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# GENERATION AND INITIAL CHARACTERIZATION OF A COLLECTION OF SPONTANEOUS STREPTOMYCES ALBUS J1074 MUTANTS RESISTANT TO RIFAMPICIN

***Aim***. Streptomyces albus J1074 is one of the most popular streptomycete chassis for heterologous expression of natural product (NP) biosynthetic gene clusters (BGCs). There is keen interest in further improvement of the strain to provide increased yields of corresponding NPs. Introduction of certain types of antibiotic resistance mutations is a proven way to improve Streptomyces strains. For example, selection for increased resistance to rifampicin is known to lead to increased antibiotic activity. Here we used available lineages of antibiotic-resistant mutants of S. albus to raise rifampicin-resistant variants (Rifr) and to study their properties. ***Methods***. Microbiological and molecular genetic approaches were combined to generate Rifr mutants and to study their properties. ***Results.*** By plating S. albus onto GYM agar supplemented with 10 mcg/mL of rifampicin, we isolated 85 stable Rifr colonies, whose resistance level was within 10-200 mcg/mL range. Sequencing revealed wide spectrum of missense mutations within rpoB gene. Bioassays demonstrated dramatically increased endogenous antibiotic activity of certain Rifr mutants. ***Conclusions.*** Selection for rifampicin resistance is a viable way to increase the yields of NPs in S. albus.

*Keywords: Streptomyces albus* J1074, antibiotic resistance, rifampicin.

*Streptomyces albus* J1074 (also known as *S. albidoflavus* J1074) is one of the most versatile and genetically amenable streptomycetes widely used for drug discovery purposes [1]. The range of applications includes heterologous expression of gene clusters and metagenomic libraries; incorporation of unnatural amino acids into ribosomally produced peptides; generation of whole-cell biosensor systems. NPs are produced by the wild type and heterologous streptomycete hosts in low yields, and this shortcoming pertains to J1074 as well. It prompts the interest in strain improvement investigations. Introduction of certain antibiotic resistance mutations into *Streptomyces* genomes has long been known to improve NP biosynthesis. Most of these mutations alter the function of ribosome (streptomycin, paromomycin, thiostrepton resistance) or β subunit of RNA polymerase (RpoB; rifampicin resistance); the approaches based on these mutations were collectively referred to as ribosome engineering [2]. We recently developed a rational genome engineering method to introduce point mutations into the genes usually targeted by the ribosome engineering, e.g. *rpsL* for S12 ribosomal protein [3]. This led to a collection of J1074 *rpsL* mutants with variable levels of activation of their indigenous secondary metabolism. The *rpsL* mutant carrying R94G substitution turned out to be one of the most productive (A. Luzhetskyy, unpublished), prompting us to generate *S. albus* strain carrying this allele in merodiploid state (e.g. *rpsL* and $rpsL^{R94G}$ alleles co-exist in J1074 chromosome; the latter was introduced on integrative actinophage VWB-based vector pTOS [4]). It is important to note that R94G allele is one of those *rpsL* mutations that do not lead to increased aminoglycoside resistance. As the accumulation of several different

resistance mutations in single genome is known to provide further increases in NP titers [2], we used R94G strains as a starting point to generate strains resistant to streptomycin, lincomycin and erythromycin. As a result, we picked quadruple pure *rpsL* mutant KO-1305 (*rpsL*$^{R94G}$ *rsmG* (Str$^r$) Lin$^r$ Ery$^r$) and merodiploid mutant KO-1307 (*rpsL rpsL*$^{R94G}$ *rsmG* (Str$^r$) Lin$^r$ Ery$^r$). The aforementioned strains exhibited increased antibiotic activity against *Staphylococcus albus* P209; their characterization will be reported elsewhere. Here we describe the generation of spontaneous rifampicin-resistant derivatives of KO-1305 and KO-1307 and their initial characterization.

## Materials and methods

*S. albus* SAM2, a J1074 derivative with deletion of φC31 pseudo *attB* site [5], was used throughout the work. The genealogy of *S. albus* strains mentioned in this work is given in Fig. 1. *Staph. aureus* 209P, *Bacillus cereus* ATCC19637, *Debaryomyces hansenii* VKM Y-9 were used in the bioassays as test cultures. We selected Rif$^r$ variants on GYM agar [6] with 10 μg/mL of rifampicin. Antibiotic resistance and activity assays, sequencing of *rpoB* alleles were carried out as described in [7]. The other bioassay conditions and media are described in [6]. KO-1408 genome has been sequenced using Illumina approach essentially as described in [8]. The quality control stage the sequence reads was performed by using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Raw Illumina short reads were quality-trimmed with Trimmomatic v.0.36 [9]. Sequencing reads were aligned to reference J1074 genome (accession number NC 020990) with Bowtie2 v.2.2.5. SNP and DIP detection was performed by means of ReadXplorer [10]. Only the SNVs and indels different from those found in SAM2 were considered true mutations in KO-1408. BLAST search tools were used to identify genes of interest in the genomes of *S. albus* J1074 and its derivatives.

