Characterization of the diastaphenazine/izumiphenazine C biosynthetic gene cluster from plant endophyte

*Streptomyces diastaticus* W2

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**Summary**

Two phenazine compounds, diastaphenazine and izumiphenazine C, with complex structures and promising antitumour activity have been isolated from the plant endophytic actinomycete *Streptomyces diastaticus* W2. Their putative biosynthetic gene cluster (dap) was identified by heterologous expression and gene knockout. There are twenty genes in the dap cluster. dap4-19 related to shikimic pathway were potentially involved in the precursor chorismic acid biosynthesis, and *dapBCDEFG* were confirmed to be responsible for the biosynthesis of the dibenzopyrazine ring, the nuclear structure of phenazines. Two transcriptional regulatory genes *dapR* and *dap4* played the positive regulatory roles on the phenazine biosynthetic pathway. Most notably, the dimerization of the dibenzopyrazine ring in diastaphenazine and the loading of the complex side chain in izumiphenazine C could be catalysed by the cyclase homologous gene *dap5*, suggesting an unusual modification strategy tailoring complex phenazine biosynthesis. Moreover, metabolite analysis of the gene deletion mutant strain *S. albus*:23C5:dap2 and substrate assay of the methyltransferase Dap2 clearly revealed the biosynthetic route of the complex side chain in izumiphenazine C.

Introduction

Natural phenazine compounds are a large class of nitrogen-containing heterotricyclic aromatic alkaloids, mainly produced by *Pseudomonas* and *Streptomyces* species (Laursen and Nielsen, 2004; Mavrodi et al., 2006; Wang et al., 2016; Guttenberger et al., 2017). The dibenzopyrazine core ring is the typical characteristic of all phenazines, while the presence of diverse decorations of functional groups on the core ring system endows them with different physiological roles and broad-specificity biological activities (Mavrodi et al., 2006). Pyocyanin, the best-studied representative of phenazines, has been demonstrated to play an important role in its host, the opportunistic and nosocomial pathogen *Pseudomonas aeruginosa* infection (Lau et al., 2004a,b). The phenazine compounds produced by plant-beneficial *Pseudomonas* spp. not only display antibiotic activities towards plant pathogens, but also contribute to the primary metabolism of phenazine-producing species, including modification of cellular redox states as electron shuttles (Price-Whelan et al., 2007), promotional effects on biofilm formation (Mavrodi et al., 2012) and bacterial survival as cell signals (Dietrich et al., 2008). Although anticancer activities of phenazines have already been discovered since 1959, their application in the development of anticancer and anti-infective agents was trapped due to non-selective DNA intercalation of small phenazine molecules with flatland structures leading to general toxicity (Cimmino et al., 2012). Until the last two decades, dimeric phenazines isolated from *Streptomyces* (such as eseraldinesA–B (Keller-Schierlein et al., 1989), phenazostatins A–D (Yun et al., 1996; Kim et al., 1997, 1999; Maskey et al., 2003), izumiphenazines A–B (Abdelfattah et al., 2010) and phenazinolin A–E (Ding et al., 2011)) and a few of synthetic and naturally occurring phenazine derivatives bearing a pendant protonatable group were demonstrated to exhibit selective inhibition against tumour cells rather than normal eukaryotic cells, which brings hope for phenazine compounds to be used as clinical drugs (Cimmino et al., 2012). Hence, discovery and characterization of more new
phenazines with complex structures and promising biological activities has attracted new attention recently.

The biosynthetic pathways of simple phenazines have been extensively studied in *Pseudomonas*. Six genes, *phzBCDEFG*, normally organized in one operon, were found to be conserved in all bacterial phenazine biosynthetic gene clusters, and their specific functions in the phenazine core structure biosynthesis have been designated (Mavrodi *et al.*, 2010; Wang *et al.*, 2013; Blankenfeldt and Parsons, 2014), whereafter researchers gradually shift in focus to the study of phenazine-modifying enzymes which are ultimate determinants of distinctive structures and activities. So far, the molecular mechanisms of complex side chain biosynthesis and dimerization of dibenzopyrazine ring were still rarely reported.

