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The exploration of high production of tiancimycins in *Streptomyces* sp. CB03234-S revealed potential influences of universal stress proteins on secondary metabolisms of streptomycetes

Huiming Liu[1], Zhiying Fan[1], Nian Tong[1], Jing Lin[1], Yong Huang[1,3], Yanwen Duan[1,2,3*] and Xiangcheng Zhu[1,4,5*]

Abstract

Background Universal stress proteins (USPs) are prevalent in various bacteria to cope with different adverse stresses, while their possible effects on secondary metabolisms of hosts are unclear. Tiancimycins (TNMs) are ten-membered endiynes possessing excellent potential for development of anticancer antibody-drug conjugates. During our efforts to improve TNMs titer, a high-producing strain *Streptomyces* sp. CB03234-S had been obtained and its possible high yield mechanism is being continuously explored to further enhance TNMs production.

Results In this work, the whole-genome resequencing and analysis results revealed a notable 583 kb terminal deletion containing 8 highly expressed *usp* genes in the genome of CB03234-S. The individual complementation of lost USPs in CB03234-S all showed differential effects on secondary metabolism, especially TNMs production. Among them, the overexpression of USP3 increased TNMs titer from 12.8 ± 0.2 to 31.1 ± 2.3 mg/L, while the overexpression of USP8 significantly reduced TNMs titer to only 1.0 ± 0.1 mg/L, but activated the production of porphyrin-type compounds. Subsequent genetic manipulations on USP3/USP8 orthologs in *Streptomyces. coelicolor* A3(2) and *Streptomyces* sp. CB00271 also presented clear effects on the secondary metabolisms of hosts. Further sequence similarity network analysis and *Streptomyces*-based pan-genomic analysis suggested that the USP3/USP8 orthologs are widely distributed across *Streptomyces*.

Conclusion Our studies shed light on the potential effects of USPs on secondary metabolisms of streptomycetes for the first time, and USPs could become novel targets for exploring and exploiting natural products in streptomycetes.

Keywords Universal stress proteins, Tiancimycins, Secondary metabolisms, Streptomycetes, Natural products

*Correspondence:
Yanwen Duan
ywduan66@sina.com
Xiangcheng Zhu
seanzhu1996@aliyun.com
[1]Xiangya International Academy of Translational Medicine, Central South University, Tongzipo Road, #172, Yuelu District, Changsha, Hunan 410013, China
[2]Hunan Engineering Research Center of Combinatorial Biosynthesis and Natural Product Drug Discovery, Changsha, Hunan 410013, China
[3]National Engineering Research Center of Combinatorial Biosynthesis for Drug Discovery, Changsha, Hunan 410013, China
[4]Center for Future Foods, Muyuan Laboratory, 110 Shangding Road, Zhengzhou, Henan 450016, China
[5]Nanyang Westlake-Muyuan Institute of Synthetic Biology, Nanyang, Henan 473000, China

Background

Environmental changes and interspecific competitions frequently impose multifarious stresses on the survival of organisms [1, 2]. Microorganisms exhibit remarkable capacities to adapt and respond to various external stresses, and these adaptive changes that involve genetic and physiological variations are often referred as stress responses [3]. Among stress-related genes and proteins, universal stress proteins (USPs) have played a pivotal role in different stress responses and widely distributed in various organisms including bacteria, archaea, and multiple eukaryotes [4–6]. USPs can be categorized into ATP-binding (UspFG-like) and non-ATP-binding (UspAs and UspA-like) groups [7]. Many organisms often possess multiple USPs, some may operate synergistically for cell growth and survival [8], or some may be redundant [9], all of which complicate functional studies of USPs. Despite extensive biochemical and structural studies [7, 10], the functions of most USPs remain elusive.

The first USP superfamily genes were discovered in *Escherichia coli*, including *uspA*, *uspC*, *uspD*, *uspE*, *uspF* and *uspG* [8, 11]. These *usp* genes are induced under various adverse stresses, such as nutrient starvation, UV exposure, metal stress, heat shock, and salt stress, to regulate bacterial growth [12]. Their functions are thought to be associated with ion scavenging, hypoxia responses, cellular mobility, and regulation of cell growth and development [13]. On the other hand, USPs also influence latency forms and antibiotic resistances of different pathogens, studies on their action mechanisms in survival and infection of pathogens are important to explore potential drug targets and develop novel treatments or therapeutic drugs [14]. For instance, the tolerance of *Mycobacterium tuberculosis* to amikacin (AK) and kanamycin (KM) has been enhanced by the overexpression of USP (Rv2005c), suggesting its involvement in the AK/KM resistant mechanism [15]. In silico docking analysis of metabolic enzymes from drug-resistant *M. tuberculosis* has also revealed that USP (Rv2623) is one of the targets of isoniazid [16]. Furthermore, USP domains have been found in certain important regulatory proteins such as the sensor kinase KdpD, which controls potassium transportation. The binding of cyclic Di-AMP with KdpD via its USP domain could downregulate the expression of the *kdp* operon in *Staphylococcus aureus* and reduce its survival under low potassium conditions [17]. Thus, USPs may link together external stimulation, signal transduction and transcriptional regulation to affect metabolic network. However, researches on microbial USPs have mainly focused on pathogenic bacteria, and the relationship between USPs and microbial secondary metabolism has not been addressed.

