Identification and Analysis of the Paulomycin Biosynthetic Gene Cluster and Titer Improvement of the Paulomycins in *Streptomyces paulus* NRRL 8115

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Abstract

The paulomycins are a group of glycosylated compounds featuring a unique paulic acid moiety. To locate their biosynthetic gene clusters, the genomes of two paulomycin producers, *Streptomyces paulus* NRRL 8115 and *Streptomyces sp*. YN86, were sequenced. The paulomycin biosynthetic gene clusters were defined by comparative analyses of the two genomes together with the genome of the third paulomycin producer *Streptomyces albus* J1074. Subsequently, the identity of the paulomycin biosynthetic gene cluster was confirmed by inactivation of two genes involved in biosynthesis of the paulomycose branched chain (*pau11*) and the ring A moiety (*pau18*) in *Streptomyces paulus* NRRL 8115. After determining the gene cluster boundaries, a convergent biosynthetic model was proposed for paulomycin based on the deduced functions of the *pau* genes. Finally, a paulomycin high-producing strain was constructed by expressing an activator-encoding gene (*pau13*) in *S. paulus*, setting the stage for future investigations.

Introduction

Natural products from *Streptomyces* are a prolific source of therapeutic agents. Many of them have already been used clinically as antibiotics, immunosuppressants and antitumor drugs. Typically, the structure, regulatory and resistance genes of those compounds are clustered in the genomes, which significantly facilitates biosynthetic investigations [1]. Over the past three decades, the biosynthetic and regulatory mechanisms of a great number of *Streptomyces* natural products have been deciphered, which enables us to generate novel compounds through combinatorial biosynthesis or synthetic biology approaches and to improve their titers in a strategic manner [2, 3].

The paulomycins are a group of glycosylated compounds isolated from several different *Streptomyces* strains (Fig. 1). Before Argoudelis et al. solved the structures of paulomycin A and B from *Streptomyces paulus* sp. 273 and named them in 1982 [4], these compounds were
reported using various names, such as U-43120 [5], NSC-163500 [6] and volonomycins [7]. Paulomycin A is composed of a quinone-like ring A, an acetylated D-allose, an unusual eight-carbon sugar paulomycose esterified by 2-methylbutyric acid at the branched hydroxyl group, and a unique paulic acid moiety containing a rare isothiocyanate group. Unlike paulomycin A, the paulomycose branched hydroxyl group is decorated by isobutyric acid in paulomycin B. A series of paulomycins with various modifications at the two-carbon branched chain of paulomycose were isolated subsequently from several *S. paulus* strains and *Streptomyces albus* J1074 (Fig. 1) [8–10]. In addition, there are some paulomycin analogs modified at the paulomycose 3'-methoxyl group, such as O-demethylpaulomycins (lack the 3'-O-methyl group) [11] and senfolomycins. Although the senfolomycins were discovered much earlier than paulomycins [12], their structures were not determined until 1988. Senfolomycin A and B are identical to paulomycin E and F respectively, but possess reversed configuration at the 3'-methoxyl groups [13]. This structural diversity is further expanded by a variety of paulomycin analogs such as the paulomenols (lacking the paulic acid moiety) U-77802 and U-77803 (the hydrogen sulfide adducts of paulomycin A and B) and the paldimycins (with N-acetyl-L-cysteine attached at the isothiocyanate moiety) [14] (Fig. 1). All paulomycins and their analogs have shown excellent antibiotic activity against Gram-positive bacteria [11, 15, 16], and some exhibit substantial activity against a range of other microorganisms that may be useful for treating urethritis and *Chlamydia* infections [17].

To our knowledge, all known paulomycin compounds have only been produced by *Streptomyces*, and no chemical synthesis has been reported. However, the limited understanding of paulomycin biosynthesis currently impedes the expansion of structural diversity through combinatorial biosynthesis. We were therefore motivated to define the paulomycin biosynthetic gene cluster by sequencing the genomes of two paulomycin producers, *S. paulus* NRRL 8115 [6] and *Streptomyces sp.* YN86 [18]. The paulomycin gene clusters were localized by
comparative genomic analyses of the two strains and another paulomycin producer *S. albus* J1074 [10]. After determining the paulomycin gene cluster boundaries in *S. paulus* NRRL 8115, we proposed a putative paulomycin biosynthetic pathway based on bioinformatic analysis. Finally, production of the paulomycins in *S. paulus* NRRL 8115 was improved considerably by overexpressing an activator encoding gene *pau13*.

**Materials and Methods**

**Bacterial strains, media and plasmids**

The strains and plasmids used in this study are summarized in S1 Table. *Escherichia coli* JM109 was used as a host to prepare plasmids. *E. coli* ET12567/pUZ8002 was used for *E. coli*-Streptomyces conjugation [19]. All *E. coli* strains were incubated in Luria-Bertani medium at 37°C. *Streptomyces paulus* NRRL8115, *Streptomyces sp.* YN86 and *Streptomyces albus* J1074 were the three paulomycin producers. For spore formation, *Streptomyces* strains were grown on mannitol/soya (MS) agar at 28°C [19]. Liquid YEME medium was used for *Streptomyces* genomic DNA isolation [19]. Medium GS-7 [7] was used as the seed culture and medium R5α was used as the paulomycin production medium for *S. paulus* NRRL8115 [20]. Plasmids pUC119::KanR, pKC1132 and pSET152::ermE were described previously [19, 21, 22].

**DNA manipulation**

Isolation of *Streptomyces* genomic DNAs was performed as previously described [19]. PCRs were performed with Taq DNA polymerase (TransGene, Beijing, China) or KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. Restriction enzyme digestions, ligations and transformations were performed following the standard methods [23]. *E. coli*-Streptomyces conjugations were carried out following the described protocols [19].

