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GENETIC ANALYSIS OF SULFATE ASSIMILATION GENE CLUSTER OF *STREPTOMYCES COELICOLOR* A3(2)

Aim. *Streptomyces coelicolor* A3(2) is the best studied species within this bacterial genus. Biosynthesis of specialized (secondary) metabolites by *Streptomyces* is of special interest. Primary metabolism, where all the precursors of specialized metabolites come from, is also studied in great detail. There are glaring gaps in our knowledge of sulfur metabolism in this species. We took genetic approach to probe the function of several genes within presumed sulfate assimilation gene cluster of *S. coelicolor* A3(2). ***Methods***. Microbiological and genetic approaches were combined to generate mutants and to study their properties. ***Results***. Sulfate assimilation gene cluster is structurally and functionally similar to that of phylogenetically close *Corynebacterium*. Most of the generated knockout strains behaved as would be expected from their molecular function inferred *in silico*. This confirms their involvement in sulfate uptake/conversion. Knockout of gene *sco6101* (having no homologs from the other bacterial sulfate assimilation operons) impaired the growth on inorganic sulfur species and L-cysteine, pointing to its association with sulfur metabolism. ***Conclusions***. Our study provides experimental evidence for the involvement of *sco6093-sco6102* segment in sulfate assimilation, and also reveals novel gene, *sco6101*, essential for sulfur cycle. Further efforts are needed to elucidate the mechanism of Sco6101 action.

Keywords: *Streptomyces coelicolor* A3(2), genetics of sulfur metabolism, sulfate assimilation.

Streptomyces is the largest genus of class Actinomycetes famous for the ability to synthesize a vast number of specialized (secondary) metabolites. Much effort has been made to understand the genetics of specialized metabolism. Less attention has been paid to the pathways and the regulation of primary metabolism, although they are the source of energy and precursors for the SM. This is especially true about sulfur metabolism [1]. Sulfur is one of the macroelements and a part of the amino acids cysteine and methionine, which in turn serve as precursors for many peptide antibiotics (glycopeptides, beta-lactams and thiopeptides to name just few) in streptomycetes. Another reduced sulfur-containing metabolite, coenzyme A (CoA), is heavily utilized for lipid metabolism, which is a branching point for production for many lipid- and polyketide-containing natural products. There is an extensive body of data on sulfur metabolism in other actinomycetes (e.g. *Corynebacterium*, *Mycobacterium*) [2], but these bacteria are distantly related to streptomycetes and harbor distinct gene sets, as will be detailed below. The situation is further exacerbated by the fact that actinobacteria carry several alternative pathways for assimilation of sulfur compounds and production of sulfur-containing amino acids [3]. In this regard, we showed previously that knockouts of two paralogous genes for rhodanese-like proteins in model species *S. coelicolor* A3(2) led to distinct phenotypes. The *sco4164* deletion blocked growth on sulfate and sulfite as a sole sulfur source, while *sco5854* knockout only suppressed production of antibiotic actinorhodin [4]. Our knowledge of sulfur metabolism in antibiotic-producing streptomycetes is very fragmentary and outdated [5] given the current advances in genomics and understanding of reduced S species in streptomycete physiology [6]. In this work we focused on a segment of A3(2) genome encompassing genes *sco6093-sco6102* bearing significant similarity to sulfate assimilation operon of *Corynebacterium glutamicum* ATCC13032. We describe functions of these *sco* genes on combining the results of bioinformatics and gene knockouts. Our results unveil the genetic control of sulfate uptake and novel role of gene *sco6101* in production of methionine by *S. coelicolor* A3(2).