Streptomycetes are important industrial microbes capable of producing diverse bioactive natural products, particularly various antibiotics [18, 19]. Despite decades of exploration, the discovery of novel natural products and titer improvement of valuable antibiotics remain challenging research areas in streptomycetes [20]. Tiancimycins (TNMs) primarily including TNM-A and TNM-D are anthraquinone-fused ten-membered enediynes originally discovered in *Streptomyces* sp. CB03234 [21, 22]. TNMs have exhibited excellent antitumor activities and possess a great potential as novel payloads of anticancer antibody-drug conjugates (ADCs) [23, 24], but their extremely low titers have limited further development. Our team had obtained a TNMs high-producing strain *Streptomyces* sp. CB03234-S through streptomycin-induced ribosome engineering [25], and then paid many efforts to explore possible high-yield mechanisms of CB03234-S for further titer improvement of TNMs [26–28]. In this work, the whole-genome resequencing of CB03234-S was conducted, and a large terminal fragment loss was found in the genome of CB03234-S. The bioinformatic analyses of this lost gene fragment revealed a region harboring 8 USP-encoding genes that is highly expressed in the original CB3234. Therefore, the potential influences of these USPs on the secondary metabolisms of CB03234 and CB03234-S were investigated, and two candidates USP3 and USP8 which significantly affect the production of TNMs were screened. Subsequently, the USP3/USP8 orthologs were identified in *Streptomyces coelicolor* A3(2) and *Streptomyces* sp. CB00271, respectively, and these USPs also showed clear effects on the secondary metabolism of individual host. Further big data analyses suggested that the USP3/USP8 orthologs were widely distributed across streptomycetes. Therefore, our studies unveiled firstly the potential effects of USPs on secondary metabolisms of streptomycetes, and provided valuable insights and prospective targets for the exploitation of natural products in streptomycetes.

Materials and methods

Strains and culture conditions

All strains and plasmids used in this study were listed in Additional file 1: Table S1. CB03234, CB03234-S (preservation number CCTCC M2017538), CB00271 (preservation number CCTCC M2021230) and *S. coelicolor* A3(2) were reserved in our laboratory. *Escherichia coli* DH5α and S17-1 were used for cloning and intergeneric conjugation, respectively [26]. As previously reported [24, 25, 29, 30], Gauze's (G1) and ISP4 solid media were used for sporulation, tryptic soy broth (TSB) medium was used as the seed medium, optimal production (OP) medium (for CB03234 and CB03234-S), M4 medium (for CB00271) and YEME medium (for *S. coelicolor* A3(2)) were applied for fermentation of corresponding stains. Mannitol soya flour (MS) solid medium supplemented with 10 mM $MgCl_2$, was used for intergeneric conjugation. All *E. coli*

strains were cultured on Luria-Bertani (LB) agar or in LB liquid medium at 37℃. Antibiotics including 50 mg/L apramycin, 25 mg/L nalidixic acid, 50 mg/L thiostrepton and 50 mg/L kanamycin were used when necessary.

DNA manipulations and genome resequencing

Genomic DNA extraction was performed using the SteadyPure Bacterial Genomic DNA Extraction Kit (Accurate Biology, Changsha, Hunan, China). Plasmid DNA was extracted using the Plasmid Miniprep Kit (Tsingke Biotech. Co., Changsha, Hunan, China). PCR amplification of the target gene was performed using the high-fidelity Golden PCR Mix TSE101 (Tsingke). Ligation and cloning of gene fragments were conducted via the Trelief SoSoo seamless Cloning Kit (Tsingke). The constructed plasmids were validated by DNA sequencing (Tsingke) and then introduced into *E. coli* S17-1 for conjugation. The intergeneric conjugation transfer of target strain was carried out according to the standard procedures as previously described [31]. Genome resequencing of CB03234-S was conducted by Biomarker Technologies Co., LTD (Beijing, China).

Bioinformatic analysis of USPs in three different streptomycetes

Based on the genome sequence and gene functional analysis of CB03234 (NCBI accession Number: NZ_LIYH00000000.1), 8 *usp* genes were located within the lost 583 kb fragment of CB03234-S. Then *usp3* and *usp8* were applied as probes for genome mining of *S. coelicolor* A3(2) (Accession Number: AL645882.2) and CB00271 (Acession Number: NZ_CP061072.1) to determine other USPs, respectively. The Conserved Domain Search (CD-Search) tool provided by the National Center for Biotechnology Information (NCBI) was applied to identify conserved domains in USPs. By performing sequence alignment using protein Basic Local Alignment Search Tool (pBLAST), the coverage and identities of USPs were obtained, which then were adopted to construct a background heat map using the E-values from the sequence alignment. The transcriptomic data of *usp* genes in CB03234 (NCBI accession: PRJNA530700) was obtained from the previous study [26], while the transcriptomic data of *usp* genes in *S. coelicolor* A3(2) was retrieved from the microarray expression raw data in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) database [32].

Sequence similarity network analysis and *Streptomyces* based pan-genomic analysis of USPs

Using USP8 (WP_073756310.1) from CB03234 as the query, a BlastP search was conducted in the nonredundant protein sequence database of NCBI (until February 28, 2024) and the top 5000 potential USP sequences were downloaded. These 5000 USP sequences were further filtered to remove the unclassified strains and narrowed down to 4920 representative sequences, which were applied to construct the USP database using the formatdb command in Blast+. After that, each USP sequence was queried against the entire database by BlastP. The Blast E-value threshold was set at 1.0×10^{-80} to enhance the species classification accuracy of proteins. Duplicates between two nodes and self-loops within each node were removed from the network. The resulting sequence similarity network (SSN) analysis results were visualized using the organic layout in Cytoscape 3.16 [33].

