UDC 575.87:577.182.82:579.873 *https://doi.org/10.7124/FEEO.v36.1717*

OSTASH B. O., VOZNYUK A. V., TSEDULIAK V.-M. S., FEDORENKO V. O.✉
Ivan Franko National University of Lviv,
Ukraine, 79005, Lviv, Hrushevskoho, 4, ORCID: 0000-0001-5904-5957
✉ *viktor.fedorenko@lnu.edu.ua, (032) 239-44-07, (032) 239-44-75*

NEW INSIGHTS INTO REGULATION OF SPECIALIZED METABOLISM OF *STREPTOMYCES VIRIDOSPORUS* ATCC14672

Aim. *Streptomyces viridosporus* ATCC14672 produces moenomycins (MOE), direct nanomolar inhibitors of bacterial peptidoglycan glycosyltransferases. Low MOE titers complicate their scaled-up purification, blurring MOE translational prospects. We took genetic approach to interrogate the involvement of several well-known pleiotropic regulators in MOE biosynthesis. ***Methods***. Bacterial genetics and bioassays were combined to generate recombinant strains harboring additional copies of selected genes, and to assess their effects on MOE production. ***Results***. The promoters of MOE biosynthetic genes harbor operators for pleiotropic transcriptional factors. We show here that, out of several tested regulatory genes, *bldD* is able to activate the production of unknown antibiotic in MOE-deficient mutant, dO5, whereas in ATCC14672 total antibiotic activity dropped in response to *bldD*. The dominant-negative allele of *lsr2* positively impacted antibiotic activity in ATCC14672 and dO5. We also tested the effects of several broad-specificity transporter genes on dO5. ***Conclusions***. Our study adds new players to the network of genes impacting MOE production. *S. viridosporus*, under certain conditions, is capable of producing an unknown antibiotic, whose elimination might be tested to improve MOE titers.

Keywords: *Streptomyces viridosporus* ATCC14672, antibiotics, genes, metabolism regulation.

Streptomyces viridosporus (formerly also known as *S. ghanaensis*) ATCC14672 is notable for production of moenomycins (MOE, fig. 1), a family of phosphoglycolipid antibiotics capable of direct inhibition of bacterial cell wall-synthesizing enzymes, peptidoglycan glycosyltransferases. MOE are active primarily against Gram-positive cocci, including vancomycin-resistant mutants [1]. Structural and biological uniqueness of MOE, their ability to overcome some types of antimicrobial resistance (an escalating issue of the last decades), set the fertile ground to re-approach this group of natural products for drug development purposes. It is true that availability of numerous other classes of small molecules, amenable for antibiotic development more than MOE, explains to a large extent a lack of enthusiasm in the latter in the second half of XXth century. Yet, even now MOE represent a formidable challenge for pharmaceutical industry. First, they are «large» (>1500 Da) small molecules based on lipid-oligosaccharide scaffold, an extremely hostile one for medicinal chemists. Second, MOE are absorbed poorly from intestine into the blood, and so oral bioavailability is not possible. Third, once in the blood, it sticks tightly to blood proteins with an unacceptably long excretion half-life [2]. However, the ominous spread of multidrug resistant pathogens in hospital settings and beyond [3] leaves us no choice but to explore every possible venue to bring new antibiotics to the market. In case of MOE, possible paths forward would include *i.* manipulations of lipid moiety to decrease its lipophilicity (thereby decreasing blood half-life) and *ii*. pursuing topical applications of MOE (thus bypassing oral availability issues).

Microbiological (submerged fermentation-based) production of MOE currently is the only viable route [2] to produce large quantities of these compounds for further studies. MOE production by ATCC14672 is around 1 mg per 1L of laboratory medium, prompting the strain and process improvement efforts. Much of this work has been done over 50 years ago at companies that manufactured MOE as an animal food additive [4]. Currently the resulting strains and protocols remain either lost or inaccessible to academic community. We cloned MOE biosynthetic gene cluster (*moe* BGC) in 2007 [2], opening up the way towards gene-based understanding of MOE production. As a result of our efforts we identified a network of regulators that activate or limit MOE production level, as summarized in [1]. There are no genes for transcriptional factors (so called cluster-situated regulators) within *moe* BGC. It is a rare feature of MOE biosynthesis that sets this pathway apart from the

majority of specialized metabolic routes operating in streptomycetes. Therefore, we focused on examination of genes for so called pleiotropic (or global) regulators of *Streptomyces* specialized metabolism. This group of genes is scattered across ATCC14672 genome and includes those encoding transcriptional factors, but also tRNA (*bldA*), putative redox-sensing DNA binding proteins (*wblA*), tRNA modification enzymes (*miaB*) and others. Our picture of MOE regulation is not complete; while we investigated around 20 genes so far, many more are known [5] to influence antibiotic production in streptomycetes. Here we report our results on several regulatory and transport genes, and elicitors, previously not studied in the MOE biosynthesis context.