**Sequencing and bioinformatics analyses**

Genomic DNA sequencing service was provided by Majorbio Company (Majorbio, Shanghai, China) using Illumina Hiseq2000 system. Analyses of the secondary metabolite gene clusters were performed with antiSMASH (http://antismash.secondarymetabolites.org/) [24]. The possible open reading frames (ORFs) were predicted by Prodigal (http://prodigal.ornl.gov/) [25]. The gene functional annotations combined the search results of NCBI and KEGG databases. Multiple alignments were performed with CLUSTALW. The sequences of the paulomycin biosynthetic gene clusters from *S. paulus* NRRL8115 and *S. sp.* YN86 have already been submitted to GenBank (accession number KJ721164 and KJ721165).

**Production of paulomycin**

For paulomycin production, 50 μL *S. paulus* NRRL8115 spores were inoculated into GS-7 medium and cultured at 28°C for 2 days. The resulting seed culture was inoculated into 50 mL medium R5α at 2% ratio (v/v) and cultured for 4 days. The fermentation broth was harvested by centrifugation and extracted with 50 mL ethyl acetate for three times. The ethyl acetate extraction was dried in *vacuo*. It was then redissolved in 1 mL acetonitrile and subjected to HPLC analysis.
Construction of mutants CIM3001 (\textit{S. paulus pau11::aph}) and CIM3002 (\textit{S. paulus pau18::aph})

An allelic replacement strategy was used to inactivate the target genes individually in \textit{S. paulus} NRRL 8115. The 2.4-kb fragment upstream of \textit{pau11} was PCR amplified with primer pair \textit{pau11-up-F} and \textit{pau11-up-R} and inserted into the \textit{PstI/XbaI} sites of pUC119::KanR to construct pCIM3001 (all primers used in this study are listed in \textit{S2 Table}. The restriction site used in plasmid construction is underlined and marked after each primer). The 1.1-kb fragment downstream of \textit{pau11} was obtained by PCR using primer pair \textit{pau11-down-F} and \textit{pau11-down-R} and cloned into the \textit{EcoRI} site of pCIM3001 via a ligation-independent cloning strategy [26] to generate pCIM3002. The fidelity of all the PCR cloned fragments was confirmed by sequencing. The 4.5-kb mutant allele containing the two fragments flanking \textit{pau11} and the kanamycin resistance gene (\textit{aph}) was excised by \textit{PstI/EcoRI} and inserted into the same sites of pKC1132 to construct pCIM3003. Plasmid pCIM3003 was then introduced into \textit{S. paulus} NRRL 8115 via \textit{E. coli-Streptomyces} conjugation. Exconjugants with kanamycin resistance and apramycin sensitivity were selected as the desired \textit{S. paulus pau11::aph} mutant strain CIM3001. The genotype of CIM3001 was confirmed by PCR with primers \textit{pau11-up-F} and \textit{pau11-down-R} (\textit{S1 Fig.}).

To inactivate the \textit{pau18} gene, the 0.7-kb upstream fragment was PCR cloned with primer pair \textit{pau18-up-F} and \textit{pau18-up-R}, and the 1.3-kb downstream fragment was cloned with primer pair \textit{pau18-down-F} and \textit{pau18-down-R}. The two fragments were inserted into the \textit{PstI/BamHI} and \textit{KpnI/EcoRI} sites of pUC119::KanR sequentially to generate pCIM3004. The 3.9-kb mutant allele containing the two fragments flanking \textit{pau18} and the kanamycin resistance cassette was then cut off from pCIM3004 by \textit{PstI/EcoRI} and inserted into the same sites of pKC1132 to construct pCIM3005. After introducing plasmid pCIM3005 into \textit{S. paulus} NRRL 8115 via \textit{E. coli-Streptomyces} conjugation, exconjugants that were kanamycin resistant and apramycin sensitive were selected as the desired \textit{S. paulus pau18::aph} mutant strain CIM3002. The genotype of CIM3002 was confirmed by PCR with primers \textit{pau18-up-F} and \textit{pau18-down-R} (\textit{S1 Fig.}).

Complementation of CIM3001 and CIM3002

The 1.0-kb fragment containing the whole \textit{pau11} gene was PCR-amplified from \textit{S. paulus} NRRL 8115 with primer pair \textit{pau11-E-F} and \textit{pau11-E-R} and inserted into the \textit{NdeI/BamHI} sites of pSET152::\textit{ermE"} to generate plasmid pCIM3006. Introduction of pCIM3006 into \textit{S. paulus} CIM3001 generated the \textit{S. paulus pau11::aph} complemented strain CIM3003.

Similarly, the 2.0-kb fragment harboring the whole \textit{pau18} gene was cloned by PCR with primer pair \textit{pau18-E-F} and \textit{pau18-E-R} and inserted into the \textit{NdeI/BamHI} sites of pSET152::\textit{ermE"} to generate pCIM3007. The \textit{S. paulus pau18::aph} complemented strain CIM3004 was obtained by introduction of pCIM3007 into \textit{S. paulus} CIM3002.

Construction of the mutants for determination of the paulomycin gene cluster boundaries

Details are described in \textit{S1 File}. The genotype of the \textit{S. paulus} mutants were verified by PCR (\textit{S2–S3 Figs.}).
Construction and complementation of the CIM3005 (S. paulus pau13::aph) mutant strain

The two fragments flanking pau13 were amplified by PCR using primer pair pau13-up-F and pau13-up-R for the 1.1-kb upstream fragment and primer pair pau13-down-F and pau13-down-R for the 1.8-kb downstream fragment. The two fragments were cloned into the EcoRI/KpnI and BamHI/PstI sites of pUC119::KanR sequentially to generate pCIM3008. The 3.9-kb mutant allele containing both the up- and down-stream fragments and the kanamycin resistance cassette was then excised by EcoRI/PstI and inserted into the same sites of pKC1132 to generate pCIM3009. Plasmid pCIM3009 was introduced into S. paulus NRRL 8115 via E. coli-Streptomyces conjugation. Exconjugants that were kanamycin resistant and apramycin sensitive were picked out as the S. paulus pau13::aph mutant strain CIM3005, the genotype of which was then confirmed by PCR with primers pau13-up-F and pau13-down-R and subsequent BamHI digestions of the PCR products (S1 Fig.).