As previously reported [34], a total of 167 *Streptomyces* complete genomes, including *S. coelicolor* A3(2) and CB03234, were downloaded from the NCBI Reference Sequence Database (RefSeq). The all-against-all BLASTp analysis of these genomes was performed by using blast 2.12.0 with an E value$<e^{-10}$ between each other. The orthologs of USP3, USP8, Sco0167 and Sco0200 presented in *Streptomyces* genomes were clustered and counted with SiLiX [35] when the alignment identity and coverage values (IC values) were set≥50%, 60% and 70%, respectively.

Construction of different *usp* related overexpression or knockout mutants

The target *usp* genes were respectively amplified from the genomic DNA of CB03234, *S. coelicolor* A3(2) and CB00271 using designed primers (Additional file 1: Table S2), and then integrated into pSET152 through *Xba*I and *Bam*HI sites. The resulting plasmids were then introduced into the corresponding strains to generate expected overexpression mutants, respectively. To generate *usp* knockout mutants, the flanking regions (each approximately 2.0 kb) of the target *usp* gene, along with the antibiotic marker (919 bp thiostrepton resistance gene *tsr* or 1490 bp kanamycin resistance gene *kan*) controlled by the constitutive promoter *kasOp**, were amplified using designed primers (Additional file 1: Table S2). These fragments were ligated together and cloned into pOJ260 through *Xba*I and *Hin*dIII sites to generate the corresponding knockout plasmid, and validated by DNA sequencing (Tsingke). After conjugation transfer, kanamycin-resistant (Kan^R) or thiostrepton-resistant (Tsr^R) but apramycin-sensitive (Apr^S) exconjugants were screened and then verified by PCR amplification to finally obtain the desired knockout mutants.

Fermentation and analysis of secondary metabolites

According to the reported procedures, the fermentations of strains and mutants related to CB03234 or CB03234-S [25], *S. coelicolor* A3(2) [30] and CB00271 [29] were conducted in OP medium, YEME medium and M4 medium, respectively. All experiments were carried out

in triplicate. The cultivation, post-processing and sample preparation procedures were executed as described previously. Samples were filtered through a 0.22 μm organic Millipore membrane syringe filter and analyzed using the Waters E2695 HPLC system equipped with a photodiode array detector and a Welch AQ-C18 column (5 μm, 250×4.6 mm, Welch Materials Inc., Shanghai, China). The mobile phase consisted of buffer A (ultrapure H_2O containing 0.1% HCOOH) and buffer B (chromatographic grade methanol) at a flow rate of 1 mL/min. For the detection of TNMs at 540 nm and the detection of actinorhodin intermediate (S)-DNPA at 254 nm, a gradient program (90% buffer A from 0 to 1 min; 90% buffer A to 5% buffer A from 1 to 15 min; 5% buffer A from 15 to 17 min; 5% buffer A to 90% buffer A from 17 to 23 min; 90% buffer A from 23 to 25 min) was applied. For the detection of β-rubromycin (β-RUB) at 254 nm, another gradient program (95% buffer A from 0 to 1 min; 95% buffer A to 5% buffer A from 1 to 15 min; 5% buffer A from 15 to 17 min; 5% buffer A to 95% buffer A from 17 to 21 min, followed by 95% buffer A from 21 to 25 min) was applied.

To assess the production of actinorhodin (ACT), 1 mL of fermentation broth was centrifuged to obtain the supernatant and the pellet. The supernatant was adjusted to pH 12 using 1 M sodium hydroxide (NaOH), and the concentration of ACT was determined by measuring the absorbance at 640 nm. For intracellular ACT analysis, the pellets were washed twice with 0.1 M hydrochloric acid (HCl) to reduce impurities, and ultrasonically extracted with 1 mL of methanol. The extract was then mixed with 1 M NaOH to adjust the pH to 12, and the absorbance was measured at 640 nm. The ACT concentration (C) in mg/L was calculated according to the Lambert-Beer law $A=K\cdot C\cdot l$, where A represents the absorbance, K represents the molar absorptivity (L/g·cm), defined as $K=\varepsilon_{640}/M_{ACT}$ (ε_{640} is the molecular extinction coefficient, 25,320 /Mol·cm), M_{ACT} is the relative molecular mass of ACT, 633.12 g/Mol·L), and l is the thickness of the absorption layer (set at 1 cm) [36].

Results and discussion

Whole-genome resequencing revealed a distinct 583 kb terminal deletion in CB03234-S

The genome of CB03234-S was resequenced and compared with its parental strain CB03234 (Fig. 1a), and two notable fragment deletions were found in the genome of CB03234-S. The 1.8 kb deletion located in *rsmG* (encoding 16 S rRNA-methyltransferase) was previously determined to be associated only with the streptomycin resistance of CB03234-S, but not with the titer improvement of TNMs [31]. Another unique 583 kb terminal deletion harbored seven secondary metabolic biosynthetic gene clusters (BGCs) and resulted in a 7.5% reduction of the whole genome of CB03234-S (Fig. 1a). Based on the previous transcriptome data of CB03234 [26] (Fig. 1b), most genes in the 583 kb lost fragment showed much lower transcription levels compared to the internal housekeeping gene *hrdB* (encoding a principal σ factor), while the highly expressed genes were mainly clustered in a 40 kb region. Further analysis showed that this region contained 8 scattered *usp* genes (named *usp1* to *usp8*) (Fig. 1c), most of which except *usp8* exhibited comparable or much higher transcription levels than *hrdB* (Additional file 1: Fig. S1a). To gain further insights into the 8 USPs, their protein sequence alignments and conserved domain (CD) analyses were conducted (Additional file 1: Fig. S1). These USPs were similar to the characterized USPs in *M. tuberculosis*, all possessed two repeat USP domains belonging to the adenine nucleotide alpha hydrolases superfamily (AANH_like superfamily) (Additional file 1: Fig. S1c), which could form an apha/beta/apha fold and bind to adenosine nucleotides to respond to various stress responses. The 8 USPs presented high coverage (ranging from 80 to 100%) but comparably low identity (ranging from 30 to 50%) with each other (Additional file 1: Fig. S1b), and their diversities were mainly

