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PROSPECTS FOR RIBOSOME ENGINEERING APPROACHES AS A WAY TO IMPROVE MOENOMYCIN PRODUCTION BY *STREPTOMYCES VIRIDOSPORUS* ATCC14672

Aim. *Streptomyces viridosporus* Pridham et al. ATCC14672 produces moenomycins (MOE), nanomolar inhibitors of peptidoglycan glycosyltransferases. MOE production titer by ATCC14672 is around 1 mg L^{-1}, necessitating the search for MOE overproducers. One promising approach is to look for overproducing variants among spontaneous mutants resistant to antibiotics-inhibitors of translation and transcription. ***Methods***. Methods of bacterial genetics were employed to raise and analyze antibiotic resistant mutants of ATCC14672. ***Results***. The wild type was quite homogeneous morphologically; frequency of atypical colonies was about 10^{-3}. One such an atypical colony, L7, was picked for further analysis and shown to produce MOE at the level of the parental strain. Antibiotics lincomycin, streptomycin, spectinomycin, thiostrepton, neomycin and kasugamycin were not suitable for ATCC1472 because of high background resistance to them. In contrast, RNA polymerase inhibitor rifampicin was successfully used to raise a small set of resistant mutants. They harbored point mutations within coding sequence of gene *rpoB* for β-subunit of RNA polymerase. Two mutants, RIFS1 and E4, harbored the same *rpoB* mutation (H437Y) but had different colony morphology and total antibiotic activity. ***Conclusions***. In this work we show that rifampicin can be used as tool to select for mutants with altered antibiotic activity. Our data point to the possibility of co-occurrence of *rpoB* and the other (as-yet-unknown) mutations in their genome.

Keywords: *Streptomyces viridosporus* ATCC14672, antibiotics, ribosome engineering.

Streptomyces viridosporus Pridham et al. ATCC14672 produces a mixture of phosphoglycolipid natural products, known as moenomycins (MOE), that are potent inhibitors of peptidoglycan biosynthesis in Gram-positive bacteria (Ostash, 2022). Moenomycin A is the archetypal representative of MOE that inspired numerous chemical and biological studies whose ultimate aim is the development of novel class of antibiotics (Ostash and Walker, 2010) in the times of worsening antimicrobial resistance crisis (Okeke et al., 2024). In addition to some pharmacokinetic issues, one major hurdle towards MOE-based drugs would be an economically viable process of their production. The MOE molecules (around 1.5 kDa in molecular mass) pose a formidable challenge for chemical synthesis. Therefore, microbiological production of MOE currently is the only way (Ostash and Walker, 2010) to access these compounds. ATCC14672 produces a few milligrams of MOE in 1L of submerged cultivation, necessitating the development of overproducers. Since the initial report on identification of MOE biosynthetic gene cluster (*moe* BGC) (Ostash and Walker, 2010), many genes were shown to impact MOE production level, as reviewed in (Ostash, 2022). One approach that has so far not been applied to ATCC14672 is ribosome engineering. This term was coined by Prof. Kozo Ochi, who discovered that missense mutations in gene *rpsL* for ribosomal protein S12 in *Streptomyces* lead to streptomycin resistance, and often increase antibiotic production (Ostash, 2023). Later on the term was used to cover the other classes of

spontaneous mutants, resistant to various translation inhibitors or rifampicin, that were raised to search for antibiotic overproducers. We decided to explore the prospects for ribosomal engineering of ATCC14672. First, we assessed the degree of natural variability of antibiotic activity and colony morphology for the wild type. Second, we determined which antibiotics can be used in this strain for the purpose of ribosome engineering. Third, we employed rifampicin to raise a small collection of resistant mutants and characterized them. The results of these experiments are reported and discussed here.

Materials and methods

S. viridosporus ATCC14672 used in this study was seeded from the spore archive set in 2004 year, after temperature-curing of the plasmid pSG5 from the original lyophilized culture of ATCC14672 obtained from American Type Culture Collection (Ostash et al., 2007). *Bacillus cereus* Frankland and Frankland ATCC19637 was used as a MOE-susceptible test culture. ATCC14672 was routinely grown on oatmeal agar and ATCC19637 on TSA (Koshla et al., 2017). Primers for *rpoB* (*SSFG_02952*) fragment PCR and sequencing: rpoB-gh_f (CATGACCACGCAGGACGTCGAG) and rpoB-gh-r (GAAGCGGAGGTCGTCCGTCAG). Solid media used to test antibiotic production by *S. viridosporus* strains will be mentioned in the experimental section; their recipes as well as bioassay protocols are given in (Koshla et al., 2017). MOE were purified from the biomass and supernatant as described in (Ostash et al., 2007). Streptomycin, neomycin, kasugamycin, lincomycin and thiostrepton were from Fluka, no less 90% pure. Rifampicin was from Borshchahivskyy enterprise. ResFinder (https://genepi.dk/resfinder) was used to predict antibiotic resistance genes in the wild-type genome (accession number DS999641.1). DNA sequencing was done at Explogen LLC.