The 1.0-kb fragment containing the whole pau13 gene was amplified by PCR using primers pau13-E-F and pau13-E-R and inserted into the same sites of pSET152::ermE/C3 to construct pCIM3010. The pau13 mutant complemented strain CIM1006 was constructed by introduction of pCIM3010 into CIM3005 via E. coli-Streptomyces conjugation.

Overexpression of pau13 in S. paulus NRRL8115

Introduction of pCIM3010 into S. paulus NRRL 8115 via E. coli-Streptomyces conjugation generated the recombination strain CIM3007, in which the expression of pau13 is under the control of the constitutive ermE’ promoter. For comparisons of the paulomycin and paulomenol titers in wild-type and CIM3007 strains, standard error values are obtained from at least three independent cultures.

Analytical and Spectroscopic Procedures

HPLC analyses were carried out with an Apollo C18 column (5 μm, 4.6 × 250mm, Alltech, Deerfield, IL, USA) with Shimadzu HPLC system (Shimadzu, Kyoto, Japan). The column was developed with a linear gradient using acetonitrile and water with 0.1% trifluoroacetic acid at a flow rate of 0.8 mL/min. For the first 5 minutes, the ratio of acetonitrile was maintained at 5%, and it was changed linearly from 5% to 90% over 5–25 min and from 90% to 100% over 25–30 min. The detection wavelength was 320 nm.

MS and tandem MS were performed on an Agilent 1260/6460 Triple-Quadrupole LC/MS system (Agilent, Santa Clara, CA, USA) with the electrospray ionization source. The high resolution MS analysis was performed on an Agilent 1200 HPLC system and 6520 QTF-MS system (Agilent, Santa Clara, CA, USA). The mass spectrometer scanned from m/z = 100–1500 in negative ion mode.

Results and Discussion

Comparative genomic analyses to define the paulomycin biosynthetic gene cluster

Three Streptomyces strains (S. paulus NRRL 8115, S. albus J1074 and S. sp. YN86) were used for searching the paulomycin biosynthetic gene clusters. The genome sequence of S. albus J1074 is available in GenBank (accession No. NC_020990). By draft sequencing the genomes of S. paulus NRRL 8115 and S. sp. YN86, we now have three sequenced genomes harboring the paulomycin gene cluster. Since paulomycin contains two sugars attached by a C- or O-glycosidic bond, we searched by comparative genomic analysis and identified several conserved gene
clusters containing glycosyltransferase-encoding genes in the three genomes as candidates. Furthermore, considering that paulomycin contains the eight-carbon sugar paulomycose with a two-carbon branched chain, we searched these clusters for the presence of genes involved in the two-carbon branched chain biosynthesis of high-carbon-chain sugars. To our knowledge, only two molecular mechanisms have been described for the formation of sugar’s two-carbon branched chain. One is catalyzed by the pyruvate dehydrogenase-like proteins (AviB1/AviB2) and the nonheme iron-dependent enzyme AviO2 involved in the methyleurekanate biosynthesis of avilamycin from *Streptomyces viridochromogenes* Tü57 [27]; the other is the TPP-dependent flavoprotein YerE involved in yersiniose biosynthesis from *Yersinia pseudotuberculosis* [28]. Further bioinformatic analysis revealed a 61-kb DNA region containing both the AviB1/AviB2 homologs and the glycosyltransferase-encoding genes in each of the three strains, which is regarded as the putative paulomycin biosynthetic gene cluster. The 61-kb region is highly conserved in both gene organization and individual gene functions among all three paulomycin producers and the paulomycin biosynthetic gene cluster from *S. paulus* NRRL 8115 (named as the *pau* gene cluster) is depicted in Fig. 2. Functions of the 53 open reading frames (ORFs) were assigned by careful bioinformatic analyses (Table 1).

**Confirmation of the pau gene cluster in S. paulus NRRL 8115**

During our study, it was observed that production of the paulomycins in *S. albus* J1074 is not stable. Given that the genetic manipulation in *S. paulus* NRRL 8115 is much easier than that in *S. sp.* YN86, *S. paulus* NRRL 8115 was used as a model system in the following paulomycin biosynthetic studies. The identities of paulomycin A, paulomycin B, paulomenol A and paulomenol B produced by *S. paulus* NRRL 8115 were confirmed by ultraviolet-visible absorption spectra, high resolution mass spectrometry (MS) and tandem MS (S4–S7 Figs.).

To obtain direct proof that the *pau* cluster is crucial for the production of paulomycins in *S. paulus* NRRL 8115, two genes in the cluster (*paul11* and *paul18*) were mutated individually by targeted gene replacement (S1 Fig.). The gene product of *paul11* is an AviB1 homologue involved in the eight-carbon sugar paulomycose biosynthesis; the gene product of *paul18* is a putative 2-amino-2-deoxyisochorismate (ADIC) synthase responsible for the biosynthesis of the
## Table 1. Homologous proteins of ORFs in the paulomycin biosynthetic gene clusters.