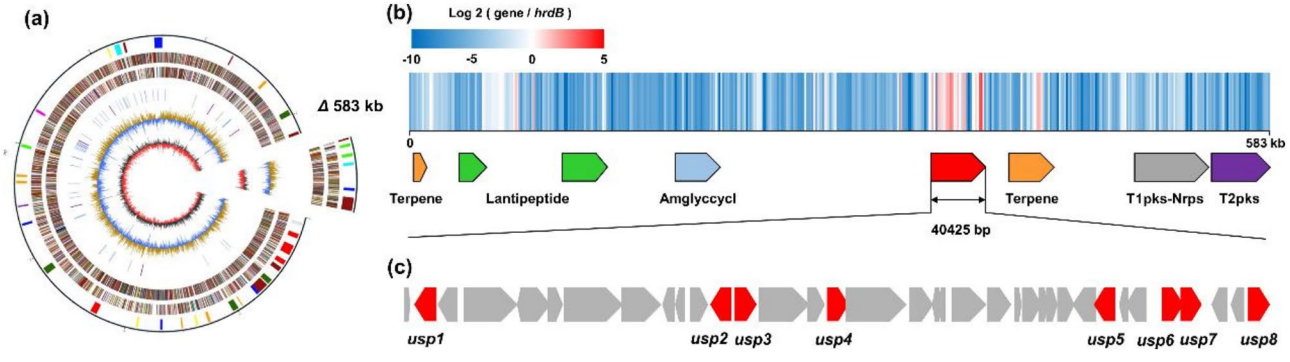

Fig. 1 Genome resequencing analysis of CB03234-S and bioinformatic analysis of the 583 kb terminal deletion. (**a**) Genome circle map of CB03234-S. (**b**) Transcriptional heat map and biosynthetic gene cluster annotation of the corresponding 583 kb fragment in CB03234. (**c**) 8 scattered *usp* genes (named *usp1* to *usp8*) were identified in the relatively high transcription 40 kb region

reflected in five variable link regions (Additional file 1: Fig. S1d). Therefore, we hypothesized that these USPs may serve distinct functions in CB03234, and their losses could exert potential influences on the metabolism of CB03234-S.

The discovered USPs showed differential effects on the secondary metabolism of CB03234-S

To assess the potential metabolic impacts of USPs, each *usp* gene was overexpressed in CB03234-S, resulting 8 mutants named S-*usp1* to S-*usp8*. During fermentation, a distinct purple color change was observed in the fermentation broth of S-*usp7* and S-*usp8*, while other mutants displayed similar characteristics to CB03234-S (Fig. 2a).

