixture solution (20 mg/mL) was added to 20 mL of BDA medium. The treatments were inoculated into the co-culture and incubated for 12 days, and then the extract of the treatments was analyzed by LC-HRMS.

Gene expression analysis

The samples were collected from the interaction zone of *A. sydowii* and *B. subtilis* for the RNA extractions. RNA-seq transcriptome library was generated following TruSeq^{TM} RNA sample preparation Kit from Illumina (San Diego, CA, USA) using 5 mg of total RNA. The expression level of each transcript was calculated according to the fragments per kilobase of exon per million mapped reads (FRKM) method. In addition, the functional enrichment analysis, including GO and KEGG, was performed to determine which differentially expressed genes were significantly enriched in the metabolic pathways at Bonferroni-corrected and GO terms P value < 0.05 compared with the whole transcriptome background. For the qRT-PCR study, PrimeScript1RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) was used for the reverse transcription. The qRT-PCR was performed by a 7500 Real Time PCR System (Applied Biosystems) using SYBR® Premix Ex Taq^{TM} II Kit (TaKaRa, Japan). There were 3 biological replicates in each sample. In order to normalize the qRT-PCR data, and *β*-tubulin (ASPSYDRAFT_53182) was used as the endogenous reference gene. The speci□c primers of target genes used in this experiment were listed in Table S1. Quantitative relative quanti□cation was calculated using the $2^{-\Delta\Delta Ct}$ method [22] The RNAseq data has been submitted to NCBI, and the accession ID is PRJNA796526.

The distribution of newly induced metabolites and intermediate products in co-culture

The co-culture plate on 12 days was separated into several growth zones: (I) the far-end from the confrontation zone on *A. sydowii* side, (II) the near-end from the confrontation zone on *A. sydowii* side, (III) the confrontation zone of the co-culture, (IV) the near-end from the confrontation zone on *B. subtilis* side and (V) the far-end from the confrontation zone on *B. subtilis* side. The width of each zone was 1 cm, and the secondary metabolites of each growth zone were compared and analyzed by LC-MS/MS to study the distribution of newly induced compounds and intermediate products. All the samples had 3 independent biological replicates.

Results

Effects of B. subtilis **on the morphology and growth of** A. sydowii

The co-culture of *A. sydowii* and *B. subtilis* showed an obvious confrontation zone on BDA plate on 12 days (Fig. 1a). At the confrontation zone, the fungal hypha interface was significantly higher than the bacterial growth interface, but the fungus did not further spread to the *B. subtilis* territory by virtue of its high superiority. Instead, the microbes formed a relatively neat boundary at the confrontation area, indicating that after the co-culture, the microbes produced chemical substances that could inhibit the growth of the opposite microorganism and prevented its further invasion. This result was also demonstrated in our previous article showing that the 7 new compounds induced by co-culture had antibacterial activity, and that these compounds were not produced in pure cultures [10]. Furthermore, *A. sydowii* presented smooth and complete conidiophores, and the spores were prolific on BDA plate (Fig. 1b). But when *A. sydowii* was co-cultured with *B. subtilis*, there were many *B. subtilis* nested in the

fungal mycelium and the conidiophores of *A. sydowii* became folded and shriveled, with the numbers of spores reduced (Fig. 1c). This phenomenon indicated that the co-culture had caused the stress response of the fungus, which could activate the silent secondary metabolic pathways of the microorganism to produce the new secondary metabolites.

