Jurnal
Teknologi

Full Paper

UNLOCKING "SILENT" GENES *VIA* COMBINE CULTURE–AN ALTERNATIVE GATEWAY TO NATURAL PRODUCTS DISCOVERY

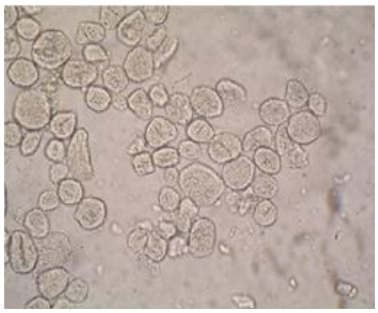
Izzatul Hidayah Azizan[a], Jalifah Latip[a*], Shotaro Hoshino[b], Ikuro Abe[b]

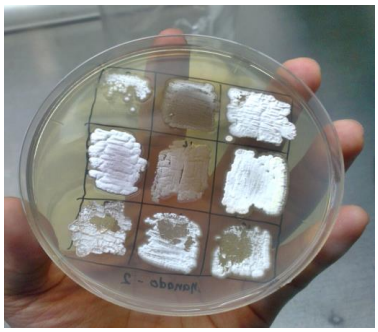
[a]School of Chemical Sciences & Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[b]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

Article history
Received
24 March 2015
Received in revised form
14 August 2015
Accepted
20 August 2015

*Corresponding author
jalifah@ukm.edu.my

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

Streptomyces sp. diketahui menghasilkan antibiotik dan sebatian semula jadi bioaktif yang lain. Walau bagaimanapun, penghasilan sebatian metabolit sekunder ini bergantung kepada keadaan kultur, di mana dalam kebanyakan kes, gen yang berkaitan dengan penghasilan metabolit sekunder tidak diekspresi dalam kultur pengeraman. Baru-baru ini, kaedah pengeraman novel yang dikenali sebagai pengkulturan-kombinasi telah diperkenalkan untuk mengaktif gen tersebut, dan seterusnya menyebabkan penghasilan metabolit kriptik. Kami melaporkan kajian awal ke atas kaedah pengkulturan-kombinasi menggunakan dua strain bakteria tanah; *Streptomyces* and *Tsukamurella.* Keputusan kajian mendapati, kehadiran *Tsukamurella* iaitu sejenis bakteria penghasil asid mikolik menyebabkan penghasilan metabolit baru dalam *Streptomyces*. Tambahan lagi, penghasilan sebatian yang diketahui berkait dengan *Streptomyces* didapati bertambah melalui kaedah pengkulturan-kombinasi berbanding dengan pengkulturan tunggal ke atas strain *Streptomyces*. Keputusan kajian ini menunjukkan kebolehupayaan pengkulturan-kombinasi dalam mengaktif gen mikroorganisma dan seterusnya berpotensi dalam menemukan metabolit novel.

Kata kunci: *Streptomyces*, *Tsukamurella,* pengkukturan-kombinasi

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 Erlenmayer flask *(Streptomyces*) and 500 ml Erlenmayer 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% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.3% $CaCO_3$ (pH = 7.0). *Streptomyces* strain was cultured at 30℃ 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 Erlenmayer 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 Rensui) 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 Erlenmayer flasks containing 100 mL of A-3M medium, as control cultures. All microorganisms were cultured at 30℃ 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 CH_3OH-$CHCl_3$ mixture (50:50, v/v). The extracts were then subjected to HPLC analysis on HPLC, performed on a 4.6 x 250 mm Cosmosil $5C_{18}$-MS-II column (Nacalai Tesque, Kyoto, Japan), in a CH_3CN (solvent A/H_2O-containing 1% acetic acid (solvent B) gradient system using a JASCO PU2080 pump to control the flow rate at 1.0 $mLmin^{-1}$. 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 AP1165 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).