Materials and methods

S. viridosporus ATCC14672 and its MOE-deficient derivative, dO5, were used throughout the work. Strain dO5 harbors deletion of key MOE biosynthetic gene *moeO5*, its construction and properties will be described separately. *Bacillus cereus* ATCC19637 was used as a MOE-susceptible test culture. *Acinetobacter jostii*, *Mycobacterium smegmatis*, *Myxococcus xanthus* biomass was kindly provided from the collection of JSC Arterium (Lviv). Following expression plasmids were used in the work: pTOSbldA [6] carrying leucyl tRNA gene *bldA*; pMC109 [7] harbors dominant negative allele of *lsr2*; pVMC [8] carries multidrug transporter gene *xnr_2146* of *S. albus* J1074; pOOB92a carries *adpA* gene of ATCC14672. Three plasmids were constructed in this work using standard T4 DNA ligase-based methods. pTESbldD carries ATCC14672 gene *bldD* under control of *ermEp* promoter of integrative vector pTES [9]. pAVi21 is pTES-based plasmid expressing multidrug transporter gene *xnr_4573*. The latter was amplified with primers xnr4573_up (AAATCTAGACGATGGACCTACGTCCCTCC) and Xnr4573_rp (AAAGAATTCGGAG GAAGACGAGGGAGGTC). Plasmid pTOS_1511 is based on vector pTOS [9], it carries gene *xnr_1511* for LonA protease along with its native promoter. Primers for cloning: 1511XbaIKpnI_up (AAATCTAGAGGT ACCAGCCCGGTCAGGAACATC) and 1511XbaIEcoR_rp (AAATCTAGAATTCTCAGC AAGGTGGCCGC). The plasmids were maintained/constructed in host strain *Escherichia coli* DH5α and transferred conjugally from *E. coli* ET12567 (pUZ8002) into *S. viridosporus* strains. *S. viridosporus* strains were grown on oatmeal medium at 37 °C to obtain spores for conjugations. Solid media used to test antibiotic production by *S. viridosporus* strains will be mentioned in the experimental section; their recipes as well as bioassay protocol are given in [6] Standard bioinformatics tools and databases were used to describe genes and retrieve genomes of ATCC14672 and J1074 (BLAST, Genbank), and to annotate operator sequences within *moe* BGC (MEME Suite).

Results and discussion

Positive effects of pleiotropic transcriptional factor (TF) AdpA [1] encouraged us to take a broader look at possible involvement of global regulators in MOE production level. A detailed mapping of putative operator sites for TFs BldD and DasR within *moe* BGC of ATCC14672 has therefore been undertaken. As a control we also remapped the locations of AdpA operators. The final result, summarized in fig. 2, shows the presence of operators within the intergenic regions of the gene cluster. We were able to detect numerous sites for the tested TFs. Upstream regions of key gene *moeO5* and its neighbors were especially enriched with the operators. In all, our data support the idea that BldD and DasR might be involved in MOE production regulation.

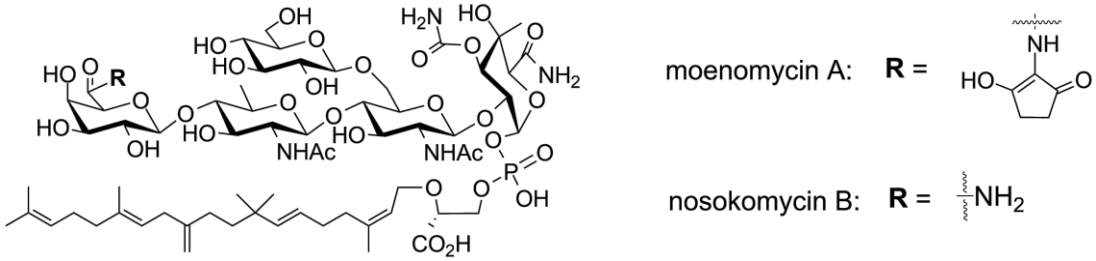

Fig. 1. Structures of the major components of MOE complex produced by ATCC14672.

