UNLOCKING “SILENT” GENES VIA COMBINE CULTURE—AN ALTERNATIVE GATEWAY TO NATURAL PRODUCTS DISCOVERY

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Graphical abstract

Abstract
Streptomyces sp. has been known to produce antibiotics and other bioactive natural products. However, the production of these secondary metabolites depends on the culture conditions, where in most cases the secondary-metabolite genes are not expressed in fermentation culture. Recently, a novel fermentation method known as combined-culture has been introduced to unlock these “silent” genes, hence induces the production of cryptic metabolites. We report herein, our preliminary work on combined-culture using two soil-borne bacterial strains; Streptomyces and Tsukamurella. From the results, it is shown that the presence of Tsukamurella, a mycolic acid-containing bacterium induces the production of new metabolites in Streptomyces. Moreover, the production of compounds associated with Streptomyces was enhanced via combination-culture as compared to culture of Streptomyces strain alone. These findings may promote the feasibility of combined-culture in unlocking the “silent” genes of microorganisms which could lead to the discovery of novel metabolites.

Keywords: Streptomyces, Tsukamurella, combined culture

Abstrak

Kata kunci: Streptomyces, Tsukamurella, pengkulturan-kombinasi

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1.0 INTRODUCTION

Streptomyces species are well known for their great sources of bioactive secondary metabolites such as antifungals, antivirals, antitumors, anti-hypertensives, antibiotics and immunosuppressives [1-6]. Streptomyces belongs to the family of Actinobacteria, a group of Gram-positive whose genetic material is GC-rich (70%) when compared with other bacteria such as Escherichia coli (50%). Until now, two species of Streptomyces have been particularly well studied. They are S. coelicolor, the most widely used in genetic study, and the first Streptomyces used for industrial production of streptomycin, S. griseus [7].

Single-strain culture is the common method for cultivating microorganism. However, most microbial metabolites remained unexplored in fermentation culture as their biosynthetic genes are cryptic. The growth condition in a single-strain flask culture is unlike from the natural environment due to the absence of interacting microorganisms in the natural environment. Production of secondary metabolite is influenced by environmental factors such as temperature, presence of hormone-like chemicals and medium composition [8]. To date, various method have been tried to overcome this limitation [9-11]. Co-culture; a method which use another bacterial strain as an activator in cultivating microorganisms has been developed [12-13]. However, this method usually is specific, due to the specific mutual interaction between the two bacterial strains thus unable to unlock the silent genes.

Preliminary studies by Onaka and co-workers have developed a novel fermentation method named combined-culture to unlock these “silent” genes. This method applied the co-culture of two soil-borne bacterial strains, i.e. Streptomyces and mycolic acid-containing bacteria to induce the production of secondary metabolites [14-15].

In this study, we investigated the interaction between Streptomyces strains isolated from several sources and Tsukamurella, a mycolic acid-containing bacterium via HPLC analysis. The genus Tsukamurella belongs to the family Corynebacteriaceae; where the members of this family show the presence of mycolic acid in the outer layer of the cells.

2.0 EXPERIMENTAL

2.1 Microbes Sources

Streptomyces strain was isolated from soil samples collected from Tako, Tomisato (Chiba prefecture, Japan) and Fujino (Kanagawa prefecture, Japan). Streptomyces cinnamoneus and Tsukamurella pulmonis were purchased from National Institute of Technology and Evaluation, Biological Resource Centre (Japan). Tsukamurella pulmonis was previously isolated by Onaka’s group [14] and gifted by his group.

2.2 Microorganisms Cultivation

All Streptomyces strains and T. pulmonis were separately inoculated into a 500 mL baffled Erlenmeyer flask (Streptomyces) and 500 mL Erlemeyer flask (T. pulmonis), each containing 100 mL of V-22 medium, composed of 1.0% starch, 0.5% glucose, 0.5% Bacto™ Tryptone (Difco), 0.3% NZ case (Wako), 0.2% Yeast Extract (Difco), 0.1% K2HPO4, 0.05% MgSO4 • 7H2O and 0.3% CaCO3 (pH = 7.0). Streptomyces strain was cultured at 30°C for 3 days on a rotary shaker at 220 rpm, and T. pulmonis was cultured using the same method for 2 days.