The metabolite transfer and biosynthetic pathway of compound N2

The newly induced compounds by co-culture have often been reported, but the studies on the biosynthetic pathways of the newly induced compounds in the co-culture are still rare. To investigate the biosynthetic pathways of the major newly induced compounds **N1-N4** (orsellinic acid, sydonic acid, (7S)-(-)-10-hydroxysydonic acid and (R)-(-)-hydroxysydonic acid) and **N7** (serine sydonate), the pure culture of microbe was firstly treated with heat-inactivated confrontational microbe. No new compounds that had been produced in the co-culture were observed in either *A. sydowii* or *B. subtilis* pure culture, suggesting that the synthesis of those compounds required live interacting microbes or the presence of induced signal molecules. Furthermore, the pure culture of microbe was treated with the EtOAc extract, aqueous extract, petroleum ether extract, and ethanol extract of the confrontational microbe, respectively. There were still no new compounds that had been produced in the co-culture detected in pure cultures with the aqueous, petroleum ether or ethanol extract, respectively. However, a newly induced compound was detected in the pure culture of *B. subtilis* with the EtOAc extract of *A. sydowii* (Fig. 2). The molecular formula of this compound was $C_7H_6O_2$, which was indicated by the ESI-HRMS at *m/z* 121.2675 $[M-H]^-$ (calculated for 122.3452), and was identified as benzoic acid by comparing the MS/MS data with the public MS/MS spectrum library through MS-FINDER and confirmed by the comparison with commercial standard (Fig. S1). Considering the fact that benzoic acid was the common sub-structure of **N1**-**N4** and **N7** (Fig. 3), it could be reasonably inferred that benzoic acid might act as the precursor and contribute to the biosynthesis of these newly induced metabolites.

To test this hypothesis, benzoic acid was added to the pure cultures of *A. sydowii* and *B. subtilis*, respectively. The production of compound **N2** was observed in the pure culture of *A. sydowii*, but was not detected in the pure culture of *B. subtilis*, suggesting that **N2** was biosynthesized by the culture of *A. sydowii* with benzoic acid as the precursor (Fig. 4a). Interestingly, when adding benzoic acid to the culture of *A. sydowii*, another new compound was detected with the retention time at 8 min. The ESI-HRMS data indicated that the formula of this compound was $C_7H_6O_3$ with *m/z* 137.0247 $[M-H]^-$ (calculated for 138.2364), and it was identified as 3-OH-benzoic acid using MS-FINDER and confirmed by comparison with the commercial standard (Fig. S2). Thus, 3-OH-benzoic acid was also supplemented to the cultures of *A. sydowii* and *B. subtilis*, and the production of **N2** was only detected in the culture of *A. sydowii* (Fig. 4b), suggesting that 3-OH-benzoic acid acted as the intermediate product in the biosynthesis of **N2**. Therefore, the biosynthetic pathway of **N2** was determined as follows: benzoic acid, which was produced by *B. subtilis*, was bio-transformed to 3-OH-benzoic acid by *A. sydowii*, and then utilized by *A. sydowii* to produce **N2**.

The metabolite transfers and biosynthetic pathways for the production of compounds N3, N4, N7 and N1

It should be noticed that adding either benzoic acid or 3-OH-benzoic acid to the culture of *A. sydowii* did not induce the production of **N1**, **N3**, **N4** or **N7**, indicating that these metabolites cannot be induced by *A. sydowii* alone. Thus, the benzoic acid was exogenously added to the co-culture of *A. sydowii* and *B. subtilis*. The contents of **N3**, **N4** and **N7**, together with **N2**, were increased by 1.9, 1.4, 1.4 and 1.9 times respectively (Fig. 4c), but the content of **N1** remained at the original level. Similarly, the content of 3-OH-benzoic acid was increased by 2.67 times as expected (Fig. 4d). These data suggested that benzoic acid was also the precursor of the compounds **N3**, **N4** and **N7**, and then biosynthesized by the co-culture of *A. sydowii* and *B. subtilis.*

To exclude the false positive in precursor determination, a mixture of benzoic acid and stable isotope labeled benzoic acid-d_5 (2:1) was added to the co-culture. The unique mass charge ratios (*m/z*) of unlabeled products and associated ions of the labeled counterparts were easily detected by LC-HRMS. The *m/z* of labeled and unlabeled transformation products of benzoic acid exhibited the ion pair pattern and fixed ratio of intensity, and had the same retention time and chromatographic peak shape. For example, the signal of the two monoisotopic ions *m/z* 281.1396 and *m/z* 284.1448 of compound **N3** exhibited a 2:1 intensity ratio and differed by 3.0052 Da. However, there was 2 Da less than benzoic acid-*d5* due to the substitution of two deuterated hydrogen on the benzene ring in compound **N3** (Fig. 5). Similarly, the 3-OH-benzoic acid, **N2**, **N4** and **N7** exhibited the aforesaid ion pair and diagnostic characteristics in the co-culture (Fig. S3-S6). These data indicated that benzoic acid was the precursor of these compounds.