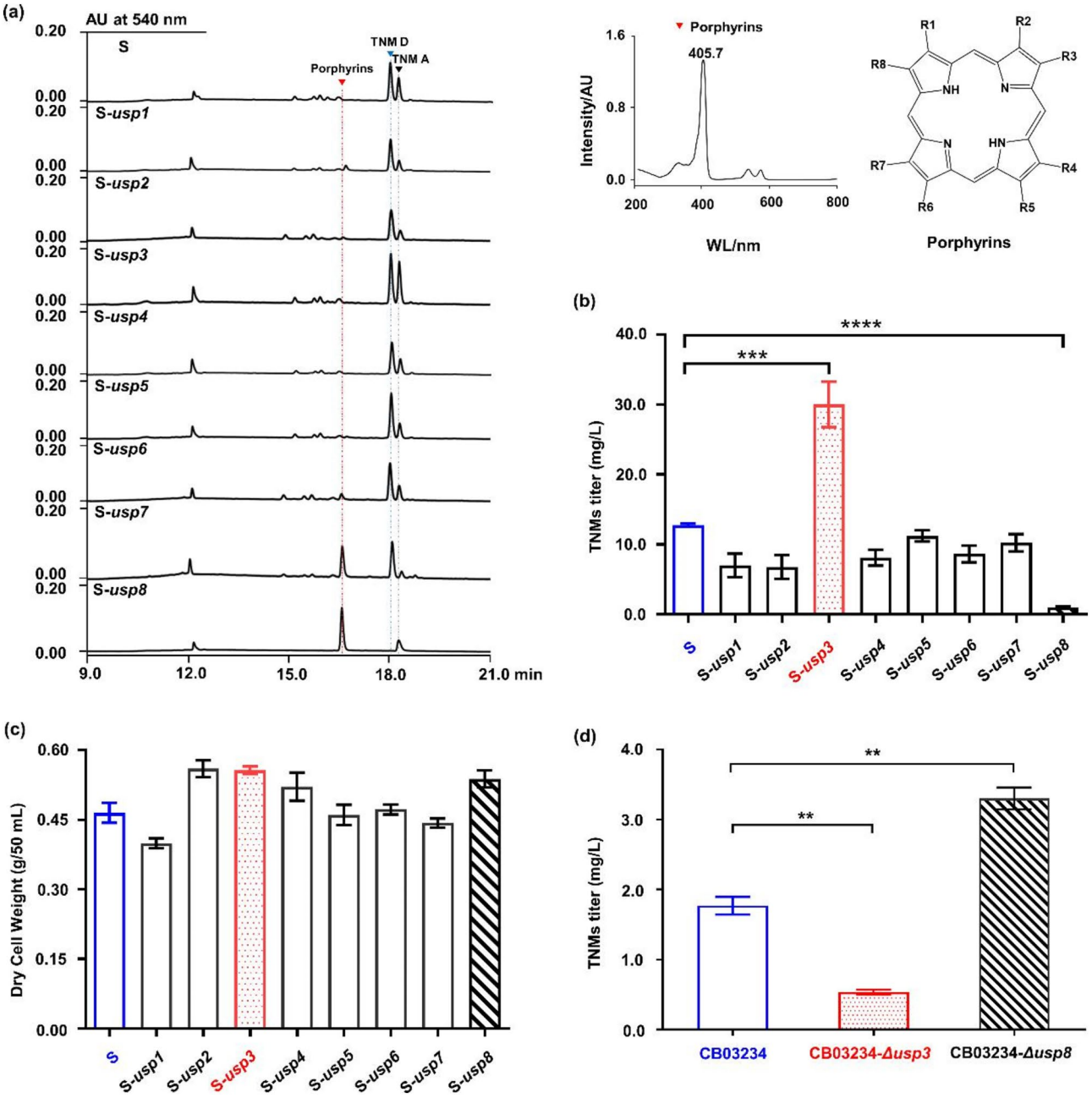

Fig. 2 Fermentation results of the corresponding strains and mutants. (**a**) HPLC analysis of CB03234-S (S) and its USP overexpression mutants in OP medium (left), ultraviolet spectrum and structure of porphyrin-type compounds (right). (**b**) The comparison of TNMs titer from CB03234-S (S) and its USP overexpression mutants. (**c**) The comparison of dry cell weight from CB03234-S (S) and its USP overexpression mutants. (**d**) The TNMs titer of CB03234-*Δusp3* and CB03234-*Δusp8* in OP medium (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.0001$)

The metabolites analyses suggested that the appeared purple color was due to the presence of porphyrin-type compounds, identified by their characteristic UV absorption at 405 nm [37] (Fig. 2a). Notably, the overexpression of *usp8* led to a drastic reduction in the TNMs titer, decreasing from the original 12.8±0.2 mg/L to merely 1.0±0.1 mg/L, with porphyrin-type compounds becoming the dominant metabolites. In contrast, the overexpression of *usp3* significantly increased the titer of TNMs to 31.1±2.3 mg/L (Fig. 2b). The other *usp* mutants showed varying degrees of reduction in the TNMs titer, ranging from 12 to 50% (Fig. 2b). Further analysis of the fermentation broth indicated that the dry cell weights of mutants, except for S-*usp1*, were comparable to or even higher than that of CB03234-S, suggesting the changes of the TNMs titer among the mutants were unrelated to their biomasses (Fig. 2c).

To further validate the effects of *usp3* and *usp8* on TNMs biosynthesis, each candidate gene was inactivated in CB03234 via homologous recombination (Additional file 1: Fig. S2a). No significant changes were found in the metabolic profiles of either knockout mutant, except for the changes of TNMs titer (Additional file 1: Fig. S2b). Compared to the original TNMs titer of 1.8±0.1 mg/L in CB03234, the TNMs titer decreased to 0.5±0.0 mg/L in CB03234-*Δusp3*, whereas it increased to 3.3±0.1 mg/L in CB03234-*Δusp8* (Fig. 2d). The overexpression and knockout experiments suggested that USP3 and USP8 played major but opposite roles in TNMs production, with USP8 exerting a distinct negative effect. Since the biosynthesis of porphyrins is closely related to the tricarboxylic acid cycle (TCA cycle) [38], and shares a common precursor acetyl-CoA with TNMs, we proposed that USP8 may regulate metabolic flux to enhance the central carbon metabolism, thereby restricting TNMs production. The silence of porphyrins production in CB03234 could be attributed to the low expression level of USP8 and the more than 7-fold higher expression level of USP3 (Additional file 1: Fig. S1a). Thus, the overexpression of USP8 in CB03234-S without USP3 significantly activated the biosynthesis of porphyrins and substantially reduced TNMs production. Since only USP3 showed a promotional effect, the loss of 8 USPs (especially USP8) overall made a positive contribution and increased TNMs production in CB03234-S. Furthermore, the subsequent complementation of USP3 could further enhance TNMs titer in CB03234-S. However, the regulatory mechanisms of USP3 and USP8 on TNMs production, as well as the effects of other USPs in CB03234 remain to be verified. In summary, these results revealed for the first time potential regulatory influences of USPs on the secondary metabolism of streptomycetes, and suggested a novel route to improve titers of TNMs and other anthraquinone-fused ten-membered enediynes.