| Genes | Sizes [a] | Proposed Functions | Protein Homologs [b](identity/similarity) in S. sp. YN86 [c] | Protein Homologs in S. albus J1074 [d] |
|-------|-----------|--------------------|-------------------------------------------------------------|----------------------------------------|
| pau1  | 382       | RNA polymerase sigma factor | (NP_628619, 82/89) PauY1 (98/99) YP_007744020 (98/99) |                          |
| pau2  | 267       | M23 family peptidase | (WP_018522979, 82/87) PauY2 (100/100) YP_007744019 (99/99) |                          |
| pau3  | 337       | aminotransferase | (WP_019069167, 63/69) PauY3 (90/90) YP_007744018 (91/91) |                          |
| pau4  | 187       | TetR-family transcriptional regulator | (NP_630249, 59/76) PauY4 (96/97) YP_007744017 (96/97) |                          |
| pau5  | 173       | LuxR family regulator | (NP_005479035, 51/69) PauY5 (52/52) YP_007744016 (50/50) |                          |
| pau6  | 389       | isovaleryltransferase | (AAU34206, 37/52) [31] PauY6 (99/99) YP_007744015 (100/100) |                          |
| pau7  | 272       | Aldo-keto reductase | (NP_630632, 64/77) PauY7 (98/99) YP_007744014 (100/100) |                          |
| pau8  | 162       | hypothetical protein | (NP_625096, 55/69) PauY8 (100/100) YP_007744013 (100/100) |                          |
| pau9  | 480       | EmrB/QacA subfamily transporter | AsuM1 (ADI58655, 35/54) [48] |                          |
| pau10 | 709       | elongation factor G | (NP_628821, 90/95) PauY10 (100/100) YP_007744011 (100/100) |                          |
| pau11 | 325       | pyruvate dehydrogenase α subunit | AviB1 (AAK83190, 47/47) |                          |
| pau12 | 345       | pyruvate dehydrogenase β subunit | AviB2 (AAK83191, 34/45) [27] |                          |
| pau13 | 286       | SARP family transcriptional regulator | SnZ (WP_023415585, 51/65) [49] |                          |
| pau14 | 361       | glycosyltransferase auxiliary protein | DesVIII (AAC68676, 28/37) [46] |                          |
| pau15 | 426       | O-glycosyltransferase | DesVII (AAC68677, 43/58) [46] PauY15 (100/100) YP_007744006 (100/100) |                          |
| pau16 | 461       | NDP-hexose 2,3-dehydratase | SnogH (CAA12009, 54/64) [50] PauY16 (99/99) YP_007744005 (100/100) |                          |
| pau17 | 389       | 3-hydroxybenzoate 6-hydroxylase | XlnD (Q9F131, 44/60) [51] PauY17 (100/100) YP_007744004 (100/100) |                          |
| pau18 | 651       | phenazine biosynthesis protein PhzE | PhzE (NP_250594, 53/65) [33] YP_007744003 (100/100) |                          |
| pau19 | 219       | phenazine biosynthesis protein PhzD | PhzD (NP_250593, 56/70) [33] YP_007744002 (100/100) |                          |
| pau20 | 261       | 2, 3-dihydro-2, 3-dihydroxybenzoate dehydrogenase | MxcC (AAG31126, 58/67) [52] |                          |
| pau21 | 402       | 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase | PtmI (AAQ84163, 50/60) [53] PauY21 (100/100) YP_007744000 (100/100) |                          |
| pau22 | 331       | dTDP-glucose 4,6-dehydratase | CpzDII (ADI52027, 65/76) [54] PauY22 (100/100) YP_007743999 (100/100) |                          |
| pau23 | 289       | hexose-1-phosphate thymidylyltransferase | DesIII (AAC68682, 59/73) [55] |                          |
| pau24 | 404       | acyltransferase | MppN (AAU34207, 36/50) [31] PauY24 (100/100) YP_007743997 (100/100) |                          |
| pau25 | 375       | C-glycosyltransferase | SaqQT5 (ACP19370, 50/64) [56] PauY25 (99/99) YP_007743996 (100/100) |                          |
| pau26 | 145       | glyoxalase | (WP_005631538, 29/38) [57] PauY26 (100/100) YP_007743995 (100/100) |                          |
| pau27 | 547       | FAD-binding monooxygenase | TcmG (AA67511, 37/49) [58] PauY27 (100/100) YP_007743995 (100/100) |                          |
| pau28 | 255       | reductase | AntM (AGG37759, 48/64) [59] PauY28 (98/99) YP_007743994 (100/100) |                          |
| pau29 | 349       | 3-oxoacyl-ACP synthase III | CosE (ABC00733, 43/58) [35] PauY29 (100/100) YP_007743993 (100/100) |                          |
| pau30 | 380       | UBA/THIF-type NAD/FAD binding protein | MoeZ (CAB08310, 40/58) [42] PauY30 (99/99) YP_007743992 (100/100) |                          |
| pau31 | 455       | cysteine desulfurase | SufS (AHG14743, 27/43) [41] PauY31 (99/99) YP_007743991 (100/100) |                          |
| pau32 | 290       | putative regulatory protein | PhpR (AAU00090, 23/36) PauY32 (100/100) YP_007743990 (100/100) |                          |
| pau33 | 496       | oxidoreductase | KedU31 (AFV52193, 26/38) [50] PauY33 (99/99) YP_007743989 (100/100) |                          |
| pau34 | 478       | acyl-CoA synthase | (AFV52195, 43/57) PauY34 (100/100) YP_007743988 (100/100) |                          |
| pau35 | 98        | acyl-carrier protein | KedU34 (AFV52196, 24/43) [60] PauY35 (100/100) YP_007743987 (100/100) |                          |
| pau36 | 250       | dihydrolipoyl dehydrogenase | (DapB (AAW38176, 18/32) [61] |                          |
| pau37 | 249       | ribulose-5-phosphate 4-epimerase | AraD (NP_414603, 19/30) [30] PauY37 (99/99) YP_007743985 (99/99) |                          |
| pau38 | 228       | 4'-phosphopantetheinyl transferase | AlpN (AAR30158, 19/30) [62] PauY38 (99/99) YP_007743984 (99/99) |                          |