When adding the EtOAc extract of *A. sydowii* to the culture of *B. subtilis* to induce the production of benzoic acid, the component in the EtOAc extract of *A. sydowii* which acted as the inducer was investigated. The EtOAc extract was divided into 7 fractions by preparative HPLC (Fig. S7-S13) and added to the pure culture of *B. subtilis*. Benzoic acid was only observed in the pure culture of *B. subtilis* supplemented with the fraction 6 of the extract (Fig. S14), indicating that the component in the fraction 6 induced *B. subtilis* to produce benzoic acid. As only one component was included in the fraction 6, the structure of this component was analyzed by ESI-HRMS. The molecular formula of this component was identified as $C_{15}H_{14}O_5$ based on the *m/z* 273.0770 $[M-H]^-$ (calculated for 274.7532), this compound was identified as 10-deoxygerfelin (2-Hydroxy-4-(3-hydroxy-5-methylphenoxy)-6-methylbenzoic acid) by MS/MS through MS-FINDER (Fig. S15). This compound was further isolated and purified to obtain the pure compounds and confirmed by NMR data compared with published data (Supplementary material). Thus, these data revealed that the compound 10-deoxygerfelin produced by *A. sydowii* induced *B. subtilis* to produce benzoic acid, and then benzoic acid was further utilized by microorganisms in the co-culture. Interestingly, by comparing the structures of **N1** and 10-deoxygerfelin, it could be inferred that **N1** might be the product of 10-deoxygerfelin by breaking the ether bond, which was consistent with the fact that benzoic acid was not the precursor of **N1** in the substrate feeding and stable isotope labeling studies. To verify this conjecture, the purified 10-deoxygerfelin was added to co-culture. As expected, the content of

N1 was increased by 2.5 times (Fig. S16), which confirmed the fact that the 10-deoxygerfelin was the precursor of the **N1**.

Gene expression of enzymes related to the biosynthetic pathways of newly induced metabolites

The structure analysis indicated that **N3** and **N4** were the hydroxyl substitution derivates of **N2**, **N7** was the serine substitution derivate of **N2**, and that **N1** was the hydrolysate of the 10-deoxygerfelin produced by *A. sydowii*. Thus, RNA-Seq was performed to study the global gene expressions of *A. sydowii* in the pure culture and in its co-culture with *B. subtilis*, which was helpful for the better understanding of the biosynthetic pathways of compounds **N1**, **N3**, **N4** and **N7**. In the co-culture with *B. subtilis*, 11 transcripts encoded the enzymes which was related to the synthesis of newly induced compounds, of which 4 transcripts were functionally annotated as hydroxylase related to the biosynthesis of **N3** and **N4**, and the expression of these transcripts was up-regulated by 2.3-4.2 folds compared with that in the pure culture. Six transcripts were functionally annotated as acyltransferase synthesis related to the biosynthesis of **N7** and compared with pure culture, the expression of these transcripts was up-regulated by 2.2-5.8 folds in the co-culture. The remaining one transcript was functionally annotated as hydrolase-isochorismatase related to the synthesis of **N1** and the expression of the transcript was up-regulated by 2.2-folds in the co-culture (Table 1). To confirm the results of the RNA-Seq analysis, parts of the transcripts (Table S1) were selected for quantitative RT-PCR analysis. The transcripts with the IDs of ASPSYDRAFT_148263, ASPSYDRAFT_53314 and ASPSYDRAFT_46545, putatively overexpressed in the data sets, were all overexpressed in the co-culture, with expression levels 3.23, 2.14 and 5.52 times higher than that in the control samples, respectively. The qRT-PCR and RNA-Seq values were of similar orders of magnitude, which indicated that the RNA-Seq data were reliable and correctly represented the variations of expression that occurred during the co-culture of *A. sydowii* and *B. subtilis*.