Validating influences of USPs on the secondary metabolisms of other streptomycetes

The changes of TNMs titer causing by USP3 and USP8 inspired us to explore the potential regulatory effects of their orthologs in other streptomycetes. The model strain *S. coelicolor* A3(2) commonly used for growth and metabolic studies, and the characteristic CB00271 with the high production of β-rubromycin (β-RUB) found in our lab [29], were selected for investigations. Through the genome mining using USP3 and USP8 as probes, 9 USPs (Sco0167, 0172, 0180, 0181, 0198, 0200, 0207, 7247 and 7299) were identified in *S. coelicolor* A3(2), and 6 USPs (271USP1 to USP6) were found in CB00271. CD analysis indicated that Sco0172 and Sco0207, as well as 271USP1 to USP5, all had only one USP domain (Additional file 1: Fig. S3). The rest of USPs were respectively compared with USP3 and USP8 through sequence alignment (Additional file 1: Fig. S4a), and the E-values of the alignment results were utilized to generate heat maps indicating the level of identity (Additional file 1: Table S3). 271USP6 showed a moderate identity of less than 40% to both USP3 and USP8 and was therefore regarded as their common ortholog. On the other hand, Sco0167 showed the highest identity of 48% to USP8, while Sco0200 and Sco7299 both showed the highest identity of 45% to USP3. The time-dependent transcriptional changes of the remaining 7 *usp* genes in *S. coelicolor* A3(2) were then extracted from the Gene Expression Omnibus database (GEO accession number: GSE18489), and only *sco0167*, *sco0180* and *sco0200* consistently showed expression levels comparable to or higher than that of the reference *hrdB*, of which the level of *sco0200* was the highest (Additional file 1: Fig. S4b). Consequently, Sco0200 and Sco0167 were determined as the USP3/USP8 orthologs, respectively.

Referring to the regulatory effects of USP3 and USP8 in CB03234, *sco0200* was overexpressed in *S. coelicolor* A3(2), while *sco0167* was knocked out (Additional file 1: Fig. S5a). The fermentation results indicated that the production of the representative metabolite actinorhodin (ACT) was completely suppressed by the overexpression of Sco0200 (Additional file 1: Fig. S6a), while the deletion of Sco0167 increased the ACT titer from the original 79.8±2.3 to 108.0±3.7 mg/L (Fig. 3a). In addition, a distinct peak was eluted at 16.621 min in the HPLC profile of *S. coelicolor* A3(2)-*Δsco0167* (Fig. 3b). HRESIMS analysis of this substance identified a molecular ion peak corresponding to $C_{16}H_{14}O_5$ (m/z $[M+H]^+$ calcd for $C_{16}H_{15}O_5$, 287.2800, found 287.0883) (Additional file 1: Fig. S6b), and subsequent UV spectral characterization (Additional file 1: Fig. S6c) revealed it to be the ACT intermediate (S)-DNPA [39]. Similarly, the overexpression of 271USP6 in CB00271 also abolished the productions of the major metabolite β-RUB, as well as the