## Results and discussion

The spores of *S. albus* KO-1305 and KO-1307 strains were spread and incubated on GYM plates supplemented with 10 μg/mL rifampicin, and Rif$^r$ mutants were observed after 3 to 7 days at a frequency of $10^{-7}$ to $10^{-8}$. After three passages under nonselective conditions the resulting mutants (about 80 clones) were again plated onto Rif-containing agar; all strains exhibited abundant growth. This confirmed the stability of their Rif$^r$ phenotype.

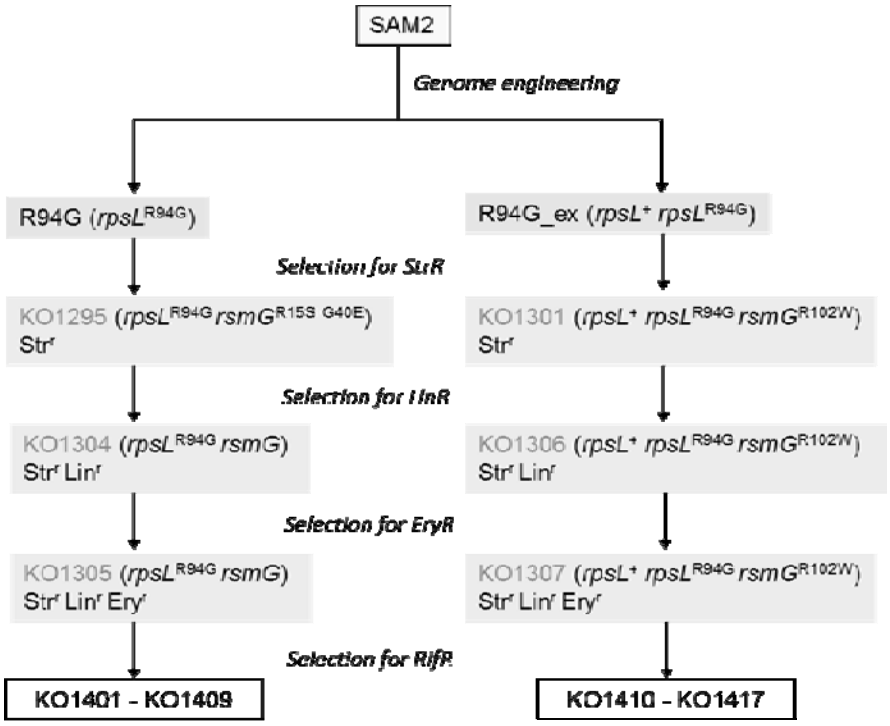


Fig. 1. Scheme of generation of spontaneous Rif$^r$ mutants KO1401-KO1417 (bottom of the figure). Abbreviations: StrR, resistance to streptomycin; LinR, resistance to lincomycin; EryR, resistance to erythromycin. Other abbreviations and strain names, please see the main text.

Most often the Rif$^r$ phenotype arises from mutations in *rpoB* gene for the RNA polymerase (RNAP) β-subunit; hence we amplified *rpoB* (*xnr_3712*) from our mutants. In all but one cases the *rif* mutations were located within the so called "*rif* cluster" of RNAP, encompassing the 422-443 amino acid stretch. The only exception was KO-1412, which was revealed to carry missense mutation within *rpoB* leading to Pro475→Leu substitution. Along with this mutation, two other novel mutations within "*rif* cluster" were uncovered: Ser433→Trp (KO-1403) and Arg440→ Cys (KO-1407). We used spore dilution spot tests to determine Rif resistance level of the mutants; these data, along with sequencing results, are summarized in Table.