Table 1
The upregulated genes with annotation in *A. sydowii* under the interaction with *B. subtilis*

Transcript_ID (ASPSYDRAFT_)	Fold change	Putative function
148263	3.9	Hydroxylase
94541	2.8	Hydroxylase
81146	2.3	Hydroxylase
53618	4.2	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
49120	3.3	Acyl-coenzyme
46545	5.5	Acyltransferase
53314	2.7	Acyltransferase
150919	4.8	Acyltransferase
165232	5.8	Acyltransferase family
43440	2.2	Acyltransferase
54616	2.2	hydrolase-isochorismatase

Therefore, the data from substrate feeding, stable isotope labeling and RNA-Seq study demonstrated that the biosynthetic pathways of **N1**, **N2** and its derivates (**N3**, **N4** and **N7**) were as follows: the compound 10-deoxygerfelin produced by *A. sydowii* induced *B. subtilis* to produce benzoic acid, and 10-deoxygerfelin was hydrolyzed by hydrolase to generate **N1** simultaneously. The benzoic acid was bio-transformed to 3-OH-benzoic acid firstly, and then was converted to **N2** by *A. sydowii*, after which **N2** was further converted to **N3** and **N4** by the hydroxylase produced by *A. sydowii*, and **N2** was also bio-transformed to **N7** by the acyltransferase produced by the co-cultured microbes (Fig. 6). These data demonstrated the microbial crosstalk in the biosynthesis of secondary metabolites during the co-culture.

The distribution of newly induced metabolites and the main intermediate products

To better elucidate the microbial interaction and crosstalk of the microbes in the co-culture, the distribution of newly induced metabolites and intermediate products in the co-culture were analyzed. The co-culture plate was divided into 5 different zones, including the far-end from the confrontation zone on *A. sydowii* side (I), the near-end from the confrontation zone on *A. sydowii* side (II), the confrontation zone of the co-culture (III), the near-end from the confrontation zone on *B. subtilis* side (IV) and the far-end from the confrontation zone on *B. subtilis* side (V) (Fig. 7a), and the contents of the newly induced metabolites and intermediate products at different zones were analyzed by LC-MS/MS (Fig. 7b).

Generally, the concentrations of the compounds were highest in the confrontation zone of the co-culture (III) and decreased with the distance from the confrontation zone, indicating that co-culture played an important role in the production of these newly induced metabolites and intermediate products. However, the distribution profiles of these compounds were different and can mainly be divided into 3 types: the first type included the intermediate product, benzoic acid, whose concentration was higher on the *B. subtilis* side than that on the *A. sydowii* side at the same distance. These data supported the conclusion that benzoic acid was biosynthesized by *B. subtilis*, and the highest concentration of benzoic acid in the confrontation zone indicated that both the substrate (10-deoxygerfelin) and the enzyme activities for benzoic acid biosynthesis were high. The concentration of benzoic acid was lower on the *B. subtilis* side and decreased with the distance from confrontation zone. The possible reason may be that the substance (10-deoxygerfelin) produced by *A. sydowii* was transmitted to the *B. subtilis* zones through active diffusion and passive transportation to complete the biosynthesis. The farther the distance was, the lower the concentration of the substance, so the concentration of the benzoic acid was also lower. The distribution of benzoic acid on the *A. sydowii* side was also attributed to the product transmit through active diffusion and passive transportation. The secondary type of distribution included the substrate 10-deoxygerfelin, the intermediate product 3-OH-benzoic acid, and the newly induced metabolites **N1**, **N2**, **N3** and **N4**, whose characteristics was that at the same distance, the concentrations of compounds on the *A. sydowii* side were higher than that on the *B. subtilis* side. *A. sydowii* was regarded as the most important factor in the biosynthesis of these compounds. Benzoic acid, which was biosynthesized and transmitted from the *B. subtilis* side, was utilized as the substrate to be biosynthesized into 3-OH-benzoic acid, **N2**, **N3** and **N4** consecutively. Admittedly, some signal factors produced by *B. subtilis* were also supposed to be transmitted to *A. sydowii* side to elevate the expression levels of hydroxylase, hydrolase and other related key enzymes which contributed to the biosynthesis of the intermediate products and the newly induced metabolites, because our substance feeding study indicated that these compounds could not be biosynthesized by *A. sydowii* with benzoic acid only. The third type of distribution included the newly induced compound **N7**, which was mainly distributed in the confrontation zone, and the content was very low on both *A. sydowii* side and *B. subtilis* side. The results suggested that the biosynthetic enzymes related to **N7** produced by *A. sydowii* or *B. subtilis*, such as acyltransferase, were mainly distributed in the confrontation zone, and the co-existence of *A. sydowii* and *B. subtilis* was important for the production of **N7**. Therefore, our data demonstrated the biosynthetic process and described the profile of microbial crosstalk in the co-culture. Neverteless, which compounds contribute to the elevated expression levels of the biosynthetic enzymes involved in the co-culture still needs to be studied in the future.