Results and discussion

As the ribosome engineering approaches often result in colonies with altered morphogenesis, we first checked the degree of variability of morphological traits within the natural population of original ATCC14672. Serial dilutions of ATCC14672 spores were plated onto oatmeal agar and morphological variants were observed after 7 days of incubation at 30 °C (Fig. 1). A typical ATCC14672 colony is round, uniformly covered with dark-green spores. Such colonies represented a vast majority of the population we observed on the plated serial dilutions. A second major morphotype consisted of light-green colonies, likely due to a retarded developmental cycle. However, these colonies did not turn dark green even after a month of growth. Finally, we observed rare cases of colonies impaired in their morphology, as indicated in the Fig. 1. They occurred at a frequency of $(3 \pm 1) \times 10^6$.

Because of genetic links between morphogenesis and specialized metabolism, it could be that morphologically altered colonies are affected in MOE production. However, it was not the case for the defective colonies we observed in natural population, as shown for one variant in Fig. 1 B. Thus, our analysis revealed a low level of occurrence of morphologically impaired colonies, yet such aberrations were not coupled to the ability of ATCC14672 to produce MOE.

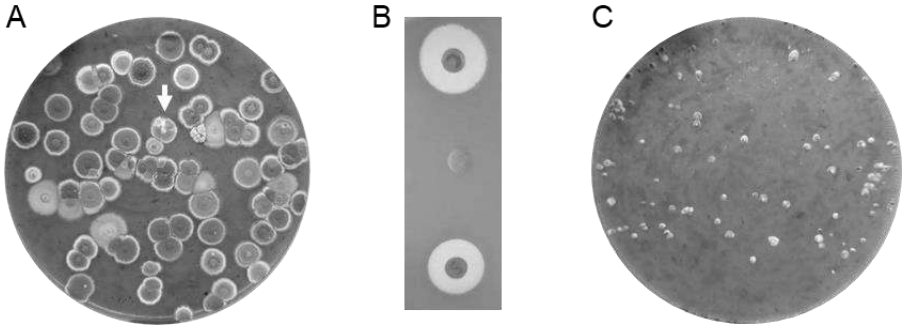

Fig. 1. *S. viridosporus* ATCC14672 exhibits variability in colony morphology and resistance to certain antibiotics. Serial spore dilution was plated onto oatmeal agar (**A**) revealing colonies with derailed formation of aerial mycelium and spores. The normalized (relative to the 10 mg of dry biomass) total antibiotic activity of one such colony (marked with arrow in part **A**), grown in liquid STSB, was higher (top disk, part **B**) than that of the wild type (bottom disk, part **B**). The identity of antibiotic activity to MOE was confirmed via biochromatography (data not shown). Colonies of ATCC14672 grown on oatmeal agar supplemented with 150 mcg/mL lincomycin (**C**).

We determined the ability of ATCC14672 to grown in the presence of certain concentrations of lincomycin, streptomycin, kasugamycin, thiostrepton, neomycin, and rifampicin. For the first four antibiotics the frequency of occurrence of spontaneous resistant colonies was too high (up to 10^{-4}) to consider these drugs useful for ribosome engineering. *In silico* analysis of ATCC14672 genome with ResFinder tool (see the Methods) provided clues about the gene candidates to confer resistance to lincomycin (efflux protein (Tseduliak et al., 2023)) and thiostrepton (thiostrepton-inducible protein TipA). Yet, we did not reveal plausible determinants of resistance to aminoglycoside antibiotics. Neomycin was very effective at killing ATCC14672, yet we failed to isolate spontaneous colonies resistant to this antibiotic, even after testing approximately 10^{10} spores in total.