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Materials and methods

S. coelicolor M145, a prototrophic variant of A3(2) [4], was used throughout the work. Cosmid 3F08 carrying *sco6093-sco6102* stretch was kindly provided by Prof. M. Bibb (John Innes Centre, UK). Genome browser at StrepDB database https://strepdb.streptomyces.org.uk__was used to locate and retrieve the sequences of *sco* genes. Integrative vector pIJ6902 [4] was used to construct complementation plasmids. The *sco* genes of interest were individually replaced with apramycin resistance cassette *oriT-aac(3)IV* from plasmid pIJ774 using recombineering approach [7]. Below is the brief description of *sco6101* replacement in M145; the same approach (with a different set of primers) was used to generate the other knockouts. The *oriT-aac(3)IV* was amplified with primers 6101red_up (GCCTGGGCGGCCCGGGCC TCCGAGGAGGCCCTGTCGTGAGATTCCGGG GATCCGTCGACC) and 6101red_rp (GA GTTCCTCGGTCGTACGCTCTTCCTGAACCGC CGTCATATGTAGGCTGGAGCTGCTTC). The generated cosmid 3F08Δ6101 was PCR-verified with primers flanking the deletion (SK72-6101-k-for (AAAGAATTCCACGTCCATCACGGCCTC) and SK72-6101-k-rev (AAAGGATCCTCTG-CAAGCTCGCCATCGTC)), alone or in combination with the primers to internal *aac(3)IV* sequences. The cosmid was transferred conjugally into M145 from *E. coli* ET12567 (pUZ8002) [4]; transconjugants were selected for apramycin resistance and kanamycin sensitivity (double crossover, loss of vector sequences). Strain verification was done as described above for the cosmid. Marker gene *oriT-aac(3)IV* was excised from replacement site with the help of recombinase aCre expressed from plasmid pUWLCre, as described in [8]. In this way we generated eight *S. coelicolor* mutants: Δ*sco6094*, Δ*sco6095*, Δ*sco6096*, Δ*sco6097*, Δ*sco6098*, Δ*sco6099*, Δ*sco6100* and Δ*sco6101*. For growth test we used minimal medium [4] supplemented with various sulfur sources (final concentration of 2 mM), as in [2]. Standard bioinformatics tools such as BLASTP, HHpred, TMHMM, STRING were used to mine for M145 sulfur metabolism genes and predict their function.

Results and discussion

As previously noted [2], the *sco6093-sco6102* segment of M145 genome bears significant similarity to known sulfate assimilation operons of the other bacteria (Table 1).

The M145 and *C. glutamicum* sulfate assimilation genes being similar in terms of genetic organization (Fig. 1) show a few differences. In M145 these genes likely form a cluster, not an operon as in *C. glutamicum*. M145 has no gene *cysZ* (Fig. 1) for sulfate uptake. Instead, a set of M145 genes, *sco3704-3705* and *sco3453,* were detected showing similarity to *cysPUWA-sbp* (for sulfate/thiosulfate transporters) of *E. coli* and *cysTWA-subI* genes of *Mycobacterium tuberculosis*. They are not clustered with M145 *sco6093-6102* cluster. Yet, the latter harbors *sco6096-6094*, homologs of the *ssuABC* genes of *C. glutamicum* involved in sulfonate uptake. Genes beyond *sco6093-6102* stretch do not seem to show any association with sulfur cycle. An exception is the gene *sco6103*, frequently annotated as *cysE* responsible for L-serine acetylation. This gene shows no similarity to studied *cysE* genes of *E. coli* or *M. tuberculosis* and most likely is not involved in sulfur metabolism.

Table 1. Functional annotation of *S. coelicolor* M145 genes *sco6093 – sco6102*

Gene	Deduced protein function	*C. glutamicum* ortholog[1]	ID/SI aa, %
sco6102	Nitrite/sulfite reductase	*cg3118* (*cysI*)	24%/40%
sco6101	Hypothetical protein	*cg3117* (*cysX*)	50%/57%
sco6100	PAPS reductase	*cg3116* (*cysH*)	33%/50%
sco6099	Adenylylsulfate kinase	*cysC E.coli*[2]	41%/57%
sco6098	Sulfate adenylyltransferase subunit 2	*cg3115(cysD)*	47%/65%
sco6097	Sulfate adenylyltransferase subunit 1	*cg3114* (*cysN*)	46%/61%
sco6096	Sulfonate ABC transporter substrate binder	*cg1380 (ssuA(*	25%/46%
sco6095	Sulfonate ABC transporter ATPase	*cg1379 (ssuB)*	36%/53%
sco6094	Sulfonate ABC transporter permease	*cg1377 (ssuC)*	32%/46%
sco6093	Putative sirohydrochlorin ferrochelatase	*cg3113* (*cysY*)	30%/46%

Notes: [1] reciprocal best BLASTP hit (*E* value < 3×10^{-9}), [2] No *C. glutamicum* homolog for Sco6099, *E. coli* ortholog was compared instead.

