Research Paper

Cultivable soil actinomycete communities in some areas of western China

ABSTRACT

In order to obtain much more unknown actinomycetes for discovery of new drug leads and other industry products, 1065 soil samples were collected from 8 areas in west of China. 8159 purified cultures of actinobacteria were isolated from these samples by using 27 media. The 16S rRNA gene sequences of 1064 strains were analyzed, and the phylogenetic analysis was carried out. Cultivable actinomycete communities in different areas and vegetations were analyzed. Total 64 genera of the class actinobacteria were identified. From research results, it is showed that actinomycete communities in tropical rain forest of Xishuangbanna is the highest compared with all studied areas in this study, and that of secondary forest were relative monotonous. A large number of unknown actinomycetes exist in saline and alkaline soil in Qinghai and Xinjiang. Selective isolation methods for unknown actinomycetes from soil samples, including medium, inhibitors and pretreatment are discussed in this paper.

Key words: Actinomycete communities, western China.

INTRODUCTION

Actinomycetes (Actinobacteria) have been paid a great attention owing to their production of various natural drugs and other bioactive metabolites including antibiotics, enzyme inhibitors and enzymes for a long time. Over 25000 bioactive secondary metabolites (including antibiotics) were published in the scientific and patent literature, and about a half of them were produced by actinomycetes. Now about 150 antibiotics have being applied in human therapy and agriculture, 100-120 of them were produced by actinomycetes (Berdy, 2005). Actinomycete is still an important source for development of bioactive compounds (Goodfellow and Fiedler, 2010; Raja and Prabekarana, 2011; Tiwari and Gupta, 2012). Baltz showed a proposition of "renaissance in antibacterial discovery from actinomycetes" (Baltz, 2008). However, the development of new drugs from actinomycetes and other microbes and plants is more and more difficult in the whole world due to too many known compounds and microbes. In order to overcome these challenges, some new concepts based on genome were described, including "new habitats, new methods, new species, new gene cluster, new products and new use" (Goodfellow and Fiedler, 2010; Jensen, 2010; Jiang et al., 2009; Xu et al., 2010). In other words, novel microbial species should contain new gene cluster synthesizing new secondary metabolites, as long as getting new species is an important premise for obtaining new compounds. Many companies and laboratories focused on new actinomycete resources from new habitats, such as oceans, extreme environment, plants and animals, to develop new drugs.

In recent years, research works on uncultivable microorganisms has been carried out in many laboratories (Hughes et al., 2001; Joseph et al., 2003; Zengler et al., 2002). But in our view, obtaining pure cultural actinomycetes is still an important premise and new hope for discovery of novel compounds for drug development. Since 2003, a large number of test samples were collected from 8 selective areas of western China. Actinomycetes were isolated and identified from these samples.

MATERIALS AND METHODS

Collection and preparation of soil samples

A total of 1065 soil samples were collected from 8 selective
Figure 1. Sites of sampling areas in China. 1, Kanasi Integrated Nature Landscape Protect Region; 2, Grand Shangri-La; 3, Jiuzhaigou National Nature Protect Region; 4, Emei and Qingcheng Mountains; 5, Huangjing National Nature Protect Region; 6, Wulin Mountain Chain National Nature Protect Region; 7, Xishuangbanna National Nature Protect Region; 8, Saline and alkaline soil in Qinghai and Xinjiang.

areas of western China (Figure 1 and Table 1) from 2003 to 2012. Each sample of mixture was obtained by mixing the soil collected from 5 to 10 sampling holes with depth range from 5 to 20 cm. The samples were put in sterile glass dish immediately, and dried for 10 days at 28°C. Two grams of each dried sample were pre-treated at 80 or 100°C for 1 h, respectively put in 18 ml sterile water containing 0.1 % Na₄P₂O₅, and shaken for 60 min at 220 rpm/min. The suspension was diluted from 10⁻² to 10⁻⁵ which solution was used for dilution.

Isolation media of actinobacteria

The following 5 media were used for isolating common actinobacteria in soil samples:

**YIM 7 = HV medium (Hayakawa and Nonomura, 1987)**

Humic acid 1 g, Na₂HPO₄ 0.5 g, KCl 1.7 g, MgSO₄·7H₂O 0.05 g, FeSO₄·7H₂O 0.01 g, CaCl₂ 1 g, B-vitamins (0.5 mg each of thiamine-HCl, riboflavin, Niacin, pyridoxin, Ca-pantothenate, inositol, p-aminobenzoic acid, and 0.25mg of biotin), agar 18 g, water 1000 ml, pH 7.4.