The endophytic actinomycete, *Streptomyces diastaticus* W2, isolated from the herb *Artemisia annua*, was found to produce two complex phenazine compounds, diastaphenazine (1) and izumiphenazine C (2) (Fig. 1), with selective biological activities in our previous article (Li *et al.*, 2015). Besides antibacterial activity against the human pathogen *Staphylococcus aureus*, the newly identified dimeric phenazine diastaphenazine showed extraordinary cytotoxicity against carcinoma cells of human liver, lung, colon, cervix and stomach (Li *et al.*, 2015). Izumiphenazine C contains a phenazine core scaffold attached by an *N*-methyl-para-aminobenzoic acid side chain, which was first identified from the fermentation products of *Streptomyces* sp. IFM 11204 and displayed a synergistic effect with phenazine dimers against human gastric adenocarcinoma (Abdelfattah *et al.*, 2010). In this study, we successfully cloned a phenazine biosynthetic gene cluster from the chromosome of *S. diastaticus* W2 through genome analysis and library construction and demonstrated it responsible for both diastaphenazine and izumiphenazine C biosynthesis. Further functional research of the biosynthetic genes in the cluster disclosed the assembly line of the complex side chain in izumiphenazine C and the possible dimerization pathway to produce diastaphenazine. These results paved the way for elucidation of the biosynthetic mechanisms of complex phenazines and provided the future possibilities for the rational design and modification of phenazine derivatives with antitumour activity based on the combinatorial biosynthesis.

**Results and discussion**

*Identification of the diastaphenazine/izumiphenazine C biosynthetic gene cluster*

In order to seek out the putative gene cluster responsible for these two complex phenazine-products biosynthesis, the genome DNA of *S. diastaticus* W2 was completely sequenced. A 20 kb-sized gene cluster (*dap*, GenBank accession number MK685983) with 34% homology to the lomofungin biosynthetic gene cluster derived from *Streptomyces lomondensis* (Zhang *et al.*, 2015) was selected out by bioinformatics analysis (Fig. 1A and Table S1). The *dap* cluster consists of 20 open reading frames: 6 shikimate pathway related genes, 6 conserved phenazine biosynthetic genes, 2 regulator genes, 2 methyltransferase genes and several genes with unknown functions.

Further experimental data to authenticate the biological function of *dap* were achieved by construction of the *S. diastaticus* W2 genomic library. The cosmid 23C5 carrying the entire *dap* cluster was screened out from the library and then introduced into *Streptomyces albus* for heterologous expression. High-resolution electrospray ionization mass spectrometry (ESI-HRMS) results showed that the heterologous expression strain *S. albus*:23C5 started to produce the target compounds, diastaphenazine (calcd for *m/z* 437.1254 [M+H]+) (1) and izumiphenazine C (calcd for *m/z* 346.1224 [M+H]+) (2) as formed by the wild-type producer strain (Fig. 2), while the negative control strain *S. albus*::pMSB carrying the empty vector did not. It is clear that the *dap* cluster possesses requisite genes for the biosynthesis of diastaphenazine and izumiphenazine C.

**Regulatory factors**

Specific regulatory factors are usually closely aligned with biosynthetic genes. There are two putative regulatory genes, *dapR* and *dap4*, discovered in the *dap* cluster by bioinformatics. DapR, containing a C-terminal helix-helix domain and a PucR superfamily domain, displayed high sequence similarity with the known transcriptional activator AdeR for sporulation of *Bacillus subtilis* (Oudega *et al.*, 1997; Lin *et al.*, 2012). The *dapR* gene was in-frame deleted from the cosmid 23C5 by using λ Red recombinase system, and then, the resulting plasmid p23C5ΔdapR was introduced into *S. albus* to yield the gene deletion mutant strain *S. albus*::23C5ΔdapR (Fig. S1). To exclude the putative polar effect of gene deletion, the entire open reading frame of *dapR* was subsequently cloned into the *Streptomyces* expression vector pWHM4S and introduced into *S. albus*::23C5ΔdapR to construct the gene complementation mutant strain *S. albus*::23C5::pdapR (Fig. S1). HPLC and ESI-HRMS analyses both revealed the absence of all phenazine compounds including 1 and 2 in *S. albus*::23C5::dapR and regeneration of them in *S. albus*:23C5::pdapR (Figs 2 and S2). Dap4, containing the DDE superfamily motif, shared similarity to *Streptomyces* TyIR regulatory proteins (Stratigopoulos *et al.*, 2005). Similar results were obtained in the *dap4* gene knockout and complementation experiments, which demonstrated the positive
regulatory roles of two regulatory genes in the biosynthesis of phenazines (Figs 2 and S2).