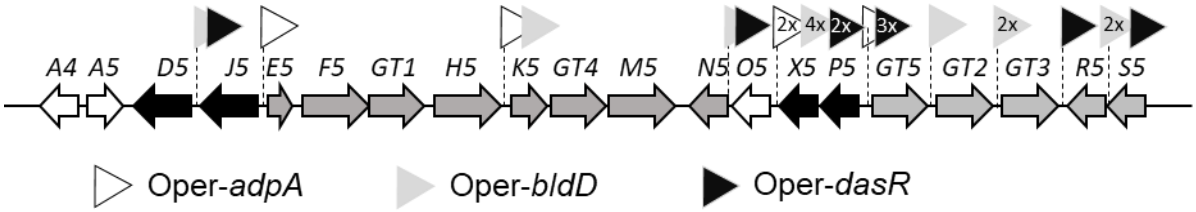

Fig. 2. Organization of *moe* BGC and location of AdpA, BldD and DasR operator sequences in promoter regions. Genes are shown as arrows where black color denotes transporter genes, grey and white – structural ones. Triangles on top of dashed lines mark operator sites. Different triangles are for different operators, as explained in the graphical legend, bottom of the figure. Numbers within the triangle (2x, 3x, 4x) imply the presence of several adjacent operators.

It is generally assumed that MOE is the only group of antibiotics that is produced by ATCC14672 and active against test strain *B. ereus* ATCC19637. However, there is no published evidence that ATCC14672 does not in fact produce another antibiotic capable of inhibiting ATCC19637. Furthermore, even if the latter is true, there is no guarantee that manipulations of regulatory genes in ATCC14672 would not alter the expression of the other BGCs. This would compromise the readout of simple microbiological assays. We therefore employed MOE-nonproducing strain dO5 to scrutinize its ability to produce compounds active against ATCC14672. After 5 days of growth on tryptic soy agar, the dO5 colonies inhibited ATCC19637 growth to a small extent (Ø6÷8 mm on average), as compared to the wild type colonies (Ø 20÷24 mm). On R5 medium dO5 strain showed no activity against ATCC19637. Hence, dO5 indeed produces antibiotics unrelated to MOE and active against ATCC19637, although it requires specific growth conditions. Care therefore is to be taken when interpreting bioassay results. We then introduced into dO5 several global regulators of specialized metabolism, such as *lsr2* (plasmid pMC109), *bldD* (pTESbldD, *bldA* (pTOSbldA), *adpA* (pOOB92a), and *lonA* (pTOS_1511) and tested the resulting strains on different agar media, such as NL5 and R5. None of the strains exhibited antibiotic activity against ATCC14672 under these conditions. One exception was dO5 pTESbldD$^+$ strain which yielded sizable growth inhibition zone. However, we cannot rule out a possibility of workup error in this case (e.g. strain mixing). The activity of nucleoid-associated protein Lsr2 (plasmid pMC109) is modulated by divalent cations [10], therefore we grew dO5 pMC109$^+$ on TSA supplemented with different metal salts. Out of the number of metals tested (Pb^{2+}, Cr^{6+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}) magnesium (30 mM) and copper (0.3 mM) led to a slight increase in antibiotic activity of dO5 pMC109$^+$ versus dO5 against ATCC19637 (ΔØ around 5–6 mm).

We then wondered as to whether it is possible to induce antibiotic production by dO5 with chemical elicitors. There are recent reports on successful use of biomass of mycolic acid-producing bacteria as inducing agents [11]. By analogy to the referred method, we grew dO5 together with elicitor cultures in liquid cultures and on agar plates. Of various combinations tested, there was reproducible increase in antibiotic activity of dO5 paired with *Acinetobacter*, both on agar plates and in liquid cultures.

Potential of transporter genes as a tool for manipulations of specialized metabolism remains poorly studied in *Streptomyces* and there are no data at all for ATCC14672. We constructed two ATCC14672 derivatives, one expressing transporter gene *xnr_2146*, and another – *xnr_4573*. A rationale behind this part of work is as follows. Both aforementioned genes of *S. albus* J1074 encode transporters of broad specificity. The Xnr_2146 is major facilitator superfamily antiporter, that contributes to elevated lincomycin resistance [8], while Xnr_4573 is homologous to Rv1819 of *M. tuberculosis*, an ABC transporter involved in vitamin B_{12} and bleomycin uptake [12]. By either exporting some autotoxic compounds from ATCC14672 cells, or importing nutrients/signals, these proteins might stimulate antibiotic production. Presence of pVMC shut down endogenous antibiotic production by dO5, while pAVi21 boosted the latter to some extent (fig. 3).