(Continued)
ring A moiety (see the following text). The two S. paulus mutants CIM3001 (S. paulus pau11::aph), and CIM3002 (S. paulus pau18::aph) were then cultured in the same condition as that for paulomycin production in the wild-type strain and checked by HPLC (Fig. 3). The production of paulomycins (A and B) and paulomenols (A and B) was totally abolished in the two mutants confirming that the pau cluster is involved in paulomycin biosynthesis. Recent mining of the S. albus J1074 genome identified the same region for paulomycin biosynthesis by comparing the metabolic profiles of the wild-type strain and a mutant control with two genes sshg_05327 (encoding Pau18 homolog) and sshg_05328 (encoding Pau19 homolog) deleted [29]. Complementation with constitutively expressed pau11 and pau18 genes in trans restored production of paulomycins and paulomenols in the two complemented strains CIM3003 and CIM3004, respectively, excluding a possible polar effect.

Determination of the pau cluster boundaries

To determine the paulomycin biosynthetic gene cluster boundaries, selective ORFs at both ends of the pau cluster were inactivated systematically by targeted gene replacement (S2–S3 Figs.). Inactivation of pau7 and pau43 dramatically reduced or blocked the production of the paulomycins and the paulomenols, implying that these genes are involved in paulomycin biosynthesis. In contrast, inactivation of pau1, pau3, pau45, pau48 and pau52 did not diminish

| Genes | Sizes [a] | Proposed Functions | Protein Homologs [b] (identity/similarity) | Protein Homologs in S. sp. YN86 [c] | Protein Homologs in S. albus J1074 [d] |
|-------|----------|--------------------|------------------------------------------|-------------------------------------|---------------------------------------|
| pau39 | 380      | acyl-CoA dehydrogenase domain-containing protein | AcdH (AAD44196, 32/49) [63] | PauY39 (100/100) | YP_007743983 (100/100) |
| pau40 | 156      | hypothetical protein | SAV.915 (NP_822090, 28/39) | PauY40 (99/99) | YP_007743982 (99/99) |
| pau41 | 527      | pyranose oxidase | CelL (ACH85575, 25/33) [64] | PauY41 (100/100) | YP_007743981 (100/100) |
| pau42 | 345      | TDP-4-keto-6-deoxyhexose 2, 3-reductase | LipDig3 (ABB05109, 62/73) [65] | PauY42 (99/99) | YP_007743980 (100/100) |
| pau43 | 257      | TDP-6-deoxy-L-hexose 3-O-methyltransferase | CalS11 (4GF5_A, 64/74) [66] | PauY43 (100/100) | YP_007743979 (100/100) |
| pau44 | 220      | TDP-4-keto-6-deoxyhexose 3, 5-epimerase | AveBV (NP_822124, 59/65) [45] | PauY44 (100/100) | YP_007743978 (100/100) |
| pau45 | 546      | malate synthase | AceB (AAG29597, 85/91) | PauY45 (100/100) | YP_007743977 (100/100) |
| pau46 | 181      | xanthine dehydrogenase, 2Fe-2S subunit | YagT (YP_488582, 43/53) | PauY46 (99/99) | YP_007743976 (98/98) |
| pau47 | 328      | oxidoreductase with FAD-binding domain | YagS (YP_488581, 52/83) | PauY47 (99/99) | YP_00743975 (100/100) |
| pau48 | 703      | oxidoreductase with molybdenum-binding domain | YagR (YP_488580, 37/51) | PauY48 (100/100) | YP_007743974 (100/100) |
| pau49 | 194      | YgfJ family molybdenum hydroxylase accessory protein | MobA (NP_418294, 23/34) | PauY49 (98/99) | YP_007743973 (99/99) |
| pau50 | 111      | hypothetical protein | (NP_630345, 48/59) | PauY50 (98/100) | YP_007743972 (97/99) |
| pau51 | 268      | IclR family transcriptional regulator | (NP_630346, 83/89) | PauY51 (100/100) | YP_007743971 (100/100) |
| pau52 | 444      | allantoinase | AllB (NP_630347, 76/85) | PauY52 (99/100) | YP_007743970 (100/100) |
| pau53 | 142      | guanyl-specific ribonuclease | LipX1 (ABB05092, 63/72) | PauY53 (99/99) | YP_007743969 (99/99) |

[a] Numbers are in amino acids.
[b] Given in brackets are GenBank accession numbers and percentage identity/percentage positive. References are added in case of available.
[c] Given in brackets is percentage identity/percentage positive.
[d] Not exist in S. albus J1074.
the production of paulomycin analogs (S8 Fig.), suggesting they are outside of the pau gene cluster. Consequently, the pau gene cluster was narrowed down to a 48-kb region of DNA containing 41 ORFs, from pau4 to pau44, based on the gene inactivation data and the predicted functions of the pau genes.

Biosynthesis of the D-allose moiety

A convergent model of paulomycin biosynthesis was proposed on the basis of the deduced functions of the genes within the cluster (Fig. 4). Four genes (pau23-pau25 and pau37) in the pau cluster are suggested to be responsible for the D-allose moiety biosynthesis. A plausible biosynthetic pathway is that Pau23, a putative hexose-1-phosphate thymidylyltransferase,
Fig 4. A proposed convergent model of paulomycin biosynthesis.

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activates the D-glucose-1-phosphate to TDP-D-glucose, which was then epimerized by Pau37, a homolog of ribulose-5-phosphate 4-epimerase AraD from Escherichia coli [30], to form TDP-D-allose. Subsequently, the C-glycosyltransferase homolog Pau25 transfers the activated D-allose to the paulomycin ring A at the C-5 position. Pau24 is a homolog of the acyltransferase MppN from Streptomyces hygroscopicus NRRL 30439 and is suggested to be responsible for addition of the acetyl group to the 6'-OH of D-allose [31].