Discussion

Fungi and bacteria can co-exist in the same growth environment and they will compete and communicate with each other [23]. It is generally accepted that secondary metabolites provide the producers with a biological advantage in response to the environment, which implies that there are some signal factors to control the production of metabolites [24]. Although many studies have revealed that the co-culture of

fungi and bacteria could produce newly induced metabolites that could not be produced by the fungi or the bacteria individually, the study related to the interaction-mediated crosstalk of the microorganisms which produced new compounds during co-culture was still backward. Especially, there has been no direct experimental evidence reported. In this study, the metabolite transfers and biosynthetic pathways of the newly induced structural analogues were revealed through substrate feeding, stable isotope labeling techniques and gene expression analysis. To the best of our knowledge, this is the first report on metabolite transfer and biosynthetic pathways of newly induced metabolites in the co-culture by direct experimental methods, which can provide a technical approach for related studies. In this study, the 10-deoxygerfelin and benzoic acid were determined as the inducer of benzoic acid and the precursor of **N2**-**N4** and **N7**, respectively. In the biosynthetic pathway analysis of metabolites produced by a single microorganism, the inducer has been regarded as an important factor for the biosynthesis of secondary metabolites, such as the A-factor controlling the production of streptomycin in *Streptomyces griseus* [25]. Besides, the precursor is also a key factor related to the production of secondary metabolites. For example, acetic acid and propanoic acid were the precursors of avermectin in the *Streptomyces avermitilis* based on polyketone biosynthesis pathways [26]. In previous studies, both the inducer and the precursor were produced by the same microorganism. However, the inducer and the precursor of newly induced metabolites in the co-culture of *A. sydowii* and *B. subtilis* were produced by different microbes. To be specific, the inducer produced by *A. sydowii* induced *B. subtilis* to produce the precursor, which was further utilized by *A. sydowii* to produce the newly induced metabolites. These data revealed that there was interspecific interaction and crosstalk between the two microorganisms. The interspecific crosstalk in the co-culture was also confirmed by the facts that **N3**, **N4** and **N7** were induced only in the co-culture, and that the gene expression level of hydroxylase, acyltransferase and hydrolase, which contributed to the production of **N3**, **N4** and **N7**, were higher in the co-culture than that in the pure cultures of individual microbe. Interestingly, our data also indicated that the 10-deoxygerfelin produced by *A. sydowii* was the precursor of **N1**. Therefore, 10-deoxygerfelin, acting as both the inducer and the precursor in the biosynthesis of the newly induced metabolites, was the key compound in the co-culture. Thus, from the biosynthesis point of view, the newly induced metabolites were produced through the communication between the microorganisms in the co-culture. These results can verify that the co-culture is an effective method to change the metabolite profiles and to release the potential ability of microorganisms in co-culture systems, which will be beneficial for the production of novel secondary metabolites.

The phenomenon that newly induced metabolites were distributed on different growth zones during the co-culture had been reported recently using plate zones analysis method. For example, Shang et al. divided the co-culture agar plate into several growth zones, and then analyzed and compared the metabolites in different growth zones. Their results showed that pyridoxatin, which belongs to a rare class of antifungal compounds, was detected in both the confrontation zone and the *Chaunopycnis* sp. side. However, its derivative, methyl-pyridoxatin, was only detected in the confrontation zone of the co-culture [27]. Knowles et al. analyzed the secondary metabolites at both the confrontation zone and non-confrontation zone of the co-culture plate by LC-MS and found that griseofulvin and dechlorogriseofulvin produced by *Aspergillus fischeri* were detected in the confrontation zone and the growth zones of *A.*