**Genes involved in chorismic acid biosynthesis**

The shikimate pathway widely distributed in microorganisms and plants usually links metabolism of carbohydrates to biosynthesis of aromatic compounds (Dev et al., 2012; Jiang and Zhang, 2016). It is composed of seven enzymatic reactions that convert phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) into chorismic acid (CHA) as the precursor of the aromatic amino acids and many aromatic secondary metabolites. Biosynthesis of phenazines also starts from CHA (Pierson and

Fig. 1. Gene organization of the dap cluster (A) and the proposed biosynthetic pathway (B) of diastaphenazine (1) and izumiphenazines C (2). E4P: D-erythrose 4-phosphate; PEP: phosphoenolpyruvate; DAHP: 3-deoxy-D-arabinoheptulosonate-7-phosphate; CHA: chorismic acid; ADIC: 2-amino-2-desoxyisochorismate; DHHA: trans-2,3-dihydro-3-hydroxyanthranlic acid; AOCHC: 6-amino-5-oxo-cyclohex-2-ene-1-carboxylic acid; HHPDC: hexahydrophenazine-1,6-dicarboxylic acid; PCA: phenazine-1-carboxylic acid; PDC: phenazine-1,6-dicarboxylic acid; DHP: 1,6-dihydroxyphenazine; 3: para-aminobenzoic acid; 5: 4-methylaminobenzoic acid.
Pierson, 2010), and thus, a copy of chorismic acid biosynthesis related genes was frequently found in the gene clusters for the biosynthesis of phenazines, e.g. endophenazines from Streptomyces cinnamomensis DSM 1042 (Seeger et al., 2011) and esmeraldin from Streptomyces antibioticus Tu 2706 (Rui et al., 2012). The same situation occurred in the dap cluster.

Bioinformatics analysis showed that Dap19 shared 64% identity with the transketolase Tkt which catalysed the biosynthesis of erythrose 4-phosphate (E4P), one precuror of the shikimate pathway (Sprenger, 1995). DapC with 55% similarity to 3-deoxy-D-arabinohexulosonate-7-phosphate (DAHP) synthase PhzC from Pseudomonas fluorescens could catalyse the first step of the shikimate pathway (Mavrodi et al., 1998). dap14, dap16 and dap18 shared high sequence homology with the shikimic pathway related genes araA, araC and araB from Escherichia coli respectively. Additionally, Dap17 harbouring two

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conserved motifs, shikimate 5-dehydrogenase and shikimate kinase domains, probably exerted dual functions of \( \text{AroE} \) and \( \text{AroK} \). The homolog of \( \text{AroD} \) (dehydroquinic acid dehydratase) is absent in the \( \text{dap} \) gene cluster. \( \text{dap15} \) homologous with the hydroxylase \( \text{RdmE} \) gene was found to share the same transcription unit with other shikimate pathway genes and hence speculated to function as \( \text{AroD} \) in the shikimate pathway. The existence of these genes ensured the precursor supply for phenazine production. However, in-frame deletion of single genes including \( \text{dap15}, \text{dap17} \) and \( \text{dap19} \), did not destroy the production of phenazines, but only slightly affected the yield (Figs 2 and S2). Similar result was reported in the esmeraladin biosynthetic research for three shikimic acid pathway related genes \( \text{esmA6-A8} \) as well (Rui et al., 2012). The most likely reason is that the homologous genes involved in the shikimate pathway of primary metabolism took over their duty.

**Enzymes for the biosynthesis of dibenzopyrazine ring**

Five genes (\( \text{dapEDFBG} \)) in the \( \text{dap} \) cluster were considered to be responsible for the phenazine core formation due to their high sequence similarity with the gene set (\( \text{phzEDFBG} \)) of phenazine biosynthesis in \( \text{Pseudomonas} \) (Blessy and Filion, 2018). As shown in Fig. 1B, \( \text{dapE} \) homologous to the phenazine biosynthetic gene \( \text{phzE} \) was supposed to catalyse the conversion of chorismate acid (CHA) into 2-amino-2-desoxyisochorismate (ADIC) (Mavrodi et al., 1998; Li et al., 2011), which initiated the biosynthesis of phenazine core ring. When the \( \text{dapE} \) gene was in-frame deleted from the cosmid 23C5, the corresponding gene deletion mutant strain \( S. \text{albus}::23C5\Delta \text{dapE} \) lost the capacity for all phenazines production including diastaphenazine, izumiphenazine C and PCA (Figs 2 and S3). The phenazine biosynthetic pathway was resumed once \( \text{dapE} \) was complemented into \( S. \text{albus}::23C5\Delta \text{dapE} \) to give rise to the mutant strain \( S. \text{albus}::23C5\text{pc-dapE} \) (Figs 2 and S3). These results undoubtedly confirmed the critical role of \( \text{dapE} \) in phenazine core production.