Thus much of our work was eventually focused on rifampicin, as ATCC14672 was reasonably susceptible to it. Starting from concentration of 10 μg/mL of the antibiotic, we observed the survival rate of $(4.2 \pm 1.5)\times10^{-8}$ and this rate dropped two-threefold at concentrations 50 and 100 μg/mL. We therefore attempted to raise rifampicin-resistant (Rif^r) mutants, initially using oatmeal agar plates supplemented with 50 μg/mL of the antibiotic. After three selection campaigns we were able to isolate 30 Rif^r clones in total, of which five exhibited stable inheritance of Rif^r phenotype. We then reasoned that using lower rifampicin concentrations might help isolate clones harboring distinct set of *rpoB* missense mutations. Thus one round of selection for resistance to 25 μg/mL of the antibiotic was undertaken, and 20 Rif^r colonies were isolated. Of these colonies nine displayed almost 100% inheritance of Rif^r phenotype.

Some of the isolated mutants differed significantly in their morphology from parental strain. This was true for mutant RIFS1, isolated in course of selection for resistance to 50 μg/mL of rifampicin, and E6 (25 μg/mL of the drug). Both had white colonies, most likely due to arrested spore formation (see examples in Fig. 2).

The antibiotic activity of the Rif^r mutants was determined under agar and liquid media-grown cultures. In agar plug assays two (RIFS1, T1) out of nine mutants showed diminished activity, the rest exhibited growth inhibition zones equal or slightly larger than that of the wild type (see also Fig. 2 B). In liquid medium STSB, however, none of the strains exceeded the production level of the parental strain ATCC14672 (normalized relative to the dry biomass; data not shown).

We isolated total DNA from the nine Rif^r mutants and sequenced a region of *rpoB* gene known to be the primary hotspot for missense mutations leading to rifampicin resistance (Dolya et al., 2023). We succeeded to obtain quality sequencing data for seven out of nine samples, these data (as well as summary of the phenotypes of the mutants) are given in Table.

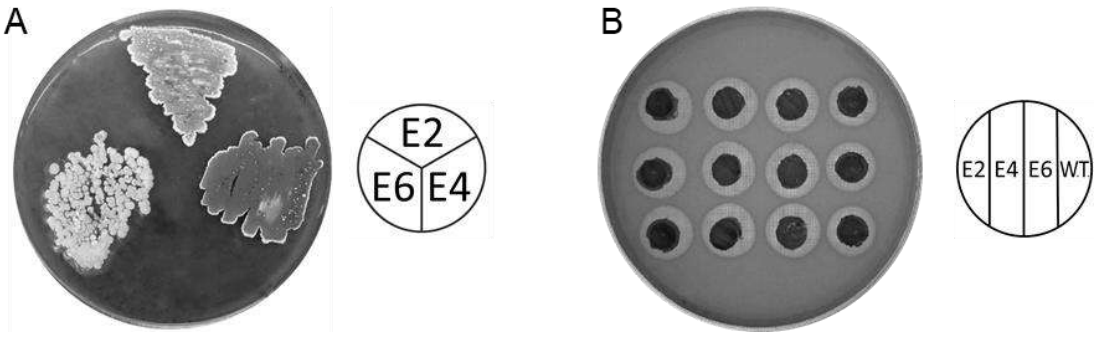

Fig. 2. Morphology and antibiotic activity of selected Rif^r *S. viridosporus* mutants of ATCC14672. Strain E6 (**A**) had stable White phenotype, and it appeared to be more antibiotically active against *B. cereus* ATCC19637 than the parental strain (**B**). The graphical legend to the right of the plates shows the positions of strains or plugs. For the agar plug assay, mutants were grown on nutrient agar (NA; Sanimed) for five days, then the plugs were stacked on ATCC19637 lawn in soft NA.

Table. Properties of Rif^r *S. vididosporus* mutants

Strain	Phenotype	RIF[1] conc., μg mL^{-1}: used for selection/resistance	*rpoB* mutation[3]
ATCC14672	Dark-green or pale green colonies (WT)	-/0.05	No mutation
RIFS1	White colonies (WH)	50/200	His437Tyr
RIFS2	WT	50/100	Thr427Asn+Ser442Phe
RIFS3	WT	50/200	ND[4]
T1	WT	50/100	Ser442Phe
T2	WT	50/200	His437Tyr
T3	WT	50/200	His437Tyr
E2	WT	25/100	Ser442Phe
E4	WT	25/200	His437Tyr
E6	WH	25/200	ND

Notes: [1] Rifampicin, [2] Determined by spotting serial dilutions of spores on plates supplemented with different amounts of RIF. Strain showing a CFU titer within the order of magnitude of that for control (no antibiotic) was considered resistant, [3] Amino acid residue numbering as shown in (Dolya et al., 2023), [4] Not determined.