fischeri in the co-culture, but were not detected in the growth zones of *Xylaria cubensis* [28]. However, no microbial crosstalk or relationships between these metabolites and biosynthesis were revealed, possibly because no biosynthetic pathways were discovered. In this study, the newly induced compounds **N1**-**N4** were mainly distributed in the confrontation zone, and their contents on the *A. sydowii* side were higher than that on the *B. subtilis* side, suggesting that *A. sydowii* played an important role in the biosynthesis of these compounds. This result, together with the biosynthetic pathways data, revealed the basic profile of microbial crosstalk during the co-culture. To the best of our knowledge, this is the first report to elucidate the biosynthetic pathways and to disclose the microbial crosstalk process by the combined approach including substance feeding, isotope labeling experiment, plate zones analysis and gene expression study. This approach might help to reveal more biosynthetic pathways in the co-culture. On the other hand, our data also demonstrated that the plate zones analysis method, with the characteristics of easy operation and unrestricted by treated microorganism, was an effective method to distinguish the different roles of microorganisms in the co-culture, contributing to the understanding of the microbial crosstalk process and the synthetic pathways of newly induced compounds in the co-culture.

Benzoic acid was detected in the pure culture of *B. subtilis* by adding the compound 10-deoxygerfelin of *A. sydowii*. Benzoic acid is a platform and industrially important aromatic compound, which has been widely used in various industrial sectors. The main benzoic acid salts, including sodium benzoate, potassium benzoate, and calcium benzoates are industrially produced by neutralizing benzoic acid with their corresponding hydroxides or by heating with their corresponding concentrated carbonates. Due to the anti-microbial property, benzoic acid and its various salts can be used as preservatives and flavoring agents in food, cosmetic, hygiene, and pharmaceutical products [29]. Besides, benzoic acid can be used as a precursor to synthesize many important commodity chemicals such as epsilon-caprolactam via the SNIA Viscosa process [30] and phenol via decarbonation mechanism [31]. Recently, benzoic acid has been commercially produced by the chemical oxidation of toluene oxidation, which is operated at high pressure and temperature with heavy metal catalysts such as manganese naphthenate and cobalt [32]. Moreover, toluene is derived from non-renewable petroleum resource, making the overall process unsustainable. The benzoic acid production from renewable biomass holds significant potential because the mild bioprocesses is good for the environment. In addition, the biologically-obtained benzoic acid is considered to be safe, which is a major advantage for its applications in food, pharmaceuticals, and cosmetics industries. The biosynthesis of benzoic acid by native microorganisms has also been reported, but only in one bacterium, i.e. *Streptomyces maritimus*, in which benzoic acid was synthesized via a plant-like β-oxidation pathway as an intermediate during enterocin biosynthesis [33]. Besides, microbial benzoic acid production using a heterologous host has been attempted, but the workload of this method is large and the strain is easy to mutate [34]. To our best knowledge, the microbial benzoic acid production using *B. subtilis* or its genetic recombination strains has not been reported. In this study, considerable amount of benzoic acid was produced by *B. subtilis* by supplementing the extract of *A. sydowii*, and the safety of *B. subtilis* has already been demonstrated for a long time. These data suggested that the production of benzoic acid by individual *B. subtilis* alone could be achieved by activating silent genes under proper conditions, which would provide an alternative approach to produce

benzoic acid and its deviates. Moreover, as the *B. subtilis* is a biocontrol bacterium with bioavailability [35], the production of benzoic acid by *B. subtilis* can also enhance its antibacterial activity and expand the utilization value of *B. subtilis*.

In summary, this study clearly introduced the metabolites transfer and biosynthetic process of the newly induced benzoic acid derivatives symbiotically in the co-culture of *A. sydowii* and *B. subtilis*, laying a solid foundation for further exploration of the interspecific interaction mediated crosstalk during the co-culture.

Declarations

Funding

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis, the first draft of the manuscript was written by Yu Sun, Yu Sun and Yue-Sheng Dong conceived and designed the work, Xuan Shi, Liang-Yu He, Quo-Feng Guo and Zhi-Long Xiu participated in the study, Yan Xing polished the language of the paper, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.