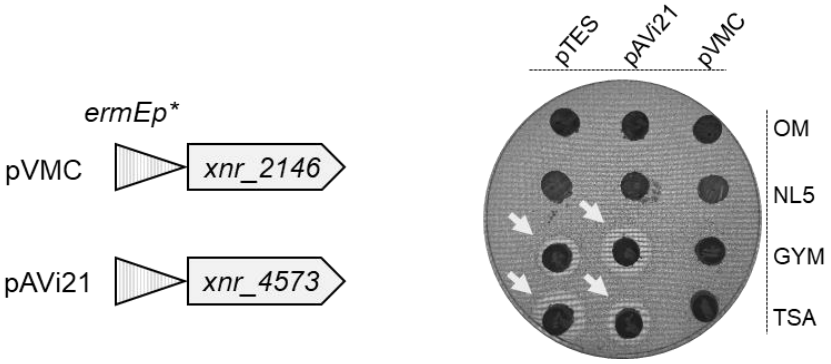

Fig. 3. Expression of transporter genes in dO5 impacts antibiotic production. Agar plug assay (right) of dO5 derivatives harboring pTES (empty vector), pAVi21 and pVMC. Notation of the plasmids is shown to the left of bioassay photo. Arrows on the plate indicate samples showing antibiotic activity against *B. cereus* ATCC19637.

In summary, our results attest to the fact that ATCC14672 is able to produce antibiotic(s) other than MOE, at least under conditions when MOE production is eliminated. This production takes place in certain media (hereafter referred to as «favorable conditions»), such as TSA and GYM, while the other media (such as R5, NL5) do not support the production. Under non-favorable conditions (e.g., medium R5) we practically were not able to induce antibiotic production by dO5. Thus, we learned the conditions under which bioassays are a reliable readout of MOE production. In the same time, we have means to increase the production of that as-yet-unknown antibiotic, which might be used to purify it enough for structural elucidation.

We introduced plasmids pMC109 and pTESbldD into the wild type ATCC14672 strain. The *lsr2*-expressing pMC109 increased antibiotic activity of ATCC14672 much like it did in dO5, in a cation-dependent manner (up to 6 mm of increase in diameters of growth inhibition zones). The pTESBldD decreased antibiotic activity of the wild type (from around Ø18 mm to 12 mm). The latter result is not unexpected in the light of mechanisms of BldD action as a repressor of metabolic processes in the absence of secondary messenger c-di-GMP [13].

Conclusions

Our experiments demonstrate that *S. viridosporus* is a source of an antibiotic other than MOE, whose chemical identity remains to be established. While the production of this antibiotic might distort the interpretation of bioassay-based measurement of MOE production, we reveal also the conditions where the impact of unwanted bioactivity will be absent or minimal. Of the tested regulatory genes, we single out two, *lsr2* (its dominant negative allele) and *bldD* as promising target for deeper studies in MOE producer. BldD is deeply seated regulator of numerous processes, and its knockout in ATCC14672 leads to derailed MOE biosynthesis [13]. Practical use of BldD for strain improvement will require a fine balance of BldD expression and supply of its effector molecules.

This work was supported by grant BG-24F from Ministry of Education and Science of Ukraine (to B.O.). We thank Marie Elliot for kind gift of the plasmid pMC109.

References

1. Ostash B., Makitrynskyy R., Yushchuk O., Fedorenko V. Structural diversity, bioactivity, and biosynthesis of phosphoglycolipid family antibiotics: recent advances. *BBA Adv*. 2022. Vol. 2. P. 100065. https://doi.org/10.1016/j.bbadva.2022.100065.
2. Ostash B., Walker S. Moenomycin family antibiotics: chemical synthesis, biosynthesis, and biological activity. *Nat. Prod. Rep.* 2010. Vol. 27. P. 1594–1617. https://doi.org/10.1039/c001461n.
3. Okeke I. et al. The scope of the antimicrobial resistance challenge. *Lancet*. 2024. Vol. 403. P. 2426–2438. https://doi.org/10.1016/S0140-6736(24)00876-6.
4. Schuricht U. et al. The biosynthesis of moenocinol, the lipid part of the moenomycin antibiotics. *Tetrahedron Lett*. 2001. Vol. 42. P. 3835–3837. https://doi.org/10.1016/S0040-4039(01)00569-X.
5. Augustijn H., Roseboom A., Medema M., van Wezel G. Harnessing regulatory networks in *Actinobacteria* for natural product discovery. *J. Ind. Microbiol. Biotechnol*. 2024. Vol. 51. P. kuae011. https://doi.org/10.1093/jimb/kuae011.
6. Koshla O., Lopatniuk M., Rokytskyy I., Yushchuk O., Dacyuk Y., Fedorenko V., Luzhetskyy A., Ostash B. Properties of *Streptomyces albus* J1074 mutant deficient in $tRNA^{Leu}_{UAA}$ gene *bldA*. *Arch. Microbiol*. 2017. Vol. 199. P. 1175–1183. https://doi.org/10.1007/s00203-017-1389-7.