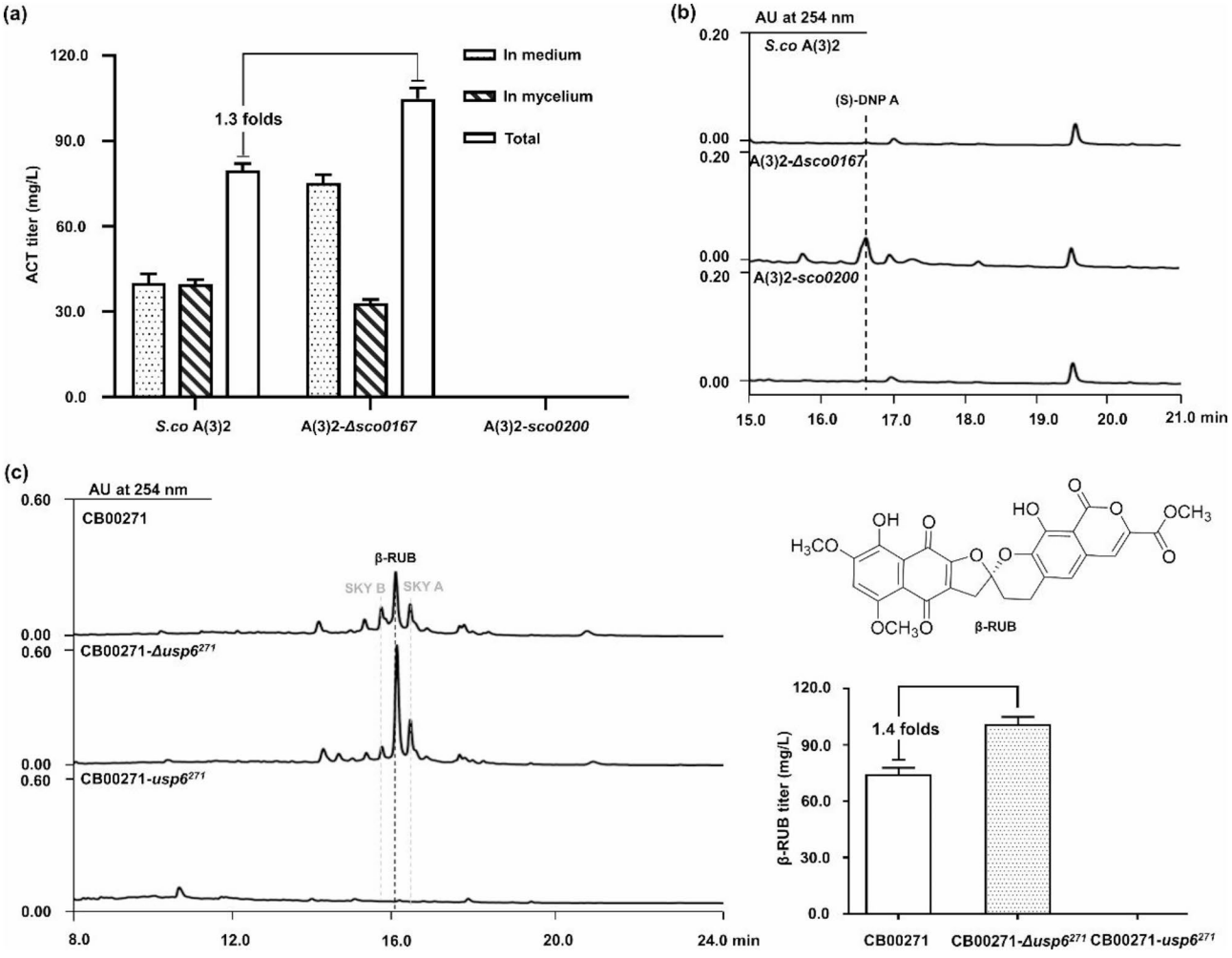

Fig. 3 Effects of USPs on the secondary metabolism of *S. coelicolor* A3(2) (*S. co* A3(2)) or CB00271. (**a**) The comparison of ACT titer from *S. co* A3(2), *S. co* A3(2)-*sco0200* and *S. co* A3(2)-*Δsco0167* in YEME medium. (**b**) HPLC profiles of fermentation extracts from *S. co* A3(2), *S. co* A3(2)-*sco0200* and *S. co* A3(2)-*Δsco0167*. (**c**) HPLC profiles of fermentation extracts from CB00271, CB00271-*Δusp6*^{271} and CB00271-*usp6*^{271} in M4 medium (left), and the comparison of their β-RUB titer (right)

minor metabolites such as skyllamycins (SKY), whereas the deletion of 271USP6 increased the β-RUB titer from 74.4±3.4 to 101.4±3.8 mg/L (Fig. 3c). The above results suggested that the USP3/USP8 orthologs could also affect secondary metabolisms in other different streptomycetes.

In comparison, the regulatory influence of USP3 in CB03234 did not appear consistent with the effects of its orthologs in other streptomycetes, while the influence of USP8 and its orthologs was consistent. Since USPs can respond to various stresses and enable cell self-adjustment for survival [40], we speculated that USP3 and USP8 could redistribute metabolic fluxes and induce more energy consumption such as ATP and NADPH to cope with stresses, thereby affecting the secondary metabolism. Taking USP8 as an example, its overexpression probably enhanced the flux of acetyl-CoA towards the TCA cycle, so the production of porphyrins was activated to support this metabolic change, as porphyrins have been reported to be involved in the transport and utilization of oxygen and facilitate the respiration [41]. These metabolic changes strongly shunted CoA precursors and restricted TNMs production. Our previous studies revealed that TNMs production is orchestrated by a pathway-specific cascade regulatory network, which was proposed to be activated by reduced intracellular ATP levels [27]. Therefore, USP3 might induce the increased consumption of intracellular energy such as ATP, thereby strengthening the biosynthesis of TNMs. In contrast to TNMs, the biosynthesis of ACT is primarily regulated by the supply of CoA precursors and required sufficient energy [42], which explains why both Sco0167 and Sco0200 exhibited similar trends in regulating the production of ACT. Overall, our findings suggested that USPs, such as USP3, USP8 and their orthologs, may serve as new regulatory targets for manipulation of secondary metabolisms in streptomycetes.

Sequence similarity network and pan-genomic analyses revealed a wide distribution of USP3/USP8 orthologs in *Streptomyces*

Next, the SSN analysis was carried out to investigate the possible species distribution of USP3/USP8 orthologs (Fig. 4a). Among the 4920 screened USP sequences, most originated from *Actinomycetes*, of which 85.7% belonged to *Streptomyces*, suggesting a conservation of USP3/USP8 orthologs in streptomycetes. The visualization of SSN analysis result also illustrated a difference between USP3 and USP8, in which USP8 and its ortholog Sco0167 were gathered together and formed the largest cluster, while USP3 and its ortholog Sco0200 were gathered in another separate cluster. Interestingly, 271USP6 did not cluster with any other candidate and appeared independently, which coincided with its relatively low identity to both USP3 and USP8, and might hint its evolutionary divergence from other USPs. Subsequent pan-genomic analyses of USP3, USP8, Sco0167 and Sco0200 were respectively conducted (Fig. 4b) in 167 *Streptomyces* genomes collected from our previous work [34]. Due to the low identities among four USP candidates (less than 50%, Additional file 1: Table. S3), when the alignment identity and coverage value (IC value) was set less than 50%, the representative USPs and their orthologs were indistinguishable and presented in all 167 *Streptomyces* genomes (data not shown). At the IC_50% criterion, the presences of each candidate USP or its orthologs in