The histidine to tyrosine substitution in position 437 of RpoB turned out to be the most frequent mutation encountered in our collection. It occurred when ATCC14672 was selected for resistance to either of the two rifampicin concentrations. All of the revealed so far substitutions have been described previously in actinomycetes. Nevertheless, a combination of T427N and S442F is, to our knowledge, a novel one at least for *Streptomyces*. It did not escape our attention that mutants harboring identical *rpoB* mutations may display different phenotype. Mutants RIFS1 and E4 provide a striking example. Both harbor H437Y mutations; yet, while E4 resembles the parental strain in terms of colony morphology (see Fig. 1) and total antibiotic activity, RIFS1 is impaired in colony development and antibiotic production. These points to the occurrence of the other, as-yet-undetected, mutations in the genomes of our mutants, an assumption that has long history of validated cases, including our efforts to isolate and characterize *rpsL* mutants (Shemediuk, 2022, Westhoff et al., 2017).

Conclusions

MOE are fascinating group of natural products with a long history of use as an animal growth promoter. As the use of antibiotics for animal growth promoter is gradually phased out worldwide, there is an opportunity to develop much needed MOE-based drugs to treat human infectious diseases. Our long-term research interest lies in elucidating the regulatory networks governing MOE production. While such networks are well understood for model cases (Augustijn et al., 2024), our knowledge of their operation in MOE producing strain ATCC14672 is limited (Ostash, 2022, Schuricht et al., 2001). Here we focused on attempts to understand as to whether ribosome engineering approaches would be useful to improve MOE titers. Only rifampicin was found to be efficient tool to raise resistant mutants, although their preliminary screening did not yield variants with significantly increased antibiotic activity. Tests under a wider set of submerged growth conditions are in progress to draw a definitive conclusion on this issue. We cannot help noting that our Rif^r mutants likely harbor mutations other than *rpoB*, which might impact their metabolic properties.

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Conflict of interest: The authors declare no conflict of interest.

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ПЕРСПЕКТИВИ ВИКОРИСТАННЯ РИБОСОМНОЇ ІНЖЕНЕРІЇ ЯК СПОСІБ ПІДВИЩИТИ ПРОДУКЦІЮ МОЕНОМІЦИНУ У *STREPTOMYCES VIRIDOSPORUS* ATCC14672

Мета. *Streptomyces viridosporus* Pridham et al. ATCC14672 продукує моеноміцини (МОЕ), наномолярні інгібітори пептидогліканових глікозилтрансфераз. Титр МОЕ у ATCC14672 на рівні 1 мг×л$^{-1}$, зумовлюючи інтерес до пошуку надпродуцентів. Одним із корисних підходів є пошук надпродуцентів серед спонтанних мутантів стійких до антибіотиків-інгібіторів трансляції та транскрипції. ***Методи***. Використали методи бактерійної генетики для отримання антибіотикорезистентних мутантів. ***Результати***. Штам ATCC14672 є доволі однорідним морфологічно; частота появи нетипових колоній на вівсяному агарі була приблизно 10^{-3}. При аналізі однієї такої колонії, L7, виявлено, що вона продукує МОЕ на рівні дикого типу. Антибіотики лінкоміцин, стрептоміцин, спектиноміцин, тіострептон, неоміцин та касугаміцин неефективні для ATCC14672, оскільки штам має високу фонову стійкість до них. На противагу, інгібітор РНК-полімерази рифампіцин був успішно застосований для отримання невеликої колекції стійких мутантів. Вони містили точкові мутації у кодувальній послідовності гена *rpoB*, що кодує β-субодиницю РНК полімерази. Два мутанти, RIFS1 та E4, містили ідентичну мутацію у *rpoB* (H437Y), але відмінну морфологію колоній і рівень антибіотичної активності. ***Висновки***. У цій роботі продемонмтровано, що рифампіцин може бути використаний як знаряддя для селекції мутантів ATCC14672 зі зміненою антибіотичною активністю. Наші дані вказують на можливість появи мутацій у *rpoB* одночасно з іншими, наразі невідомими, мутаціями у їхніх геномах.
Ключові слова: *Streptomyces viridosporus* ATCC14672, антибіотики, рибосомна інженерія.

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