**DapS implicated in post-modification of phenazine core**

The complicated structures of phenazines produced by \( \text{Streptomyces} \) mainly result from more diverse phenazine-modifying enzymes such as the prenyltransferases \( \text{PpzP}, \text{EpszP}, \text{Cnqpt1} \) and \( \text{Mpz10} \) (Seeger et al., 2011; Saleh et al., 2012; Zeyhle et al., 2014a,b), the hydroxylase \( \text{Mpz29} \) (Zeyhle et al., 2014a), the monooxygenase \( \text{Lomo10} \) (Zhang et al., 2015) and the methyltransferases \( \text{PpzM} \) and \( \text{EpszM} \) (Seeger et al., 2011; Saleh et al., 2012). In the \( \text{dap} \) cluster, \( \text{DapS} \), with high homology to the monooxygenase \( \text{PhzS} \) from \( \text{Pseudomonas aeruginosa} \) PA01 and the FMN-dependent monooxygenase \( \text{XpzG} \) from \( \text{Xenorhabdus szentirmaii} \), was identified adjacent to the phenazine core biosynthetic genes. \( \text{PhzS} \) was found to catalyse the oxidative decarboxylation of 5-methylphenazine-1-carboxylate (5-methyl-PCA) to pyocyanine (Stover et al., 2000), while \( \text{XpzG} \) used PDC as the substrate to form 6-hydroxyphenazine-1-carboxylic acid or 1,6-dihydroxyphenazine under the presence of NADPH or NADH (Shi et al., 2019). In consideration of the chemical structures of diastaphenazine and izumiphenazine C, the function of \( \text{DapS} \) was supposed to be more similar to \( \text{XpzG} \) for providing essential precursors for two complex final products. In-frame deletion of the \( \text{dapS} \) gene disrupted the production of diastaphenazine and izumiphenazine C rather than PCA (Fig. S4), verifying its role in the post-modification reactions of phenazine core.

**Dap5 is essential for two complex phenazine compounds production**

There are two genes, \( \text{dap5} \) and \( \text{dap9} \), with unknown functions, mingled with the above-mentioned genes. \( \text{dap5} \) encoded a NTF2-like superfamily protein harbouring a snoAL-like polyketide cyclase domain with high homology to an aklanonic acid methyl ester cyclase \( \text{DauD} \). \( \text{Dap9} \) displayed no similarity to known proteins annotated in NCBI. To investigate whether these two genes involved in phenazine synthesis, their corresponding gene deletion mutant strains \( S. \text{albus}::23C5\Delta \text{dap5} \) and \( S. \text{albus}::23C5\Delta \text{dap9} \) were, respectively, constructed for metabolite analysis. As shown in Figs 2 and S2, deletion of \( \text{dap9} \) had no effect on the biosynthesis of phenazine compounds while the absence of \( \text{dap5} \) abolished the production of diastaphenazine and izumiphenazine C, but PCA and its simple derivatives were still produced (Fig. S3). When the gene \( \text{dap5} \) was complemented into \( S. \text{albus}::23C5\Delta \text{dap5} \) to generate the mutant strain \( S. \text{albus}::23C5\text{pc-dap5} \), the biosynthesis of diastaphenazine and izumiphenazine C was restored. It is certain that \( \text{dap5} \) has been involved in the biosynthesis of two complex phenazine compounds, rather than in the formation of simple phenazines such as PCA and its derivatives. It was supposed to be the requisite gene critical for the dimerization of the dibenzopyrazine ring in diastaphenazine and the loading of the complex side chain in izumiphenazine C (Fig. 1B). \( \text{Dap5} \) belongs to the NTF2-like superfamily whose members are widely distributed in bacteria and exhibit diverse biological functions. The specific catalytic mechanism of this enzyme needs to be explored in the future.
**Proposed biosynthetic pathway of izumiphenazine C**