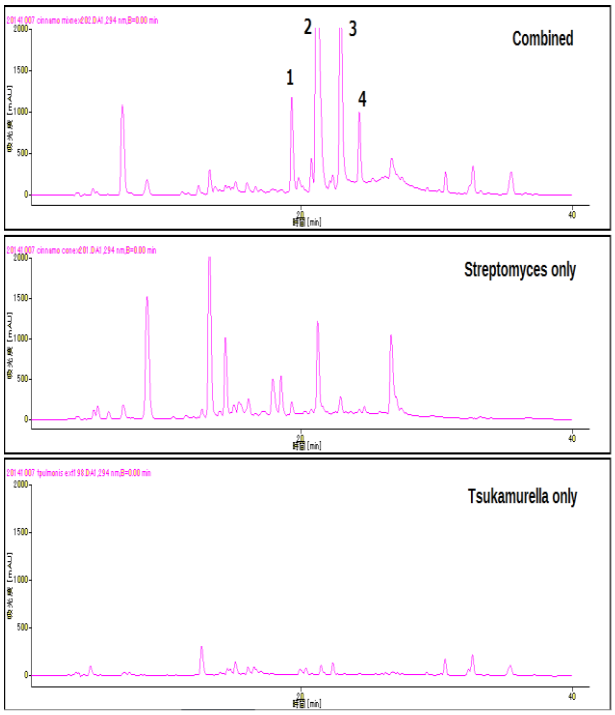


Figure 1 HPLC profiles of the extracts of *Streptomyces cinnamoneus* cultivated with *T. pulmonis*, *S.cinnamoneus* pure culture and *T. pulmonis* pure culture, monitored by UV absorption at 294 nm.

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].

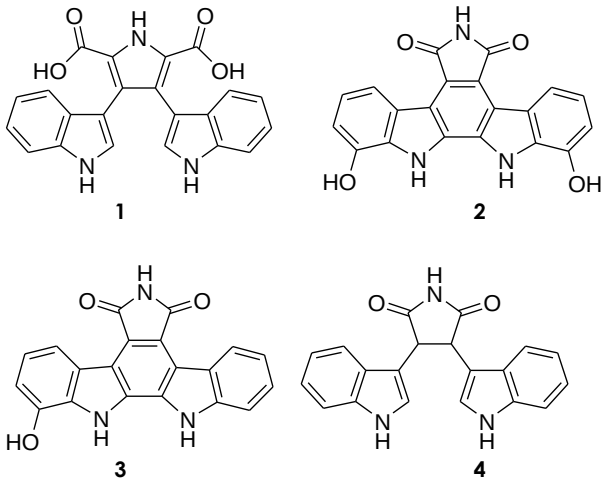


Figure 2 Chemical structures of **1** – **4** produced by combined culture of *S. cinnamoneus* and *T. pulmonis*

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).

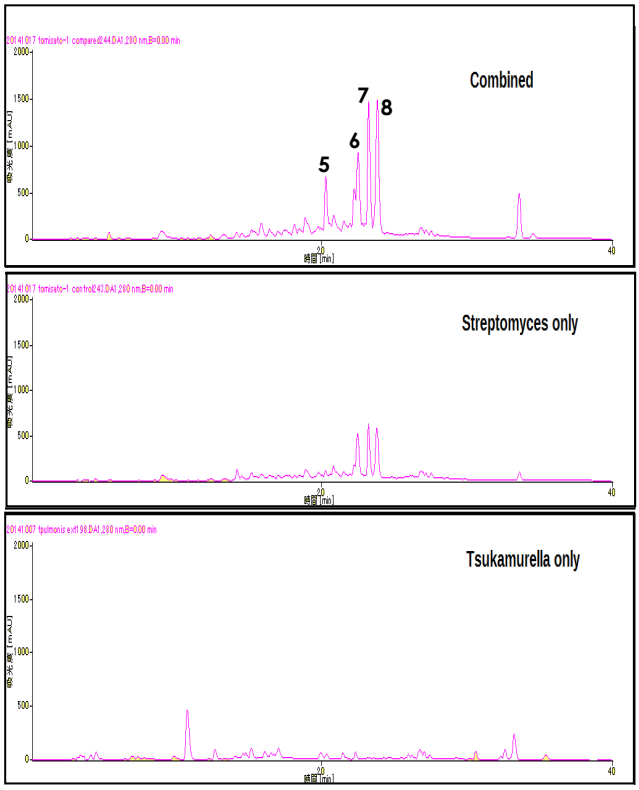


Figure 3 HPLC profiles of extract of *Streptomycesis* isolated from soils of Tomisato origin cultivated with *T. pulmonis*; *Streptomyces* pure culture and *T. pulmonis* pure culture, monitored by UV absorption at 280 nm

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.