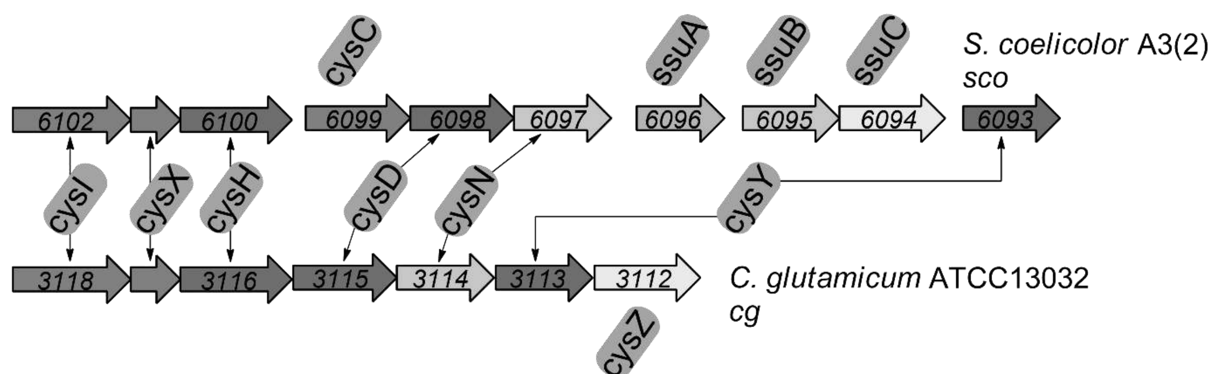

Fig. 1. Genetic organization of putative sulfate assimilation gene cluster of *S. coelicolor* M145 in comparison with its counterpart from *C. glutamicum*. Numbers on the filled arrows correspond to gene notations in respective genomes (e.g. *6102* is *sco6102*, as described in StrepDB). Trivial gene names are shown on the background (e.g. *sco6101* and *cg3117* are *cysX* orthologs); orthologous genes are connected with thin bidirectional arrows; unique genes are labeled below and above the filled arrows. Genes are drawn not to scale; abutting arrows mark transcriptional coupling of the genes.

C. glutamicum gene *mcbR* for TetR type repressor was shown to regulate the expression of genes responsible for sulfur conversion. In the *mcbR* regulon, two other regulatory genes were extensively studied: *cysR* and *ssuR*. These genes control sulfate and sulfonate utilization in *C. glutamicum* [2]. In M145 genome *sco4454* and *sco0794* are orthologs of *mcbR* and *cysR*, respectively. No *ssuR* homolog has been detected in M145. Hence, the sulfonate metabolism in streptomycetes is regulated either differently from known precedents, or it is controlled by *cysR*-like gene(s).

We knocked out eight of the genes from sulfate assimilation gene cluster of M145, and checked their growth on solid sulfur-defined media. These data are summarized in Table 2.

Overall, our data agree with the accepted route from sulfate to sulfur-containing amino acids [5], where presence of ATP sulfurylase and separate adenylylsulfate kinase was postulated (see also Table 1). There were two discoveries. Knockouts of *sco6094*, *sco6095* and *sco6096* prevented the growth on sulfate-supplemented media, revealing their involvement in sulfate uptake. The fact that the other inorganic sulfur species restored the growth of *sco6094-6096* mutants implies that M145 has the other genes for import of these (non-sulfate) ions. Knockout of *sco6101* was an illuminating one as well. Sco6101 is a short (59 aa long) protein of unknown function, whose orthologs are omnipresent across sulfate operons of actinomycetes. In *C. glutamicum* both sulfide and L-cysteine restored the growth of the mutant carrying the deletion of *cg3117* (*cysX* ortholog, see Fig. 1) [2]. Only methionine was able to restore the growth of Δ*sco6101*, pointing to a different role of this gene in M145. Our current understanding of sulfate metabolism in *S. coelicolor* is shown in Fig. 2.

