A Rational Approach to Accessing Bacterial Secondary Metabolites via Pseudo-Interbacterial Communication

Aya Yoshimura

Faculty of Pharmaceutical Sciences, Hokkaido University

Research objective

Bacterial genomes encode numerous biosynthetic gene clusters (BGCs) responsible for the production of secondary metabolites. Advances in genome sequencing and bioinformatics have enabled the estimation of BGC abundance across bacterial taxa. Notably, over 80% of these BGCs remain uncharacterized and are referred to as "silent BGCs." Unlocking the potential of silent BGCs may lead to the discovery of novel secondary metabolites. Several strategies have been developed to activate silent BGCs, including the addition of sub-inhibitory concentrations of antibiotics to cultures and co-culture with other bacterial species. These approaches suggest that mimicking the natural bacterial environment may stimulate silent BGC expression.

Recently, bacterial membrane vesicles (MVs) have been detected in both coastal and open-ocean seawater, indicating that bacteria in natural ecosystems are continuously exposed to these vesicles. MVs are lipid bilayer structures released by nearly all bacteria that carry proteins, DNA/RNA fragments, and small molecules. They have been reported to mediate horizontal gene transfer, nutrient acquisition, and bacterial interactions. Based on these insights, we hypothesized that supplementing bacterial cultures with MVs would activate the production of novel secondary metabolites. Indeed, we previously demonstrated that MVs derived from *Burkholderia multivorans* induced the production of multiple secondary metabolites across approximately 100 bacterial strains[1]. We further propose that MV-induced metabolites, along with the MVs themselves, mediate competitive interactions between *B. multivorans* and *Xenorhabdus innexi*[2]. In the present study, we broadened the scope of this approach by testing diverse combinations of MVs and susceptible strains to identify previously uncharacterized MV-induced metabolites.

Methods

First, we screened bacterial strains that produced high yields of MVs capable of inducing secondary metabolite production. Given that Gram-negative bacteria typically release more MVs than Gram-positive bacteria, approximately 400 domesticated Gram-negative strains from our laboratory collection were tested.

Bacterial strains harboring multiple BGCs were cultured in the presence of these MVs. Culture extracts were prepared under each condition and analyzed by liquid chromatography–mass spectrometry (LC-MS). Metabolomic analyses were conducted to identify the metabolites produced exclusively in MV-supplemented cultures. These results enabled us to evaluate the general potential of MVs to induce secondary metabolism and the distinct induction profiles associated with different MV sources. For the selected hit compounds, large-scale cultures were performed with MV supplementation, followed by compound purification and structure elucidation using analytical techniques such as MS and nuclear magnetic resonance (NMR).

Results

MVs are generally isolated from culture supernatants via ultracentrifugation. From the initial screening of 400 strains in 96-well plates, approximately 20 candidates exhibiting high turbidity in their supernatants were selected for further analysis. These candidates were subsequently screened based on the quantity of MVs recovered through ultracentrifugation. High-yield MV producers were evaluated for their ability to induce metabolite production in *X. innexi*, which showed the highest susceptibility to MVs derived from *B. multivorans*. Strains with MVs that induced at least a three-fold increase in the three target metabolites were selected. In total, nine MV-producing strains were identified, including several *Pseudomonas* spp.

MVs from three selected strains were tested against approximately 50 BGC-rich bacterial strains. Metabolomic analysis revealed that all tested MVs induced diverse metabolite production across multiple bacterial strains (Figure 1). Notably, the profiles of MV-induced metabolites were distinct for each MV source.