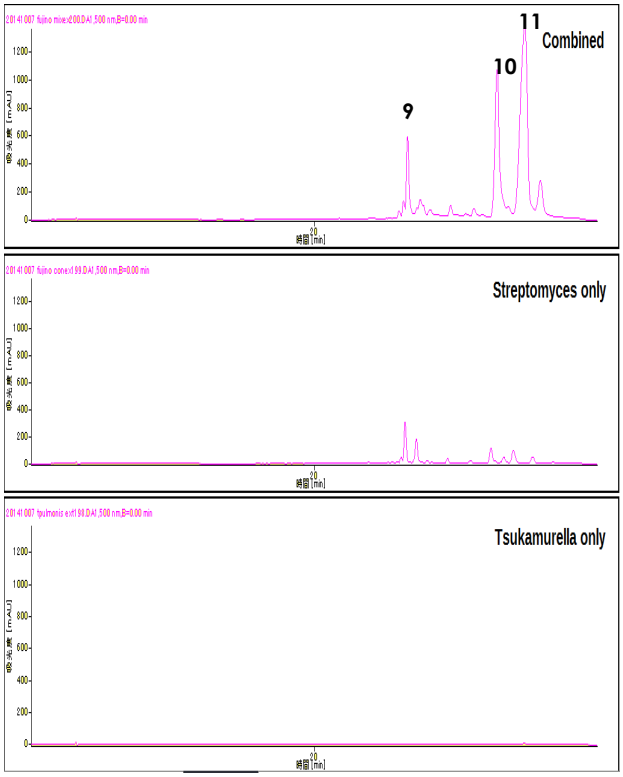


Figure 4 HPLC profiles of extract of *Streptomyces* isolated from soils of Fujino origin cultivated with *T. pulmonis*; *Streptomyces* pure culture and *T. pulmonis* pure culture, monitored by UV absorption at 500 nm

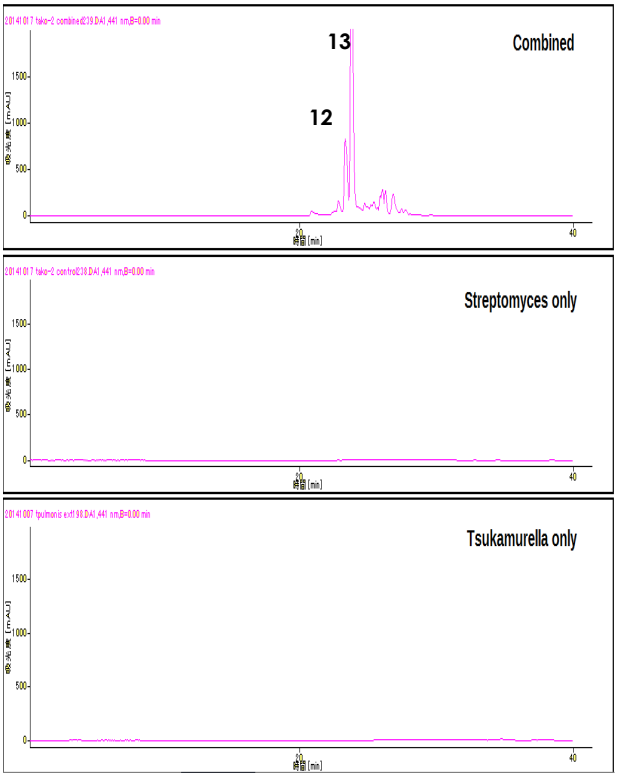


Figure 5 HPLC profiles of extract of *Streptomyces* isolated from soils of Tako origin cultivated with *T. pulmonis*; *Streptomyces* pure culture and *T. pulmonis* pure culture, monitored by UV absorption at 441 nm

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.

Acknowledgement

The International Relation Office, UKM is acknowledged for the financial support during mobility program (IHA) at the Univeristy of Tokyo, Japan. We thank all members of the Laboratory of Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, the University of Tokyo for their helpful guidance and assistance as well as their generosity in providing research consumables and facilities.

References

[1] Arasu, M.V., Duraipandiyan, V. and Ignacimuthu, S. 2013. Antibacterial and Antifungal Activities of Polyketide Metabolite from Marine *Streptomyces sp.* AP-123 and its Cytotoxic Effect. *Chemosphere*. 90(2): 479-87.

[2] Yokomizo, K., Miyamoto, Y., Nagao, K., Kumagae, E., Habib, E.S., Suzuki, K., Harada, S. and Uyeda, M. 1998. Fattiviracin A1, A Novel Antiviral Agent Produced by *Streptomyces microflavus* Strain No. 2445. II. Biological Properties. *The Journal Antibiotics*. 51(11): 1035-1039.