Table 2. Growth of *S. coelicolor* M145 mutants on sulfur-defined media

Strain \ Sulfur	SO_4^{2-}	SO_3^{2-}	S^{2-}	$S_2O_3^{2-}$	L-Cysteine	L-Methionine
Δ*sco6094*	-[1]	+[2]	+	+	+	+
Δ*sco6095*	-	+	+	+	+	+
Δ*sco6096*	-	+	+	+	+	+
Δ*sco6097*	-	+	+	+	+	+
Δ*sco6098*	-	+	+	+	+	+
Δ*sco6099*	-	+	+	+	+	+
Δ*sco6100*	-	+	+	+	+	+
Δ*sco6101*	-	-	-	-	-	+

Note. [1] No visible growth after 120h; [2] Abundant growth after 72 h.

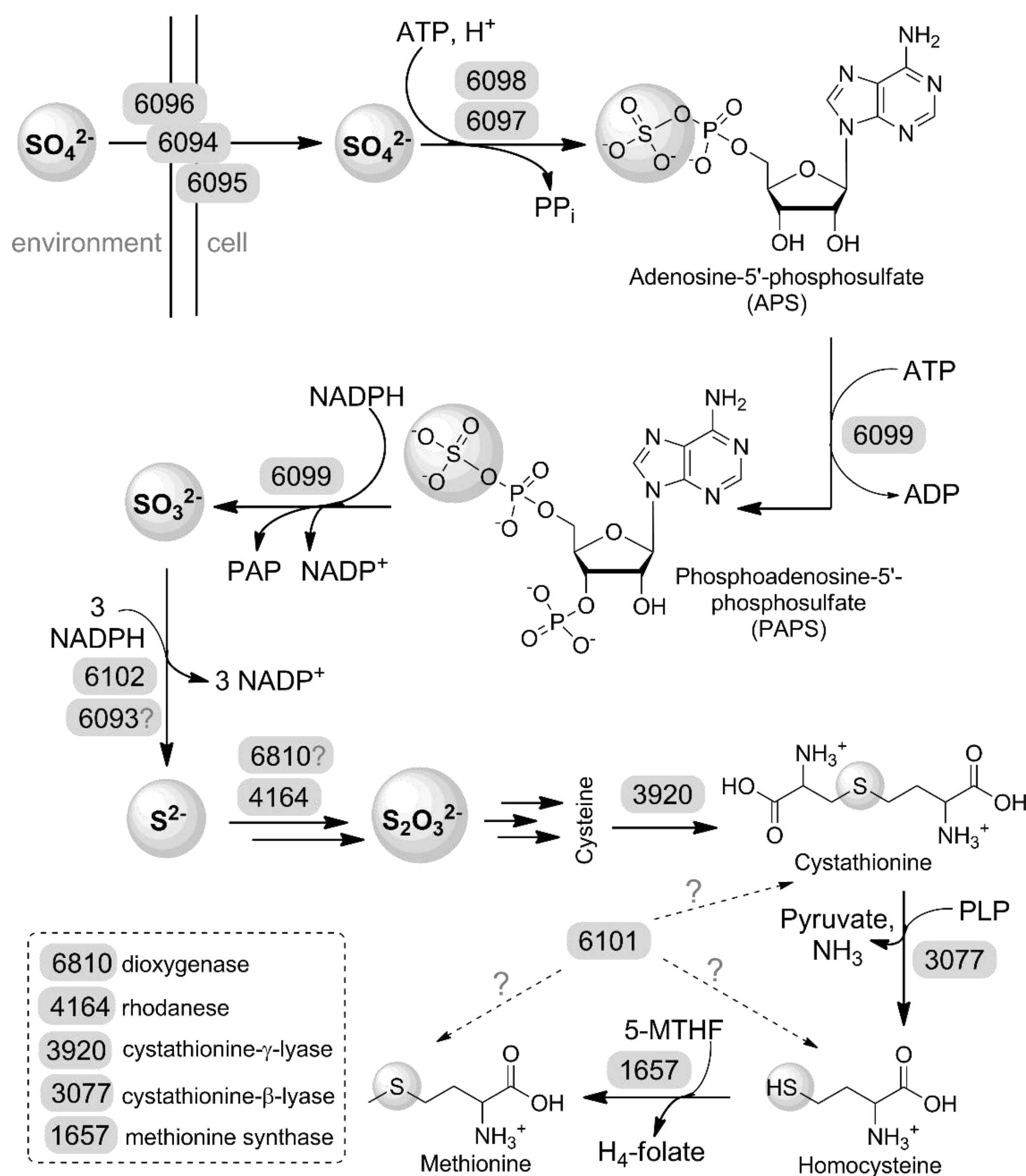

Fig. 2. A proposed pathway from sulfate uptake to methionine in *S. coelicolor* M145. M145 enzymes are shown as numbers (*e.g.*, 6094 = Sco6094; see Table 1) on rectangular background. Sulfur moiety is shown on circular background. Doubled arrows mark more than one step in the pathway. Interrogation sign marks those biosynthetic steps where the involvement of the given protein remains hypothetical. Dashed arrows around Sco6101 point to those sulfur-containing compounds whose assembly might depend on Sco6101. Graphical legend (bottom left corner) provides functions of the proteins that are not part of *sco6093-6102* gene cluster; please see Table 1 for the function of the latter.

Conclusions

Function of a single gene *S. coelicolor* A3(2), *sco6102* for sulfite reductase, has been experimentally verified so far [9]. In this work we provide first experimental evidence of the involvement of eight more genes from *sco6093-sco6102* cluster in sulfate assimilation. While participation and role of some of the genes in this process might be reliably inferred from bioinformatic predictions, the identity of genes behind sulfate uptake and association of *sco6101* with sulfur cycle remained obscure. Here we show that *sco6094-6096* genes are involved in sulfate import. Our results also point to the existence of the other genes for uptake of non-sulfate ions. We also revealed that *sco6101*, in as-yet-unknown way, is essential for production of methionine. We do not know yet which sulfur-containing amino acid intermediate to methionine depends on