7. Gehrke E., Zhang X., Pimentel-Elardo S., Johnson A., Rees C., Jones S., Gehrke S., Turvey S., Boursalie S., Hill J., Carlson E., Nodwell J., Elliot M. Silencing cryptic specialized metabolism in *Streptomyces* by the nucleoid-associated protein Lsr2. *Elife*. 2019. Vol. 8. P. e47691. https://doi.org/10.7554/eLife.47691.
8. Tseduliak V., Dolia B., Ostash I., Lopatniuk M., Busche T., Ochi K., Kalinowski J., Luzhetskyy A., Fedorenko V., Ostash B. Mutations within gene *XNR_2147* for TetR-like protein enhance lincomycin resistance and endogenous specialized metabolism of *Streptomyces albus* J1074. *J. Appl. Genet*. 2023. Vol. 64. P. 185–195. https://doi.org/10.1007/s13353-022-00738-4.
9. Siegl T., Luzhetskyy A. Actinomycetes genome engineering approaches. *Antonie Van Leeuwenhoek*. 2012. Vol. 102. P. 503–516.
10. Hindra Elliot M. Multifactorial genetic control and magnesium levels govern the production of a *Streptomyces* antibiotic with unusual cell density dependence. *mSystems*. 2024. Vol. 9. P. e0136823. https://doi.org/10.1128/msystems.01368-23.
11. Wang K., Liu N., Shang F., Huang J., Yan B., Liu M., Huang Y. Activation of secondary metabolism in red soil-derived streptomycetes via co-culture with mycolic acid-containing bacteria. *Microorganisms*. 2021. Vol. 9. P. 2187. https://doi.org/10.3390/ microorganisms9112187.
12. Rempel S., Gati C., Nijland M., Thangaratnarajah C., Karyolaimos A., de Gier J., Guskov A., Slotboom D. A mycobacterial ABC transporter mediates the uptake of hydrophilic compounds. *Nature*. 2020. Vol. 580. P. 409–412. https://doi.org/10.1038/s41586-020-2072-8.
13. Makitrynskyy R., Tsypik O., Nuzzo D., Paululat T., Zechel D., Bechthold A. Secondary nucleotide messenger c-di-GMP exerts a global control on natural product biosynthesis in streptomycetes. *Nucleic Acids Res*. 2020. Vol. 48. P. 1583–1598. https://doi.org/10.1093/nar/gkz1220.

ОСТАШ Б. О., ВОЗНЮК А. В., ЦЕДУЛЯК В.-М. С., ФЕДОРЕНКО В. О.
Львівський національний університет ім. І. Франка,
Україна, 79005, м. Львів, вул. Грушевського, 4

НОВІ ДАНІ ЩОДО РЕГУЛЯЦІЇ СПЕЦІАЛІЗОВАНОГО МЕТАБОЛІЗМУ *STREPTOMYCES VIRIDOSPORUS* ATCC14672

Мета. *Streptomyces viridosporus* ATCC14672 продукує моеноміцини (МОЕ), прямі інгібітори бактерійних пептидогліканових гілкозилтрансфераз у наномолярному діапазоні. Низький титр МОЕ ускладнює їхнє масштабоване очищення, так знижуючи перспективи його трансляційних досліджень. Ми використали генетичний підхід аби дослідити залучення кількох відомих плейотропних регуляторів біосинтезу МОЕ. ***Методи***. Бактерійна генетика та біотести поєднали для конструювання рекомбінантних штамів, що містять додаткові копії вибраних генів, і оцінити їхні ефекти на продукцію МОЕ. ***Результати***. Промотори генів біосинтезу МОЕ містять оператори плейотропних транскрипційних факторів. З кількох досліджених регуляторних генів *bldD* активує продукцію невідомого антибіотика у МОЕ-мінус мутанті, dO5, тоді як у ATCC14672 уведення *bldD* знижує сумарну антибіотичну активність. Домінантно-негативний алель *lsr2* позитивно впливає на антибіотичну активність dO5. ***Висновки***. Наше дослідження виявило нові складові у мережі генів-регуляторів продукції МОЕ. *S. viridosporus* за певних умов здатний продукувати невідомий антибіотик, інактивація якого можна дослідити як спосіб підвищення рівня продукції МОЕ.
Ключові слова: *Streptomyces viridosporus* ATCC14672, антибіотики, гени, регуляція метаболізму.