We assayed the endogenous antibiotic activity of selected KO strains using several test cultures (see Materials and methods), GYM and SG2 media, and agar plug tests. Overall, although some merodiploid-based Rif$^r$ strains showed greater activity as compared to their parent (R94G_ex) against *S. aureus* 209P, they were less active than KO strains derived from pure R94G *rpsL* mutant. One notable exception is KO-1412 carrying novel P475L mutation; it showed increased antibiotic activity after 5 days of cultivation in GYM (15-mm halo of 209P growth inhibition), while the parental strain R94G_ex showed almost no activity. Similar trend was observed when GYM-grown agar plugs of the KO strains were assayed against *B. cereus* (Fig. 2).

Table. Mutations and rifampicin resistance levels of various of *rpoB* mutants isolated from *S. albus* KO-1305 and KO-1307

| Strain | Mutation in *rpoB* | Amino acid substitution* | Mutant frequency** | Rif resistance (μg/mL) |
|---|---|---|---|---|
| J1074 | – | – | – | 3 |
| KO-1305▼ | – | – | – | 3 |
| KO-1401 | 1325C→T | Ser442→Leu | 1/48 | 30 |
| KO-1402 | 1262C-1273T→Δ | Q421-F425→L421 | 1/48 | 200 |
| KO-1403 | 1298G→C | Ser433→Trp | 1/48 | 10 |
| KO-1404 | 1310A→G | His437→Arg | 13/48 | >200 |
| KO-1405 | 1309C→T | His437→Tyr | 15/48 | 200 |
| KO-1406 | 1318C→A | Arg440→Ser | 1/48 | 20 |
| KO-1407 | 1318C→T | Arg440→Cys | 5/48 | 25 |
| KO-1408 | 1319G→A | Arg440→His | 7/48 | 25 |
| KO-1409 | 1271A→T | Gln424→Leu | 2/48 | 200 |
| | | | | |
| KO-1307▼ | – | – | – | 3 |
| KO-1410 | 1325C→T | Ser433→Leu | 1/37 | 25 |
| KO-1411 | 1310A→G | His437→Arg | 12/37 | >200 |
| KO-1412 | 1451C→T | Pro475→Leu | 1/37 | 25 |
| KO-1413 | 1309C→T | His437→Tyr | 16/37 | 200 |
| KO-1414 | 1309C→G | His437→Asp | 3/37 | 100 |
| KO-1415 | 1271A→T | Gln424→Leu | 1/37 | 50 |
| KO-1416 | 1310A→C | His437→Pro | 1/37 | >200 |
| KO-1417 | 1319G→A | Arg440→His | 1/37 | 25 |

*Notes*: * Highlighted cells correspond to substitutions that are, to the best of our knowledge [2, 7], observed for the first time in *Streptomyces*; ** Number of mutants out of total Rif$^r$ clones isolated from a given KO strain. ▼Parental strain for respective Rif$^r$ lineages.

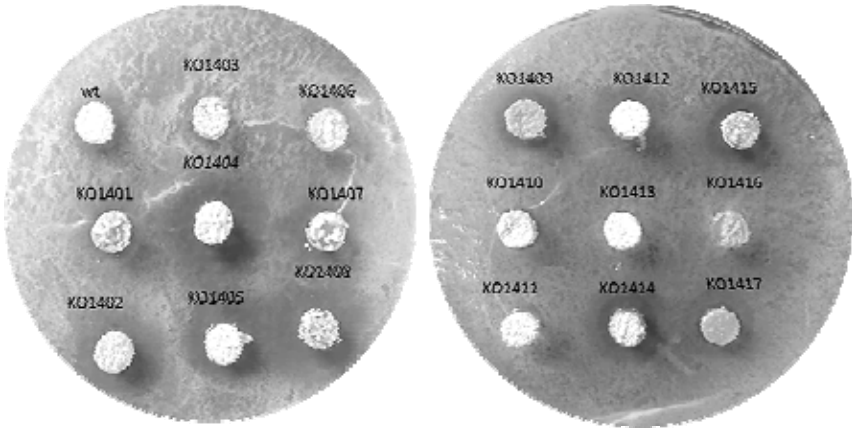


Fig. 2. Halos of *B. cereus* ATCC19637 growth inhibition around agar plugs of the KO strains cultivated for five days on SG2 agar.

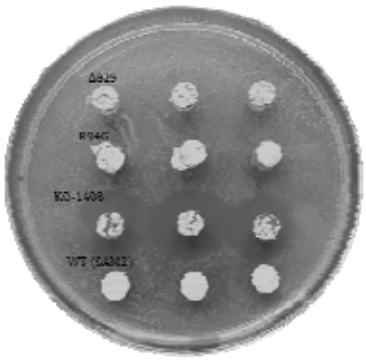


Fig. 3. Halos of *B. cereus* ATCC19637 growth inhibition around agar plugs of the B29 (paulomycin-deficient derivative of J1074 [11]), R94G, SAM2 and KO-1408 strains. Growth conditions, see Fig. 2. For each strain three agar plugs from different plates were assayed.

