The Journal of Antibiotics (2011) 64, 699–701

www.nature.com/ja

## COMMUNICATION TO THE EDITOR

# New species of actinomycetes do not always produce new compounds with high frequency

*The Journal of Antibiotics* (2011) **64,** 699–701; doi:10.1038/ja.2011.66; published online 27 July 2011

Actinomycetes have been extensively studied for their ability to produce pharmaceutically useful compounds, however, the rate of discovery of novel compounds from these bacteria has significantly decreased. New species of actinomycetes have attracted considerable attention as one of the most important resources for new bioactive compounds, but there is much controversy regarding whether these species of actinomycetes will produce new compounds with high frequency. To investigate this problem, we isolated new and known species of actinomycetes from a variety of substrates and examined the secondary metabolites produced, including potential novel compounds.

We isolated many actinomycetes from diverse resources, including terrestrial soils, mangrove soils, lichen and marine sponges, using the previously reported methods including sodium dodecyl sulfate-yeast extract method,[1] moist incubation and desiccation method,[2] membrane filter method[3] and other methods,[4] based on colony morphology. The culture isolates were identified by comparison of their 16S rRNA gene sequences with sequences in the Eztaxon[5] type strain database. The 106 strains with less than 98.5% similarity in 16S rRNA gene sequences were selected as new species of strains belonging to seven different genera (*Streptomyces*, *Agromyces*, *Gordonia*, *Nocardia*, *Nonomuraea*, *Pseudonocardia* and *Rhodococcus*). The 241 strains that showed high sequence similarity ($\geqslant$98.5%) with those in the database were picked randomly and were determined to belong to 19 different genera (Table 1).

These strains (347 strains) were cultivated in a 15-ml test tubes containing 5 ml of five production media[4,6–9] reported previously. In addition, 50% artificial seawater was used to make the media for strains isolated from marine substrates. The strains were cultured on a reciprocal shaker (320 r.p.m.) at 27 °C for 3 days. We selected butanol as an extract solvent, because butanol has a wide solubility range from hydrophilic to hydrophobic compounds, but remove high molecular compounds such as polysaccharides and proteins in order to separate into organic and water layer. The cultures were extracted with 5 ml of butanol and mixed at high speed for 30 min. After centrifugation, the butanol layers were evaporated, and the dried residues were dissolved with 150 µl of DMSO and were filtered. The 2 µl of DMSO solutions were analyzed by a UPLC-HR-electrospray ionization-MS system (Waters LCT-Premier XE, Milford, MA, USA) using an Acquity UPLC BEH $C_{18}$ column (1.7 µm, 2.1 i.d. ×100 mm; Waters) developed using an $CH_3CN$-$H_2O$ gradient system (from 5% aqueous $CH_3CN$ to 100% $CH_3CN$ for 5 min, 100% $CH_3CN$ for 1 min) containing 0.1% formic acid (flow rate, 0.8 ml $min^{-1}$) with our in-house natural product database. The peaks that were inconsistent with those in the database were selected, and those unknown compounds were purified from the large-scale cultures (1 to 2 l cultures in 500 ml Erlenmeyer flasks containing 100 ml of production medium). The structures of these compounds were determined mainly by HR-MS and NMR spectral analyses.

Of the 106 new species, seven strains belonging to *Streptomyces* produced 10 new compounds (Table 2). Two new anthracyclines, tetracenoquinocin and 5-iminoaranciamycin,[6] which showed cytotoxic activity, were isolated from the culture of Sp080513GE-26. Two new piperazic acid-containing hexapeptides, JBIR-39 and JBIR-40,[10] were isolated from Sp080513SC-24. Furthermore, we isolated new peptides, JBIR-56 and JBIR-57,[11] from RA42; a new hygrolidin derivative, JBIR-72 (manuscript in preparation), from RF31; a new butenolide, JBIR-89,[7] from RJ14; a new 1,1-dichlorocyclopropane-containing angucycline, JBIR-88,[7] which showed cytotoxic and antimicrobial activities, from RJ15; and a new antibiotic E 492 derivative, JBIR-108 (manuscript in preparation), from RJ76.

Of the 241 known species, 11 strains were *Streptomyces* species, one strain belonging to the genus *Promicromonospora* (RL26) and one belonging to the genus *Saccharopolyspora* (RL78). A total of 25 new compounds were found to be produced by these strains (Table 2). From the culture of strain RI18, we isolated two novel benzastatin derivatives, JBIR-67 and JBIR-73,[8] as well as JBIR-68,[12] a compound with anti-influenza virus activity, structure of which contained a dihydrouridine and geraniol connected by an ether bond to clarify that the structural data relates

**Table 1 Culture isolates**

| | *Number of known species* ($\geqslant$*98.5%*)[a] | *Number of new species* ($<$*98.5%*)[a] |
|---|---|---|
| *Streptomyces* | 189 | 96 |
| *Actinoplanes* | 1 | |
| *Agromyces* | | 1 |
| *Amycolatopsis* | 2 | |
| *Dactylosporangium* | 1 | |
| *Gordonia* | 2 | 2 |
| *Kitasatospora* | 3 | |
| *Micromonospora* | 4 | |
| *Mycobacterium* | 1 | |
| *Nocardia* | 18 | 3 |
| *Nonomuraea* | 1 | 2 |
| *Planotetraspora* | 1 | |
| *Pseudonocardia* | 3 | 1 |
| *Promicromonospora* | 3 | |
| *Rhodococcus* | 6 | 1 |
| *Saccharopolyspora* | 1 | |
| *Saccharothrix* | 1 | |
| *Streptosporangium* | 1 | |
| *Sphaerisporangium* | 3 | |
| *Sphingomonas* | 1 | |
| Total | 241 | 106 |

[a]Similarity in 16S rRNA gene sequences.