Sco6101 (Fig. 2). Remote structural homology detection server HHPred reveals similarity between Sco6101 and archaeal amino/thiol ligase LysW [10], suggesting mechanistic parallels. Examination of this and other hypotheses is a subject of ongoing research in our laboratories.

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ГЕНЕТИЧНИЙ АНАЛІЗ КЛАСТЕРА ГЕНІВ АСИМІЛЯЦІЇ СУЛЬФАТУ В *STREPTOMYCES COELICOLOR* A3(2)

Мета. *Streptomyces coelicolor* A3(2) – найбільш вивчений вид, яки й є фундаментальним для розуміння біології цього роду бактерій. Біосинтез стрептоміцетами спеціалізованих (вторинних) метаболітів викликає особливий інтерес. Активно вивчається також і їхній первинний метаболізм, який є джерелом попередників спеціалізованих метаболітів. Наразі є великі прогалини у розумінні метаболізму сполук сірки в цьому виді. Ми застосували генетичні методи для дослідження функцій низки генів з імовірного генного кластера асиміляції сульфату в *S. coelicolor* A3(2) M145. ***Методи***. Ми застосували мікробіологічні та генетичні підходи для отримання і вивчення мутантів. ***Результати***. Кластер генів асиміляції сульфату у M145 структурно та функціонально подібний до таких кластерів у філогенетичних родичів, як-от *orynebacterium*. Більшість створених штамів з нокаутами досліджених генів мали властивості, які очікувалися, виходячи з функцій цих генів, визначених на основі доказів *in silico*. Це підтверджує їхню участь у поглинанні та асиміляції сульфату. Делеція гена *sco6101*, який не має гомологів у інших бактерійних оперонах асиміляції сульфату, блокувала ріст на середовищах з неорганічними джерелами сірки та L-цистеїні. Це вказує на участь *sco6101* у метаболізмі сірки. ***Висновки***. Наше дослідження вперше доводить, що гени *sco6093-sco6102* залучені в асиміляцію сульфату. Ген *sco6101*, у неповністю зрозумілий спосіб, важливий для цього метаболічного шляху, і відтак заслуговує глибшого вивчення.

Ключові слова: *Streptomyces coelicolor* A3(2), генетика метаболізму сірки, асиміляція сульфату.