Izumiphenazine C has a phenazine core and a para-aminobenzoic acid (PABA) moiety modified by a methyl group. Bioinformatics analysis suggested that the remaining three genes, dap1, dap2 and dap20, of the dap cluster might be responsible for the N-methyl-para-aminobenzoic acid side chain formation of izumiphenazine C. Dap1 shared high sequence similarity to PabS, a para-aminobenzoate synthase widely distributed in microorganisms and plants for conversion of chorismate acid to PABA. Two putative methyltransferase genes, dap2 and dap20, showed high sequence homology to the ribosomal protein L11 methyltransferase protein PrmA and protein-L-isoaspartate O-methyltransferase protein Pcm respectively. Accordingly, Dap1 was supposed to be responsible for the PABA moiety biosynthesis, followed by methylation with the action of Dap2 or Dap20 to render a methyl group to izumiphenazine C. Deletion of the gene dap1 did not completely abolish the production of izumiphenazine C (Figs 2 and 3), probably because of the generally widespread existence of this kind of genes in microorganisms including the host strain *S. albus*. Meanwhile, two methyltransferase genes deletion mutant strains *S. albus::23C5Δdap2* and *S. albus::23C5Δdap20* displayed totally different results of metabolite analysis. The mutant strain *S. albus::23C5Δdap20* exhibited the same production profile as the original strain *S. albus::23C5*, still producing all phenazines. In contrast, inactivation of dap2 lost the ability to produce izumiphenazine C, but still produced diastephenazine (Figs 2 and 3), suggesting that dap2, not dap20, was involved in the biosynthesis of izumiphenazine C.

A new UV absorption peak was observed in the metabolic analysis of *S. albus::23C5Δdap2* by HPLC (Fig. 3), which was considered to be a crucial intermediate of izumiphenazine C. Large-scale fermentation of the mutant strain *S. albus::23C5Δdap2* was therefore conducted, and two compounds 3 (obsd m/z 138.0641 [M + H]⁺, calcd for m/z 138.0510 [M + H]⁺, Fig. S5) and 4 (obsd m/z 332.1024 [M + H]⁺, calcd for m/z 332.0990 [M + H]⁺, Fig. S6) were traced. The main intermediate product 3 was identified as PABA through LC-MS/MS and NMR analyses (Figs S7 and S8). However, the isolation of compound 4 was hampered by its low yield. According to the result of ESI-HRMS spectra combined with the putative metabolic pathway, it was proposed to be the demethylated derivative of izumiphenazine C, which had ever been isolated from other phenazine-producing strain and structurally identified in our previous research (Chen et al., 2019). The chemical structure of compound 4 was confirmed by comparative analyses of LC-MS/MS with the previously isolated demethylated izumiphenazine C as the standard (Fig. S9). In the fermentation products of *S. albus::23C5Δdap2*, the newly observed UV absorption peak in HPLC was formed by 3 with high yield, while a trace of 4 could be only detected by ESI-HRMS. Once dap2 was complemented into *S. albus::23C5Δdap2*, the compounds 3 and 4 disappeared, but izumiphenazine C production was restored (Figs 2, 3 and S10).

Two scenarios are generally conceivable for sequence of the side chain loading and modification in izumiphenazine C: either loading of the PABA moiety prior to methylation modification (route 1) or methylation of PABA prior to loading to phenazine core (route 2) (Fig. 4A). Massive accumulation of PABA (3) in *S. albus::23C5Δdap2* significantly supported route 2. Further evidence was provided by *in vitro* enzymatic analysis of Dap2. The gene dap2 was cloned and overexpressed in *E. coli* (Fig. S11), and the encoded protein was purified for *in vitro* reactions with the compounds 3 and 4 as substrates. In the presence of SAM, PABA (3) was completely converted into 4-methylaminobenzoic acid (5) by Dap2 in a short period of 2 h, while absence of SAM or inactivated Dap2 resulted in no product formation (Fig. 4B). On the contrary, there was little change in the reaction of 4 incubated with Dap2 and no expected product 2 was detected even if the incubation time was prolonged to

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**Fig. 3.** HPLC analysis of the heterologous expression strain of the dap cluster and its mutant strains. 3: para-aminobenzoic acid; *S. albus::23C5*: heterologous expression strain of the dap cluster; *S. albus::pMSB*: heterologous expression strain of the empty vector; Δdap1: *S. albus::23C5Δdap1*; Δdap2: *S. albus::23C5Δdap2*; Δdap20: *S. albus::23C5Δdap20*; cpdap2: *S. albus::23C5cpdap2*.