**Table 2 The producers of new compounds**

| *Strain* | *Substrate* | *New compound* | *Reference* |
|---|---|---|---|
| *New species* | | | |
| *Streptomyces* sp. Sp080513GE-26 | Marine sponge | Anthracycline; Tetracenoquinocin, 5-imino-aranciamycin | 6 |
| *Streptomyces* sp. Sp080513SC-24 | Marine sponge | Piperazic acid-containing hexapeptides; JBIR-39, JBIR-40 | 10 |
| *Streptomyces* sp. RA42 | Marine sponge | Pyrazinone-containing peptides; JBIR-56, JBIR-57 | 11 |
| *Streptomyces* sp. RF31 | Mangrove soil | Hygrolidin derivative; JBIR-72 | In preparation |
| *Streptomyces* sp. RJ14 | Lichen | Butenolide derivative; JBIR-89 | 7 |
| *Streptomyces* sp. RJ15 | Lichen | Angucycline; JBIR-88 | 7 |
| *Streptomyces* sp. RJ76 | Bark | Antibiotic E 492 derivative; JBIR-108 | In preparation |
| *Known species* | | | |
| *Streptomyces* sp. RI18 | Soil | Benzastatin derivatives; JBIR-67, JBIR-73, dihydrouridine derivative; JBIR-68 | 8,12 |
| *Streptomyces* sp. RI19 | Soil | Promothiocin derivatives; JBIR-83 and JBIR-84 | 13 |
| *Streptomyces* sp. RI24 | Soil | Naphthablin derivatives; JBIR-79 and JBIR-80 | 9 |
| *Streptomyces* sp. RI33 | Soil | Angucyclines; JBIR-90, JBIR-91, JBIR-92, JBIR-93, JBIR-116 | 14 |
| *Streptomyces* sp. RI77 | Soil | Naphtoquinone derivatives; JBIR-76, JBIR-77, JBIR-85 | In preparation |
| *Streptomyces* sp. RL23 | Mangrove soil | JBIR-94 (*N,N*′-(butane-1,4-diyl)bis(3-(4-hydroxy-3-methoxyphenyl)propanamide)) | In preparation |
| *Streptomyces* sp. RL66 | Mangrove soil | JBIR-94 (*N,N*′-(butane-1,4-diyl)bis(3-(4-hydroxy-3-methoxyphenyl)propanamide)) | In preparation |
| *Streptomyces* sp. RM23 | Mangrove soil | Indole derivative; JBIR-112 | In preparation |
| *Streptomyces* sp. RM50 | Marine sponge | Piericidin derivative; JBIR-105, JBIR-106 (13-(o-tolyl)tridec-12-enoic acid) | In preparation |
| *Streptomyces* sp. RM72 | Marine sponge | Trichostatins; JBIR-109, JBIR-110, JBIR-111 | In preparation |
| *Streptomyces* sp. RJ09 | Soil | Phenylacetylated pentapeptide; JBIR-96 | 15 |
| *Promicromonospora* sp. RL26 | Mangrove soil | Macrocyclic dilactone; JBIR-101 | 16 |
| *Saccharopolyspora* sp. RL78 | Mangrove soil | Cyclizidine derivative; JBIR-102 | In preparation |

to JBIR-68 and not to yet another new compound. New promothiocin derivatives, JBIR-83 and JBIR-84,[13] and new naphthablin analogs, JBIR-79 and JBIR-80,[9] which protected neuronal hybridoma N18-RE-105 cells from L-glutamate toxicity, were isolated from RI19 and RI24, respectively. Strain RI33 produced five new angucyclines, JBIR-90, JBIR-91, JBIR-92, JBIR-93 and JBIR-116,[14] which showed cytotoxic activity against malignant pleural mesothelioma cells. We also reported a new peptide, JBIR-96,[15] obtained from RJ09. Furthermore, we isolated 12 novel compounds from six strains of *Streptomyces*, one strain of *Promicromonospora* and one strain of *Saccharopolyspora* (all these manuscripts are in preparation).