No changes in antifungal activity have been observed for the KO strains as compared to parental ones. After a series of bioassays, we have come to conclusion that the mutant KO-1408 (R440H) exhibits the most robust increase in endogenous antibacterial activity, which is evident under different cultivation conditions and against different test cultures. Significantly, this increase appeared to be due to the presence of *rpsL*$^{R94G}$ allele in haploid ("pure") state, since isogenic *rpoB*$^{R440H}$ mutation in merodiploid *rpsL*+*rpsL*$^{R94G}$ background (KO-1417) did not result in comparable increase in antibiotic activity (Fig. 2).

Next we focused on KO-1408. First, we compared the antibiotic activity of strain lineage leading to KO-1408. The bioassays support the idea that *rif* mutation leading to *rpoB*$^{R440H}$ was a major one that induced antibacterial production by KO-1408. Indeed, initial strain R94G (carrying *rpsL*$^{R94G}$ allele) had no major effect on antibacterial activity under our cultivation conditions (Fig. 3), and following Str$^r$, Lin$^r$, Ery$^r$ mutations had no effect as well (data not shown).

One caveat is that spontaneous mutants could carry additional as-yet-unknown mutations in their genome which may contribute to the final antibiotic resistance and activity phenotypes. To rule out this scenario for KO-1408, we sequenced its genome as well as genomes of SAM2, R94G, KO-1295, KO-1304 and KO-1305. We revealed no new mutations in KO-1408 as compared to KO-1305, except for already known one leading to *rpoB*$^{R440H}$ allele. Therefore, the augmented antibiotic potency of KO-1408 most likely stems from a combination of known *rpsL*$^{R94G}$ and *rpoB*$^{R440H}$ mutations.

**Conclusions**

*S. albus* J1074 attracts interest of academic and industrial researchers as a reliable platform for drug discovery. In this work we describe a set of rifampicin resistant mutants on the basis of multiply antibiotic resistant *S. albus* KO-1305 and KO-1307 strains. Some of the identified Rif$^r$ mutations within *rpoB* were not previously reported, raising the possibility that *rpoB* mutational space leading to Rif resistance is not fully understood despite decades of research. Nevertheless, well-known

*rpoB*$^{R440H}$ turned out to be the most effective in terms of enhancement of secondary metabolism of *S. albus*. It remains to be studied the production of what compounds is activated in KO-1408 and other strains; and whether these mutants will support the increased production of compounds directed by heterologous BGCs. Experiments are underway in our laboratories to address these questions.

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**КОНСТРУЮВАННЯ І ВИХІДНА ХАРАКТЕРИСТИКА КОЛЕКЦІЇ СПОНТАННИХ МУТАНТІВ *STREPTOMYCES ALBUS* J1074 СТІЙКИХ ДО РИФАМПІЦИНУ**

***Мета***. *Streptomyces albus* J1074 – одна із найпопулярніших стрептоміцетних платформ для гетерологічної експресії кластерів генів біосинтезу природних сполук. Становить інтерес у дослідженнях, які б вели до підвищених кількостей продукції відповідних сполук. Уведення певних типів мутацій стійкості до антибіотиків – доведений шлях селекції штамів *Streptomyces*. Наприклад, селекція за стійкістю до рифампіцину веде до зростання антибіотичної активності. У цій роботі ми використали наявні лінії антибіотикорезистентні лінії *S. albus* для отримання рифампіцин-стійких варіантів (Rif$^r$) та їхнього вивчення. ***Методи***. Застосовано мікробіологічні та молекулярно-генетичні підходи для селекції Rif$^r$ мутантів та вивчення їхніх властивостей. ***Результати***. Виділено 85 стабільних Rif$^r$ клонів, чия резистентність була у межах 10–200 мкг/мл. Секвенування виявило широкий спектр міссенс-мутацій у межах гена *rpoB*. Біотести виявили сильне зростання ендогенної антибіотичної активності деяких Rif$^r$ мутантів. ***Висновки***. Селекція за стійкістю до рифампіцину – перспективний спосіб селекції високопродуктивних штамів *S. albus*.

*Ключові слова*: *Streptomyces albus* J1074, стійкість до антибіотиків, рифампіцин.