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Figures

Figure 1

The SEM images of the bacterial-fungal culture and the samples collected from *A. sydowii* and *B. subtilis* on 12 days. a. The confrontation zone b. The pure culture of *A. sydowii* c. The *A. sydowii* in the co-culture

Figure 2

HPLC profiles of extracts of microorganisms cultured in BDA plate on 12 days a. The pure culture of *B. subtilis* added EtOAc extract of *A. sydowii* b. The pure culture of *B. subtilis*

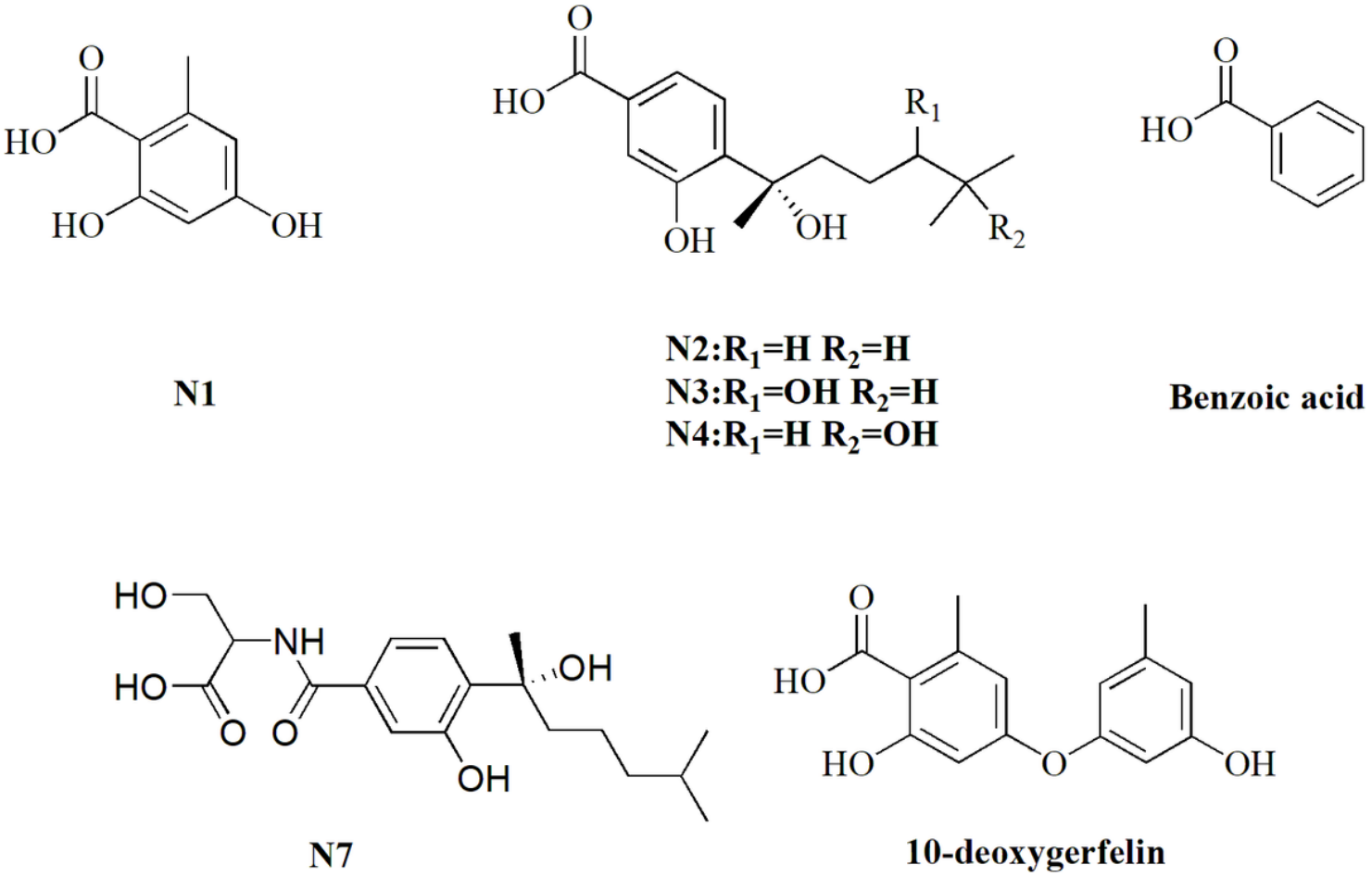

Figure 3

Structures of compounds

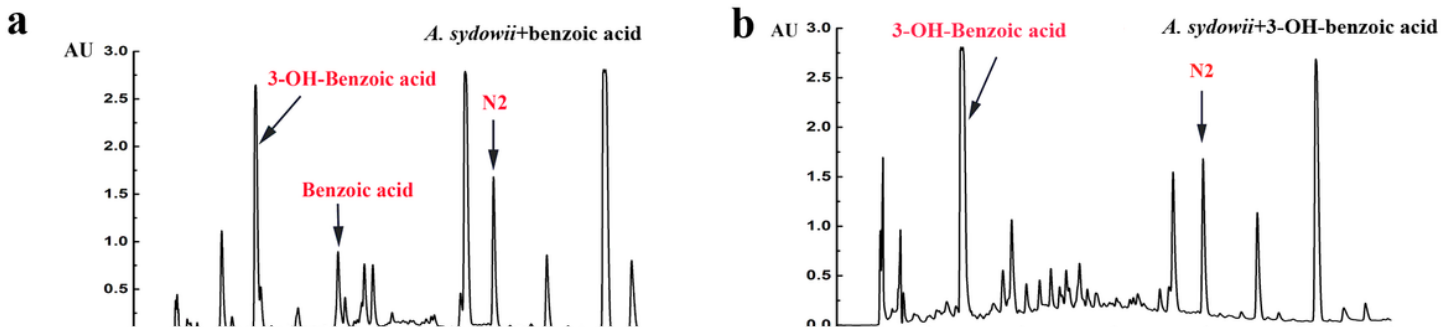

Figure 4

a. HPLC profiles of extracts of *A. sydowii* with or without exogenous addition of benzoic acid in BDA plate. b. HPLC profiles of extracts of *A. sydowii* with or without exogenous addition of 3-OH-benzoic acid in BDA plate. c. The content of the newly induced compounds in the co-culture with or without exogenous addition of benzoic acid. d. The content of 3-OH-benzoic