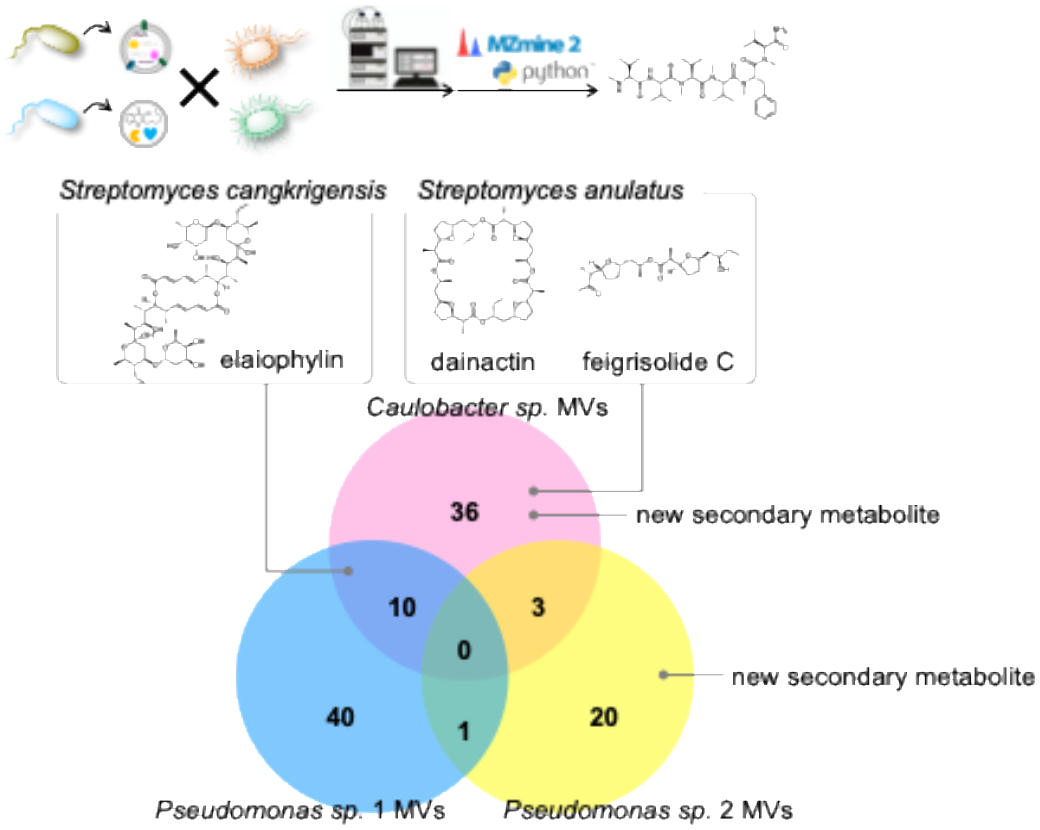

Figure 1. Results in this study.

Among the hit metabolites, MVs from *Pseudomonas* sp. 1 induced the production of elaiophylin, a known polyketide, in *Streptomyces cangkrigensis*. Similarly, MVs from *Caulobacter* spp. induced dainactin and feigrisolide C in *Streptomyces anulatus*, as well as a novel metabolite in *Mycobacterium* spp. Furthermore, MVs from *Pseudomonas* sp. 2 triggered unprecedented metabolite production in *Streptantibioticus cattleyicolor*. However, the chemical structures of these metabolites cannot be determined using conventional methods. To address this, we employed microcrystal electron diffraction (microED), an emerging technique for small-molecule structure elucidation. By analyzing electron diffraction data from the microcrystals of these metabolites, we successfully determined their complete structures, including stereochemistry.

Conclusion

This study demonstrated that, in addition to MVs from *B. multivorans*, several MVs derived from distinct bacterial strains can also induce secondary metabolism in diverse

recipient bacteria. Moreover, the profiles of MV-induced metabolites varied significantly depending on the MV source. Several MV-induced metabolites were successfully isolated and structurally characterized, including novel compounds identified using microED. These findings establish MVs as a general and versatile tool for activating silent BGCs. Expanding the repertoire of MV-susceptible strains is expected to facilitate the discovery of previously uncharacterized secondary metabolites.

References

1) Yoshimura A., Honda T., Wakimoto T. (2024) Five new analogs of streptogramin antibiotic viridogrisein isolated from *Streptomyces niveoruber*. *Chem. Pharm. Bull*. **72:** 80–85.
2) Yoshimura A., Saeki R., Nakada R., Tomimoto S., Jomori T., Suganuma K., Wakimoto T. (2023) Membrane-vesicle-mediated interbacterial communication activates silent secondary metabolite production. *Angew. Chem. Int. Ed*. **62:** e202307304.