A 3 mL portion of the Streptomyces culture and 1 mL of the T. pulmonis culture were simultaneously added to a 500 mL baffled Erlenmeyer flask containing 100 mL of A-3M medium, consisting of 2.0% starch, 2.0% glycerol, 0.5% glucose, 1.5% Pharma media (Archer Daniels Midland Co.), 1.0% HP-20 (Nihon Renrusi) and 0.3% Yeast extract (pH = 7.0). In addition, 3 mL of the Streptomyces culture and 1 mL of the T. pulmonis culture were individually inoculated in 500 mL baffled Erlenmeyer flasks containing 100 mL of A-3M medium, as control cultures. All microorganisms were cultured at 30°C for 5.5 days on a rotary shaker at 160 rpm.

The cell pellets were collected after centrifugation of the fermentation broths. The freeze-dried cells were extracted with 50 mL of a CH3OH:CHCl3 mixture (50:50, v/v). The extracts were then subjected to HPLC analysis on HPLC, performed on a 4.6 x 250 mm Cosmosil 5C18-MS-II column (Nacalai Tesque, Kyoto, Japan), in a CH3CN (solvent A)/H2O-containing 1% acetic acid (solvent B) gradient system using a JASCO PU2080 pump to control the flow rate at 1.0 mL/min. All eluates were monitored by UV absorption at respective wavelength (JASCO, Tokyo, Japan, MD-2010 Plus Multiwavelength Detector). The cell culture of Streptomyces alone and Tsukamurella alone were also analyzed with HPLC for comparative study. Selected extracts were subjected to LC-MS analysis on an AP116S machine (Applied Biosytems).

3.0 RESULTS AND DISCUSSION

In this study, the production of compounds from the association of Streptomyces and Tsukamurella was investigated. In addition, the production of compounds from Streptomyces strain alone and T. pulmonis strain alone were also investigated for comparison study.

Combination culture between known species of Streptomyces, i. e. S. cinnamoneus and T. pulmonis (3:1 ratio) was conducted for optimisation of the culture condition (temp. = 30°C for 3 days on rotary shaker at 220 rpm) prior to extraction. A total of 4
major metabolites were produced via this combination (Figure 1).

Subsequently, other *Streptomyces* species of soils origin collected from different areas were cultured with *T. pulmonis*. As shown in Figure 3 - 5, at similar culture condition, the presence of *Tsukamurella* induced the production of secondary metabolites of different *Streptomyces* species. The interaction of *T. pulmonis* with *Streptomyces* via combination culture either enhances the production of known metabolites or “unlocking” the cryptic genes which trigger the production of new metabolites. A total of six metabolites (5 – 10) associated with *Streptomyces* were produced in higher yield with the presence of *T. pulmonis* (Figure 3 and 4).

Compounds 1 - 3 were identified as chromopyrrolic acid, BE-13793C and arcyriaflavin E respectively while compound 4 is the new cytotoxic indolocarbazole alkaloid known as arcyriaflavin A (Figure 2). All compounds were identified using LC-MS and based on comparison with authentic sample [15, 16].

Moreover, three cryptic metabolites (11 – 13) were produced via combined culture of different *Streptomyces* strains with *T. pulmonis* (Figure 4 & 5). All these results indicated the interactions between those two strains (*Streptomyces* and *T. pulmonis*) are not specific thus in nature, the mycolic acid-containing bacteria may affect secondary metabolism in *Streptomyces*, which is one of the most occupant strains of soil. Thus, different *Streptomyces* will produce different metabolites when combined-cultured with *T. pulmonis*.

The isolation and identification of compound 5 – 13 from cultures of *Streptomyces* isolated from respective soils of Tomisato, Fujino and Tako origins with *T. pulmonis* are currently ongoing.
It is evident that the presence of Tsukamurella, a mycolic acid-containing bacterium is able to induce the production of new metabolites in Streptomyces while compounds associated with Streptomyces was enhanced via combination-culture as compared to culture of Streptomyces strain alone.

4.0 CONCLUSION

The combined culture is an easy, fast and effective method in searching for new and novel natural products. This novel method offer feasibility in unlocking the “silent” genes of microorganisms which could lead to the discovery of novel metabolites.

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