Biosynthesis of the ring A moiety

It is proposed that the quinone-like ring A is derived from chorismate; six genes (pau17-pau21 and pau27) are assumed to be involved in the biosynthesis of this moiety. Pau21 is a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase responsible for condensation of phosphoenolpyruvate and erythrose 4-phosphate to 2-keto-3-deoxy-D-arabinohexulonate-7-phosphate, the first intermediate of the shikimate pathway [32], which is then converted to chorismate by a number of enzymes from the primary metabolic pathway. Chorismate is the branch point of the shikimate pathway, with one branch leading to phenazines through 2-amino-2-deoxyisochorismate (ADIC) and trans-2,3-dihydro-3-hydroxyanthranilic acid (DHHA) by ADIC synthase and isochorismatase [33, 34]. The early steps of the paulomycin ring A biosynthesis should follow an analogous route that includes a same set of transformations to generate DHHA by Pau18 (ADIC synthase) and Pau19 (isochorismatase). DHHA is further converted to 3,5,6-trihydroxyl-anthranilate by the dehydrogenase Pau20 (aromatization) and the two monoxygenases Pau17 and Pau27 (C-5 and C-6 hydroxylations) in an order yet to be determined.

Biosynthesis of the paulic acid

The paulic acid is unique and not much is known about its biosynthesis. The lack of an acyl-CoA ligase-encoding gene in the pau gene cluster indicates an unusual mechanism for the paulic acid installation. Our bioinformatic analysis of Pau29 suggests it is a ketoacylsynthase III-like acyltransferase catalyzing ester bond formation between paulic acid and the D-allose moiety. Pau29 shows 43% identity to CosE from Streptomyces olindensis [35] and 33% identity to Cer from Streptomyces tendae [36]. CosE is a ketoacylsynthase III type condensation enzyme responsible for the propionyl-CoA starter unit loading in cosmomycin biosynthesis [35]; Cer is a ketoacylsynthase III-like acyltransferase appending a dimethylmalonyl moiety to the hydroxyl group of the cervimycin sugar via ester bond formation [36]. A unifying feature of the CosE homologs is that they possess a highly conserved catalytic triad Cys(Ser)-His-His, in which the first residue (Cys or Ser) is essential for transacylation and the other two His residues are indispensible for the decarboxylative condensation. In Cer, a Val substitution in the first conserved His in that catalytic triad is consistent with its loss of condensation function. Notably Cer has a new Cys-His-Asp catalytic triad that is crucial for its acyltransferase activity [36]. Careful analysis of Pau29 revealed that it also lacks the first conserved His for the decarboxylative condensation in CosE homologs and features a Ser-His-Asp motif similar to Cer (S9 Fig.), further supporting its proposed role as an acyltransferase. In addition to the aforementioned Cer, there are several examples of condensation-like enzymes catalyzing ester bond formation including XclB/XclC [37], NonJ/NonK [38] and SgcC5 [39] involved in xenocyclin, nonactin and C-1027 biosynthesis, respectively. They all use CoA, or carrier protein-tethered fatty acids, or amino acids as substrates, indicating that Pau29 requires a CoA or ACP tethered paulic acid (or an acid intermediate) as a substrate, which may plausibly be synthesized from unknown precursors by related enzymes such as Pau28 (a putative oxidoreductase.
catalyzing the acyl carrier protein (ACP)-tethered ketoreduction), Pau34 (acyl-CoA synthase), Pau35 (ACP), Pau38 (phosphopantetheinylation transferase) and Pau39 (acyl-CoA dehydrogenase).

For the biosynthesis of the unusual isothiocyanate group, the only example is from plant. In crucifer vegetables such as broccoli, cabbage and wasabi, the glucosinolates are hydrolyzed by myrosinase to generate various isothiocyanate-containing compounds [40]. However, no myrosinase homolog-encoding gene exists in the pau cluster, implying a different mechanism for the isothiocyanate biosynthesis in paulomycin. The presence of Pau50 (ThiF-like enzyme) and Pau31 (cysteine desulfurase) suggests a cysteine origin of the isothiocyanate sulfur group. Gene pau31 encodes a protein similar to the cysteine desulfurase IscS, which is a pyridoxal 5’-phosphate dependent enzyme that supplies sulfur by converting L-cysteine to L-alanine and sulfane sulfur [41]. The pau30 gene encoding a ThiF-like protein transfers the sulfur group released by cysteine desulfurase to the sulfur-carrier protein to form a thiocarboxylate group [42]. We propose that the sulfur group in paulomycin is supplied as in thiamin biosynthesis, in which the thiol group released from cysteine by cysteine desulfurase (IscS) is transferred to the sulfur-carrier protein (ThiS) by ThiF [43]. However, we could not find any ThiS homolog-encoding gene in the pau gene cluster, suggesting that a universal sulfur-carrier protein encoded by a gene outside the cluster is recruited in paulomycin biosynthesis, analogous to the 2-thiosugar moiety biosynthesis in BE-7585A [44]. The origins of the isothiocyanate carbon and nitrogen groups and the detailed biosynthetic logic of this unusual moiety are intriguing questions that require further investigation.