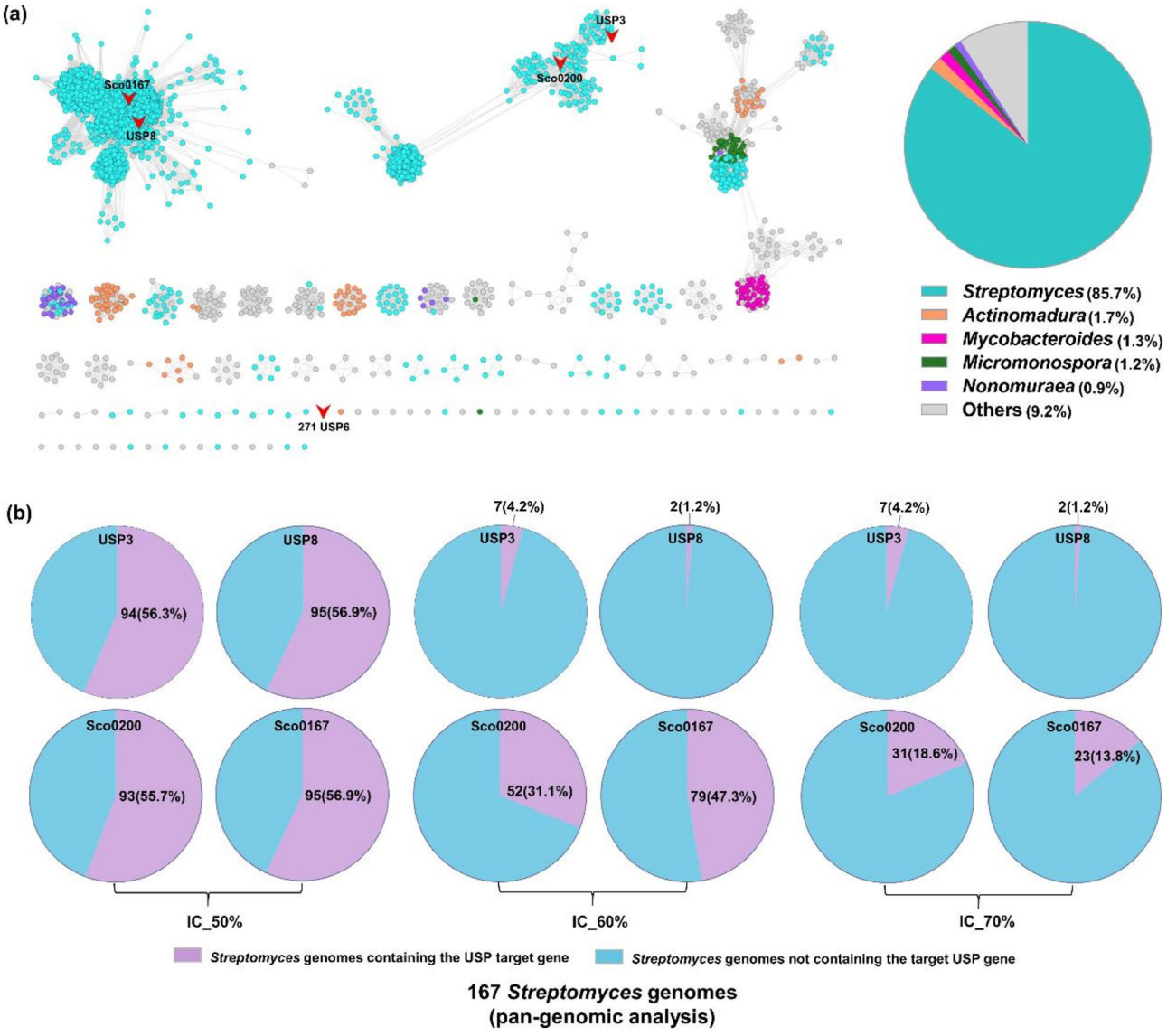

Fig. 4 (**a**) SSN analysis of USP3, USP8 and their orthologs in the NCBI database (4920 sequences, E value = 1×10^{-80}), as well as their taxonomic distributions. (**b**) Pan-genomic analysis of representative USP3, USP8, Sco0167 and Sco0200 in 167 *Streptomyces* genomes under different alignment identity and coverage values (IC values)

Streptomyces genomes were all over 55% and very similar. However, the presences of USP3 or its orthologs and USP8 or its orthologs were all drastically reduced to less than 5% when the IC value was set at 60%. On the contrary, the presences of Sco0167 or its orthologs and Sco0200 or its orthologs under the same criterion remained around 47% and 31%, respectively. Even at the IC_70% criterion, the presences of Sco0167 or its orthologs and Sco0200 or its orthologs were still over 13%. Therefore, we concluded that these USP orthologs were widely distributed in streptomycetes, while Sco0167 and Sco0200 were more conserved than USP3 and USP8 in *Streptomyces* genomes, and could serve as references to search and identify potential USP targets for manipulation of secondary metabolisms in streptomycetes.

Conclusions

In conclusion, our findings revealed for the first time the important role of USPs, especially USP3, USP8 and their orthologs, in modulating secondary metabolisms of streptomycetes. We speculated these USP candidates could redistribute metabolic fluxes and increase more energy consumption to cope with stresses, and thus substantially altered secondary metabolisms. SSN and pangenomic analyses suggested that these representative USPs and their orthologs are widely distributed across streptomycetes. Hence, USPs could become novel targets for synthetic biologic studies of streptomycetes, not only shedding new insights on deciphering metabolic networks in different streptomycetes, but also providing alternative ways to explore and exploit various natural products.

Abbreviations

USP	Universal stress protein
TSB	Tryptic soy broth seed medium
LB	Luria-Bertani medium
G1	Gauze's solid medium
MS	Mannitol soya flour solid medium
P	Original production medium
OP	Optimal production medium
ADCs	Antibody-drug conjugates
TNM	Tiancimycin
ACT	Actinorhodin
RUB-β	β-rubromycin
SKY	Skyllamycin
SSN	Sequence similarity network
TCA	Tricarboxylic acid
BGC	Biosynthetic gene cluster
CD	Conserved domain

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions

ZXC and DYW conceived and designed this project. LHM, FZY and TN contributed to the various mutants and fermentation validation. FZY and LJ conducted genetic characterization and analysis. LHM and ZXC analyzed data. LHM and ZXC co-wrote the manuscript. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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