acid in the co-culture with or without exogenous addition of benzoic acid. The plates were incubated for 12 days (Co: co-culture, Ba: benzoic acid)

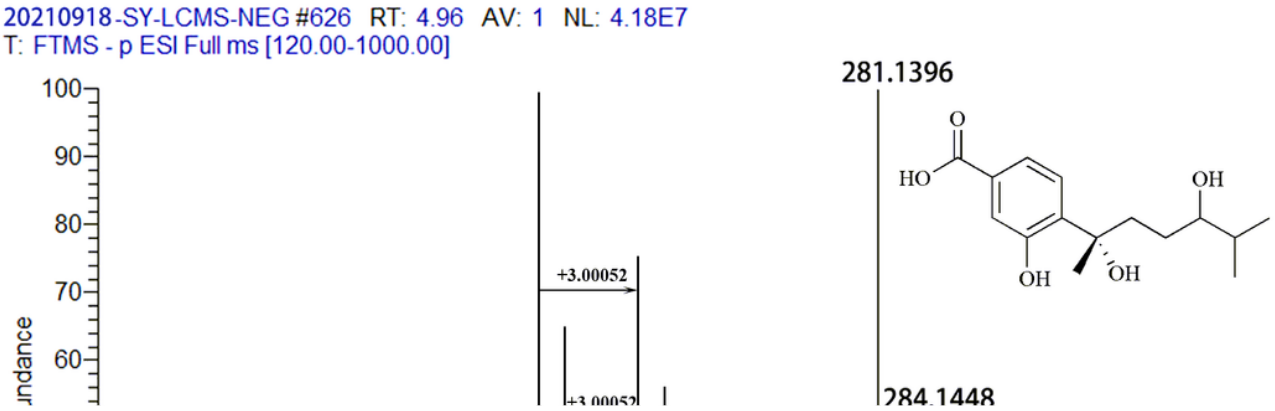

Figure 5

Representative mass spectra of a metabolite (**N3**) and its stable isotope labeled counterpart, showing a characteristic 2:1 signal intensity ratio

Figure 6

The biosynthetic pathways of newly induced compounds in co-culture

Figure 7

a. The different zones of co-culture in BDA plate on 12 days. (I) the far-end from the confrontation zone on *A. sydowii* side; (II) the near-end from the confrontation zone on *A. sydowii* side; (III) the confrontation zone of the co-culture; (IV) the near-end from the confrontation zone on *B. subtilis* side and (V) the far-end from the confrontation zone on *B. subtilis* side. b. The contents of compounds in different zones. Data were acquired from 3 independent biological replicates

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