Biosynthesis of the paulomycose moiety

Twelve genes (pau6, pau7, pau11, pau12, pau14-pau16, pau22, pau23 and pau42-pau44) were proposed to be involved in the biosynthesis of the paulomycose moiety. At first, D-glucose-1-phosphate is activated to TDP-D-glucose by Pau23, the putative hexose-1-phosphate thymidylyltransferase. The following steps from TDP-D-glucose to TDP-4’-keto-L-olivose are catalyzed by Pau22 (TDP-hexose 4,6-dehydratase), Pau16 (TDP-hexose 2,3-dehydratase), Pau42 (TDP-4-keto-6-deoxyhexose 2, 3-reductase) and Pau44 (TDP-4-keto-6-deoxyhexose 3, 5-epimerase), in a route parallel to that of deoxysugar biosynthesis in the avermectin biosynthetic pathway [45]. The TDP-6-deoxy-L-hexose 3-O-methyltransferase Pau43 is responsible for the 3’-O-methylation of TDP-4’-keto-L-olivose, and the resulting deoxysugar is then attached to the 3-O’ position of the D-allose moiety by Pau14 and Pau15. The gene products of pau14 and pau15 show high similarities to cytochrome P450 family protein DesVIII and glycosyltransferase DesVII, both of which are required for attachment of TDP-D-desosamine in pikromycin biosynthesis [46, 47]. The gene products of pau11 and pau12 resemble AviB1 (37% identity) and AviB2 (34% identity) from Streptomyces viridochromogenes Tü57, respectively. As previously mentioned, AviB1 and AviB2 are α and β subunits of the pyruvate dehydrogenase like proteins involved in the biosynthesis of the two-carbon branched chain sugar methyleurekanate [27]. We propose that the installation of the two-carbon branched chain takes place after the hexose is attached to the D-allose moiety, analogous to methyleurekanate formation in avilamycin biosynthesis, in which the 4-ketosugar precursor of methyleurekanate is attached to the sugar chain before the two-carbon unit is appended [27]. The pyruvate dehydrogenase-like proteins Pau11 and Pau12 hijack a two-carbon unit from pyruvate, which is then loaded onto the C4’ position through an unknown mechanism. Finally, the 7’-keto is reduced to a hydroxyl group by an oxidoreductase (Pau7), and an acyltransferase (Pau6) appends a variety of fatty acids to the 7’-hydroxyl group to form different paulomycins.
Genes for resistance, regulation and unassigned functions

The pau9 gene is the only apparent candidate for self-protection within the pau gene cluster, which encodes a protein exhibiting 35% identity to a drug resistance transporter AsuM1 from the asukamycin producer Streptomyces nodosus subsp. asukaensis [48].

There are four putative regulatory genes (pau4, pau5, pau13 and pau32) in the pau cluster. They belong to three transcriptional regulator families including the TetR family (Pau4), the LuxR family (Pau5 and Pau32) and the Streptomyces antibiotic regulatory protein (SARP) family (Pau13).

There are seven functionally unassigned ORFs in the pau cluster including two hypothetical proteins (pau8 and pau40) and five genes with deduced functions that cannot be assigned to paulomycin biosynthesis. The proposed functions of the five remaining genes are elongation factor G (Pau10), glyoxalase (Pau26), enoyl reductase (Pau33), dihydrodipicolinate reductase (Pau36) and pyranose oxidase (Pau41).

Improving paulomycin production by overexpressing gene pau13

With the paulomycin biosynthetic gene cluster in hand, we set out to increase production of the paulomycins by manipulating its pathway regulation, which has proved to be an efficient strategy in many rational metabolic engineering efforts. The gene product of pau13 is a SARP family regulator, which is usually functional as an activator stimulating the biosynthesis of antibiotics in Streptomyces [3]. The S. paulus pau13:aph mutant CIM3005 was constructed by replacing this gene with a kanamycin resistance cassette. HPLC analysis revealed that production of the paulomycins was almost abolished in CIM3005, suggesting that Pau13 is a positive regulator. The pau13 mutant complemented strain CIM3006 was constructed by introduction of pCIM3010, in which the pau13 gene was put under the control of a constitutive promoter ermE*, into CIM3005. When fermented in medium R5α, CIM3006 restored the production of paulomycin A, paulomenol A and paulomenol B, excluding the influence of polar effect. Subsequently, pCIM3010 was introduced into S. paulus wild-type to generate a pau13 overexpressing recombination strain CIM3007. The titers of paulomycin A, paulomycin B, paulomenol A and paulomenol B were increased 3.4±0.9, 4.2±1.3, 4.1±0.8 and 4.2±1.2 fold in S. paulus CIM3006, respectively.

Conclusions

In summary, we have defined the paulomycin biosynthetic gene cluster by comparative genome mining of three paulomycin producers. A convergent model of paulomycin biosynthesis was proposed after we confirmed the identity of the pau gene cluster and determined its boundaries in S. paulus NRRL 8115. The production of paulomycins was improved significantly by rational engineering of the pathway regulation in S. paulus NRRL 8115, establishing an excellent foundation for future investigations of paulomycin biosynthesis and engineering.

Supporting Information

S1 Fig. Construction and genotype confirmation of S. paulus mutants CIM3001, CIM3002 and CIM3005. (A) Diagram illustrating the construction of CIM3001 by replacing pau11 with a kanamycin-resistance gene (aph). (B) PCR detection of pau11 inactivation. Lane 1, fragments obtained by PCR with CIM3001 as a template and a following XbaI digestion; lane 2, fragments obtained by PCR with S. paulus NRRL 8115 as a template and a following XbaI digestion (Expected sizes of PCR fragments after restriction with the indicated enzyme are shown in panel A); Lane 3, DNA Ladder. (C) Diagram illustrating the construction of CIM3002 by replacing
pau18 with a kanamycin-resistance gene. (D) PCR detection of pau18 inactivation. Lane 1, fragments obtained by PCR with S. paulus NRRL 8115 as a template; lane 2, fragments obtained by PCR with CIM3002 as a template (Expected sizes of PCR fragments are shown in panel C); Lane 3, DNA Ladder. (E) Diagram illustrating the construction of CIM3005 by replacing pau13 with a kanamycin-resistance gene. (F) PCR detection of pau13 inactivation. Lane 1, fragments obtained by PCR with CIM3005 as a template and a following BamHI digestion; lane 2, fragments obtained by PCR with S. paulus NRRL 8115 as a template and a following BamHI digestion (Expected sizes of PCR fragments after restriction with the indicated enzyme are shown in panel E); Lane 3, DNA Ladder.