Taken together, the percentage of new compound producers (7) to new species (106) was 6.6%, new compounds (10) to new species (106) was 9.4%, new compound producers (13) to known species (241) was 5.4%, and new compounds (25) to known species (241) was 10.4%. Thus, there was no significant difference between the discovery of new metabolites between new and previously known species. These results indicate that new actinomycetes species do not always produce new compounds with high frequency. On the other hand, *Streptomyces* species that have high similarity to known species produced compounds with diverse structures (Table 2). Because recent advances in genome research have revealed that the ability of actinomycetes to produce secondary metabolites has been underestimated due to the presence of cryptic biosynthetic gene clusters, we attempted to culture these strains with several production media, but the production of new metabolites by these strains was not remarkably improved by changing production media. The strains that originated from marine sponge, mangrove soil, lichen and bark produced many new compounds (Table 2). In addition, JBIR-67, JBIR-68, JBIR-73, JBIR-83 and JBIR-84 were purified from the cultures of RI18 and RI19, isolated from soil samples using the membrane filter method,[8,12,13] which allows for selection of actinomycetes without antibiotics, unlike conventional selection methods. In conclusion, actinomycetes, especially *Streptomyces*, should still be considered attractive sources of bioactive compounds, and it is important to isolate actinomycetes from a wide variety of environmental substrates by using various isolation methods for obtaining new bioactive compounds.

## ACKNOWLEDGEMENTS

This work was supported in part by a grant from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

**Motoki Takagi[1] and Kazuo Shin-ya[2]**

*[1]Biomedicinal Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), Tokyo, Japan and [2]Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan*
*E-mail: k-shinya@aist.go.jp or motoki-takagi@aist.go.jp*

1 Hayakawa, M. & Nonomura, H. A new method for the intensive isolation of actinomycetes from soil. *Actinomycetologica* **3,** 95–104 (1989).
2 Matsukawa, E., Nakagawa, Y., Iimura, Y. & Hayakawa, M. A new enrichment method for the selective isolation of *Streptomycetes* from the root surfaces of herbaceous plants. *Actinomycetologica* **21,** 66–69 (2007).
3 Nagai, A., Khan, S. T., Tamura, T., Takagi, M. & Shin-ya, K. *Streptomyces aomiensis* sp. nov., a novel species of *Streptomyces* isolated from a soil sample using the membrane filter method. *Int. J. Syst. Evol. Microbiol.* **61,** 947–950 (2011).
4 Khan, S. T. *et al. Streptomyces* associated with a marine sponge *Haliclona* sp.; biosynthetic genes for secondary metabolites and products. *Environ. Microbiol.* **13,** 729–731 (2011).
5 Chun, J. *et al.* EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **57,** 2259–2261 (2007).
6 Motohashi, K., Takagi, M. & Shin-ya, K. Tetracenoquinocin and 5-iminoaranciamycin from a sponge-derived *Streptomyces* sp. Sp080513GE-26. *J. Nat. Prod.* **73,** 755–758 (2010).

7 Motohashi, K., Takagi, M., Yamamura, H., Hayakawa, M. & Shin-ya, K. A new angucycline and a new butenolide isolated from lichen-derived *Streptomyces* spp. *J. Antibiot.* **63,** 545–548 (2010).
8 Motohashi, K., Nagai, A., Takagi, M. & Shin-ya, K. Two novel benzastatin derivatives, JBIR-67 and JBIR-73, isolated from *Streptomyces* sp. RI18. *J. Antibiot.* **64,** 281–283 (2011).
9 Izumikawa, M., Nagai, A., Hashimoto, J., Takagi, M. & Shin-ya, K. Isolation of 2 new naphthablin analogs, JBIR-79 and JBIR-80, from *Streptomyces* sp. RI24. *J. Antibiot.* **63,** 729–731 (2010).
10 Kozone, I. *et al.* Isolation of new hexapeptides—JBIR-39 and JBIR-40—from a marine sponge-derived *Streptomyces* sp. Sp080513SC-24. *J. Marine Sci. Res. Develop.* **1,** 1000101 (2011).
11 Motohashi, K. *et al.* JBIR-56 and JBIR-57, 2(1*H*)-pyrazinones from a marine sponge-derived *Streptomyces* sp. SpD081030SC-03. *J. Nat. Prod.* (2011) (e-pub ahead of print, doi:10.1021/np200386c).
12 Takagi, M. *et al.* Anti-influenza virus compound from *Streptomyces* sp. RI18. *Org. Lett.* **12,** 4664–4666 (2010).
13 Takagi, M., Motohashi, K., Nagai, A., Hashimoto, J. & Shin-ya, K. JBIR-83 and JBIR-84, new promothiocin derivatives, isolated from *Streptomyces* sp. RI19. *J. Antibiot.* **63,** 405–408 (2010).
14 Ueda, J. Y. *et al.* New angucycline C-glycosides from Streptomyces sp. RI33. *J. Antibiot.* **64,** 367–372 (2011).
15 Ueda, J. Y. *et al.* A phenylacetylated peptide, JBIR-96, isolated from *Streptomyces* sp. RI051-SDHV6. *J. Nat. Prod.* **74,** 1344–1347 (2011).
16 Izumikawa, M., Takagi, M. & Shin-ya, K. Isolation of a novel macrocyclic dilactone–JBIR-101—from Promicromonospora sp. RL26. *J. Antibiot.* **64,** 689–691 (2011).