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overnight in the same reaction conditions (Fig. 4B). A very small amount of 5 was detected in this reaction system due to spontaneous decomposition of 4 to produce 3 as the substrate of methylation by Dap2. To obtain more direct evidence from in vivo experiments, compound 5 was fed to the gene deletion mutant strain S. albus::23C5dap2 and then the result of ESI-HRMS analysis showed the regeneration of izumiphenazine C (Fig. S12). Thus far, all data acquired from in vivo and in vitro experiments demonstrated that PABA was methylated prior to loading to phenazine core. Broad substrate specificity of the connective enzyme Dap5 might result in the error loading of 3 into the phenazine core to produce traces of 4 in S. albus::23C5dap2.

Through cloning and functional research of the dap cluster from S. diastaticus W2, the biosynthetic pathway of two complex phenazines, diastaphenazine and izumiphenazines C, was proposed. It is noteworthy that a few interesting phenazine-modifying reactions were identified, including the dimerization probably catalysed by Dap5 and methylation of PABA by Dap2 to yield 4-methylaminobenzonate for downstream incorporation. Our insights into the biosynthetic pathway of these two compounds not only revealed new modifying enzymes implicated in diversified post-modifications of complex phenazines, but also provided strategies for development of more phenazine drugs by genetic engineering in the future.

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**Experimental procedures**

**Bacterial strains, plasmids and growth conditions**

The strains and plasmids used in this study were listed in Table S2, and the primers used for PCR were listed in Table S3. All Escherichia coli strains including DH5α, BL21(DE3) and S17-1 were grown in LB medium at 37 °C with appropriate antibiotics except that E. coli BW25113/pIJ790 was grown in SOB medium at 30 °C. Streptomyces diastaticus W2, Streptomyces albus and their derivatives were cultivated at 30 °C in TSB medium for DNA extraction and grown on MS medium (2% mannitol, 2% soybean powder, 2% agar) for seed culture and fermentation.

**Genome sequencing and bioinformatics analysis**

Genomic DNA of S. diastaticus W2 was extracted using the standard method (Hopwood et al., 1985). Concentration and quality of DNA samples were determined by using Nanodrop and agarose electrophoresis. The genome of S. diastaticus W2 was sequenced by single molecule real-time three generation sequencing technique through PacBio RSII sequencing platform, and the data were assembled by HGAP (hierarchical genome-assembly process) software.

The whole genome sequence of S. diastaticus W2 was analysed using antiSMASH (https://antismash.secondarymetabolites.org) (Medema et al., 2011) to find putative secondary metabolite biosynthetic gene clusters.
The open reading frames in the newly identified phenazine gene cluster were predicted by using the 2ndFind (http://biosyn.nih.go.jp/2ndfind) and BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) programs.

**Genomic library construction and screening**

A genomic library of *S. diastaticus* W2 was constructed according to the previous literature (Li et al., 2008). After genomic DNA was partially digested by Sau3AI, ~40 kb of DNA fragments were recovered using Pulsed Field Gel Electrophoresis (PFGE) and cloned into the BglII site of the vector pMSB152B. The ligation product was packaged by MaxPlax™ Lambda Packaging Extracts (Epicentre) and transfected into the *E. coli* EPI100 to build up a genome library composed of 2400 cosmids. Subsequently, the library was screened by PCR with two pairs of specific primers, 17F1/17R1 and 17F2/17R2.

**Heterologous expression of the dap cluster**

Five positive cosmids selected out by PCR were then introduced into *S. albus* by intergeneric conjugation for heterologous expression. The corresponding heterologous expression strains were confirmed by PCR.

**Gene disruption and complementation**

The target gene was deleted from the cosmid 23C5 which contains the entire dap cluster, through PCR targeting system (Gust et al., 2003). The streptomycin resistance cassette (str) was amplified with a pair of long primers (listed in Table S2) containing 39 nt homology extensions of the target gene at the 5’ end and then introduced into *E. coli* BW25113/pIJ790+p23C5 by electroporation. λ RED recombination system promoted replacement of the target gene with the selectable marker str, and then, str was excised by digestion of the flanking Pmel sites and self-ligation. The resulting plasmid was introduced into *S. albus* by intergeneric conjugation to yield the gene deletion mutant strain.