(TIF) S2 Fig. Construction and genotype confirmation of S. paulus mutants CIM3008-CIM3010.
(A) Diagram illustrating the construction of CIM3008 by replacing pau1 with an apramycin-resistance gene \((aac(3)IV)\). (B) PCR detection of pau1 inactivation. Lane 1, fragments obtained by PCR with CIM3008 as a template; lane 2, fragments obtained by PCR with a single-cross mutant as a template; lane 3, fragments obtained by PCR with S. paulus NRRL 8115 as a template (Expected sizes of PCR fragments are shown in panel A); Lane 4, DNA Ladder. (C) Diagram illustrating the construction of CIM3009 by replacing pau3 with an apramycin-resistance gene. (D) PCR detection of pau3 inactivation. Lane 1, fragments obtained by PCR with CIM3009 as a template; lane 2, fragments obtained by PCR with S. paulus NRRL 8115 as a template (Expected sizes of PCR fragments are shown in panel C); Lane 3, DNA Ladder. (E) Diagram illustrating the construction of CIM3010 by replacing pau7 with an apramycin-resistance gene. (F) PCR detection of pau7 inactivation. Lane 1, fragments obtained by PCR with CIM3010 as a template; lane 2, fragments obtained by PCR with S. paulus NRRL 8115 as a template (Expected sizes of PCR fragments are shown in panel E); Lane 3, DNA Ladder.

(TIF) S3 Fig. Construction and genotype confirmation of S. paulus mutants CIM3011-CIM3014.
(A) Diagram illustrating the construction of CIM3011 by replacing pau43 with a kanamycin-resistance gene \((aph)\). (B) PCR detection of pau43 inactivation. Lane 1, fragments obtained by PCR with CIM3011 as a template; lane 2, fragments obtained by PCR with S. paulus NRRL 8115 as a template (Expected sizes of PCR fragments are shown in panel A); Lane 3, DNA Ladder. (C) Diagram illustrating the construction of CIM3012 by replacing pau45 with an apramycin-resistance gene \((aac(3)IV)\). (D) PCR detection of pau45 inactivation. Lane 1, fragments obtained by PCR with CIM3012 as a template and a following BlnI digestion; lane 2, fragments obtained by PCR with S. paulus NRRL 8115 as a template and a following BlnI digestion (Expected sizes of PCR fragments after restriction with the indicated enzyme are shown in panel C); Lane 4, DNA Ladder. (E) Diagram illustrating the construction of CIM3013 by replacing pau48 with an apramycin-resistance gene. (F) PCR detection of pau48 inactivation. Lane 1, fragments obtained by PCR with CIM3013 as a template; lane 2, fragments obtained by PCR with S. paulus NRRL 8115 as a template (Expected sizes of PCR fragments are shown in panel E); Lane 4, DNA Ladder. (G) Diagram illustrating the construction of CIM3014 by replacing pau52 with an apramycin-resistance gene. (H) PCR detection of pau52 inactivation. Lane 1, fragments obtained by PCR with CIM3014 as a template; lane 2, fragments obtained by PCR with S. paulus NRRL 8115 as a template (Expected sizes of PCR fragments are shown in panel G); Lane 3, DNA Ladder.
S4 Fig. Spectroscopic analyses of paulomycin A. (A) Plausible fragmentation pattern of paulomycin A in tandem MS detection. (B) Tandem MS of paulomycin A. (C) High resolution MS of paulomycin A. (D) UV-vis spectrum of paulomycin A. (TIF)

S5 Fig. Spectroscopic analyses of paulomycin B. (A) Plausible fragmentation pattern of paulomycin B in tandem MS detection. (B) Tandem MS of paulomycin B. (C) High resolution MS of paulomycin B. (D) UV-vis spectrum of paulomycin B. (TIF)

S6 Fig. Spectroscopic analyses of paulomenol A. (A) Plausible fragmentation pattern of paulomenol A in tandem MS detection. (B) Tandem MS of paulomenol A. (C) High resolution MS of paulomenol A. (D) UV-vis spectrum of paulomenol A. (TIF)

S7 Fig. Spectroscopic analyses of paulomenol B. (A) Plausible fragmentation pattern of paulomenol B in tandem MS detection. (B) Tandem MS of paulomenol B. (C) High resolution MS of paulomenol B. (D) UV-vis spectrum of paulomenol B. (TIF)

S8 Fig. HPLC traces of S. paulus CIM3008-CIM3014 for the pau cluster boundaries determination. The inactivated gene of each mutant is bracketed. Paulomycin A (□); Paulomycin B (○); Paulomenol A (○); Paulomenol B (△). (TIF)

S9 Fig. Multiple alignments of Pau29 and its homologs. The catalytic triad Cys-His-Asp for ketoacylsynthase III-like acyltransferase CerJ is marked with inverted triangles; and the conserved catalytic triad Cys(Ser)-His-His for ketoacylsynthases DpsC and CosE are marked with asterisks. It is notable that the first conserved His in ketoacylsynthases is substituted by Val in CerJ and Asn in Pau29, PauY29 and YP_007743993. (TIF)

S1 File. Construction of the mutants for determination of the paulomycin gene cluster boundaries. (DOC)

S1 Table. Strains and plasmids used in this study. (DOC)

S2 Table. Primers used in this study. (DOC)

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Author Contributions
Conceived and designed the experiments: YC JL. Performed the experiments: JL GA. Analyzed the data: YC JL MW. Contributed reagents/materials/analysis tools: JL. Wrote the paper: JL ZX YC.

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