[3] Schleissner, C., Perez, M., Losada, A., Rodriguez, P., Crespo, C., Zuniga, P., Fernandez, R., Reyes, F. de la Calle, F. 2011. Antitumor Actinopyranose Produces by *Streptomyces albus* POR-04-15-053 Isolated from Marine Sediment. *Journal of Natural Products*. 74(7): 1590-1596.

[4] Cioni, J.P., Doroghazi, J.R., Ju, K.-S., Yu, X., Evans, B.S., Lee, J. and Metcalf, W.W. 2014. Cynohydrin Phosphonate Natural Products from *Streptomyces regensis*. *Journal of Natural Products*. 77(2): 243-249.

[5] De Lima Procopio, R. E., da Silva, I. R., Martins, M. K., de Azevedo, J. L. and de Araujo, J. M. 2012. Antibiotic produced by *Streptomyces*. *Brazilian Journal of Infectious Diseasess*. 16(5): 466-471.

[6] Kino, T., Hatanaka, H., Miyata, S., Inamura, N., Nishiyama, M., Yajima, T., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. 1987. FK-506, A Novel Immunosuppressant Isolated from a *Streptomyces*. II. Immunosuppressive Effect of FK-506 in vitro. *The Journal of Antibiotics*. 40(9): 1256-1265.

[7] Waksman, S. A. 1953. Streptomycin: Background, Isolation, Properties and Utilization. *Science*. 118: 259-266.

[8] Scherlach, K. and Hertweck, C. 2009. Triggering Cryptic Natural Product Biosynthesis in Microorganisms. *Organic and Biomolecular Chemistry*. 7(9): 1753-1760.

[9] Hosaka, T., Ohnishi-Kameyama, M., Muramatsu, H., Murakami, K., Tsurumi, Y., Kodani, S., Yoshida, M., Fujie, A. and Ochi, K. 2009. Antibacterial Discovery in

Actinomycetes Strains with Mutations in RNA Polymerase or Ribosomal Protein S12. *Nature Biotechnology*. 27(5): 462-464.

[10] Onaka, H., Tabata, H., Igarashi, Y., Sato, Y. and Furumai, T. 2001. Goadsporin, A Chemical Substance Which Promotes Secondary Metabolism and Morphogenesis in *Streptomycetes*. I. Purification and Characterization. *The Journal of Antibiotics.* (Tokyo). 54(12): 1036-1044.

[11] Onaka, H., Taniguchi, S., Ikeda, H., Igarashi, Y. and Furumai, T. 2003. pTOYAMAcos, pTYM18, and pTYM19, actinomycete-*Eschericia coli* Integrating Vectors for Heterologous Gene Expression. *The Journal of Antibiotics*. (Tokyo). 56(11): 950-956.

[12] Cueto, M., Jensen, P.R., Kauffman, C., Fenical, W., Lobkovsky, E. and Clardy, J. 2001. Pestalone, A New Antibiotic Produced by a Marine Fungus in Response to Bacterial Challenge. *Journal of Natural Products*. 64(11): 1444-1446.

[13] Schroeckh, V., Scherlach, K., Nutzmann, H.W., Shelest, E., Schmidt-Heck, W., Schuemann, J. Martin, K., Hertweck, C. and and Brakhage, A.A. 2009. Intimate Bacterial-fungal Interaction Triggers Biosynthesis of Archetypal Polyketides in Aspergillus *nidulans*. *Proceedings of The National Academy of Sciences*. U. S. A. 106(34): 14558-14563.

[14] Onaka, H., Mori, Y., Igarashi, Y. and Furumai, T. 2011. Mycolic Acid Containing Bacteria Induce Natural Product Biosynthesis in *Streptomyces* species. *Applied and Environmental Microbiology*. 77(2): 400-406.

[15] Hoshino, T., Kojima, Y., Hayashi, T., Uchiyama, T. and Kaneko, K. 1993. A New Metabolite of Tryptophan, Chromopyrrolic Acid, Produced by *Chromobacterium violaceum*. *Bioscience Biotechnology and Biochemistry*. 57(5): 775-781.

[16] Hoshino, S., Zhang, L., Awakawa, T., Wakimoto, T., Onaka, H. and Abe, I. 2015. Arcyriaflavin E, A New Cytotoxic Indolocarbazole Alkaloid Isolated by Combined-Culture Of Mycolic Acid-Containing Bacteria and *Streptomyces cinnamoneus* NBRC 13823. *The Journal of Antibiotics*. 68: 342-344.