The target gene cassette containing its complete ORF and RBS was amplified with primers listed in Table S3 and then cloned into pWHM4S. The resulting plasmid was introduced into the corresponding gene deletion mutant by conjugation to obtain the target gene complementation mutant strain. All gene deletion and complementation mutant strains were verified by PCR with primers listed in Table S3.

**Fermentation and analysis of phenazine compounds**

*Streptomyces* strains carrying phenazine biosynthetic genes were inoculated on MS medium plates and cultured at 30 °C for 5 days. The solid medium was chopped and extracted with equal volume of ethyl acetate. The crude extract dissolved in methanol was analysed by HPLC using an Agilent 1260 liquid chromatograph system with an Agilent Eclipse Plus C18 column (250 × 4.6 mm, 5 μm) at a constant flow rate of 1 ml min⁻¹, with UV detection at 250, 270 and 310 nm. The mobile phase consisted of solvents A (0.1% formic acid) and B (methanol), which were used with the following gradient profile: 0–20 min, 40–90%B; 20–25 min, 90–40%B; and 25–30 min, 40%B. The LC-MS analysis was performed following the method at a flow rate of 0.4 ml min⁻¹ with a linear gradient from 10% to 90% of solvent B in 20 min (solvent A: water/formic acid (99:1); solvent B: acetonitrile/formic acid (99:1)).

**Purification of compounds 3 and 4**

To isolate and purify compounds 3 and 4, 20 l fermentation of Δdap2 was carried out with MS medium. The crude extract of ethyl acetate was concentrated and loaded onto a column filled with silica gel and flushed with chloroform and different proportions of chloroform–methanol mixture. Outflows were detected by HPLC, and the fractions containing the target compounds were collected. Second purification was conducted on a Sephadex LH20 column with methanol. The purified compounds were finally prepared by semi-preparative HPLC using the same elution method as mentioned earlier.

**Expression and purification of Dap2**

The encoding gene of Dap2 was amplified from the genomic DNA of *S. diastaticus* W2 using the primers dap2forward/dap2reverse (Table S3). The purified PCR product was cloned into the EcoRI/Ndel sites of the vector pET28a, and the constructed pET-dap2 was then transformed into *E. coli* BL21(DE3) for protein overexpression. The expression of Dap2 fused with His-tag was induced by IPTG and purified by Ni-affinity chromatography.

**In vitro catalytic reaction of Dap2**

The enzyme activity of Dap2 was assayed in a 100 μL reaction system containing 10 μM Dap2, 400 μM substrate (compound 3 or 4), 180 μM SAM and 50 mM Tris buffer (pH 7.8). The reaction mixture was incubated at room temperature for 90 mins and then terminated by mixing 100 μL ethyl acetate. The organic phase was collected and evaporated to dryness. The residue was then dissolved in 100 μL methanol and detected by HPLC and LC-MS analyses.
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Conflict of interest

We declare that we have no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Deduced functions of genes in the *dap* cluster

**Table S2.** Bacterial strains and plasmids used in this study

**Table S3.** Primers used in this study

**Fig. S1.** Verification of the gene deletion and complementation mutant strains by PCR

**Fig. S2.** HPLC analysis of metabolite profile of the gene deletion mutant strains

**Fig. S3.** EIC traces of PCA (m/z 225.1513) in the heterologous expression strain and mutant strains

**Fig. S4.** EIC traces of the compound 1 (m/z 437.1254), compound 2 (m/z 436.1224) and PCA (m/z 225.1513) in the heterologous expression strain and mutant strain

**Fig. S5.** ESI-HRMS spectra of compound 3

**Fig. S6.** ESI-HRMS spectra of compound 4

**Fig. S7.** High-resolution LC-MS/MS analysis of the compound 3 (ii) and the para-aminobenzoic acid standard (i)

**Fig. S8.** 1H NMR (600 MHz, DMSO-d6) spectrum of compound 3

**Fig. S9.** High-resolution LC-MS/MS analysis of the compound 4 (ii) and the demethylated izumiphenazine C standard (i)

**Fig. S10.** EIC traces of the compounds 3 (m/z 138.0641) and 4 (m/z 332.1024) in the heterologous expression strain and mutant strain

**Fig. S11.** SDS-PAGE analysis of the purified Dap2 protein

**Fig. S12.** LC-MS analysis of the gene deletion mutant strain *S. albus*: 23C5::Δdap2 feeding with compound 5