**YIM 171 = Improvement Glycerol-Asparagine medium**

Glycerol 10 g, asparagine 1 g, K₂HPO₄·H₂O 1 g, MgSO₄·7H₂O 0.5 g, CaCO₃ 0.3 g, vitamin mixture of HV medium 3.7 mg, agar 15 g, water 1000 ml, pH 7.2.

**YIM 212 = Mycose-Proline medium**

Mycose 5 g, proline 1 g, (NH₄)₂SO₄ 1 g, NaCl 1 g, CaCl₂ 2 g, K₂HPO₄·H₂O 1 g, MgSO₄·7H₂O 1 g, vitamin mixture of HV medium 3.7 mg, agar 15 g, water 1000 ml, pH 7.2.

**YIM 37 = Raffinose-Histidine medium**

Raffinose 5 g, histidine 1 g, K₂HPO₄·H₂O 1 g, MgSO₄·7H₂O 0.5 g, Agar 15 g, water 1000 ml, pH 7.2 ~ 7.4.

**Improvement HVG**

Humic acid 1.0 g, keratin 0.5 g, CaCl₂ 0.3 g, 10 mM 3-N-Morpholino propanesulfonic acid (MOPS), trace salts 0.1 ml, Gellan Gum 7 g, water 1000 ml, pH 7.2 ~ 7.4.

All of media were supplemented with filter sterilized potassium dichromate 50 mg, or mixture solution of 100 mg cycloheximide, 50 or 100 mg nystatin and 20 or 40 mg nalidixic acid, or K₂Cr₂O₇ 50 or 75 mg for 1000 ml medium, as inhibitors against fungi and Gram negative bacteria.

The following 22 media were used for isolating special group of actinobacteria:

**YIM 1**

Gauze No.1: Sol. starch 20 g; KNO₃ 1g; K₂HPO₄ 0.5 g; MgSO₄·7H₂O 0.05 g; FeSO₄ 10 mg; Agar 20 g; pH 7.2 ~ 7.4. 8h 30 min.

**YIM 2**

Glucose-asparagine agar: Glucose 10 g; asparagines 0.5 g; K₂HPO₄ 0.5 g; agar 15 g; pH 7.2 ~ 7.4. 8h 30 min.

**YIM 3**

Casein agar (for isolation of thermophilic actinomycetes): Glucose 10 g; casein 1 g; K₂HPO₄ 0.5 g; MgSO₄·7H₂O 0.5 g; FeSO₄ 10 mg; Agar 20 g; pH 7.2.

**YIM 4**

Chitin agar (Hsu and Lockwood, 1975. for isolation of aquatic act): Colloidal chitin 2 g; K₂HPO₄ 0.7 g; KH₂PO₄ 0.3
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YIM 5

Arginin agar: Arginin 1 g; glycerol 12.5 g; MgSO₄·7H₂O 0.5 g; FeSO₄·10mg; K₂HPO₄ 1g; Trace salt 1 ml; Agar 20 g; pH 7.2; 8° 30 min.
Trace salt: FeSO₄·7H₂O 0.2 g; MnCl·2H₂O 0.1 g; ZnSO₄·7H₂O 0.1 g; water 100 ml.

YIM 6

Starch-casein agar (Kiisber and Williams, 1964): Sol. starch 10 g; casein 0.3 g; KNO₃ 2 g; MgSO₄·7H₂O 0.05 g; NaCl 2 g; K₂HPO₄ 2 g; CaCO₃ 0.02 g; FeSO₄ 10 mg; agar 20 g; pH 7.2~7.4.

YIM 7

HV agar (Hayakawa, 1990): Humic acid 1.0 g; Na₂HPO₄ 0.5 g; KCl 1.7 g; MgSO₄·7H₂O 0.05 g; FeSO₄·7H₂O 0.01 g; CaCl₂ 1 g; agar 18 g; B-vitamins (0.5 mg each of thiamine-HCl (B1), riboflavin, Niacin, pyridoxin, Ca-pantothenate, inositol, p-aminobenzoic acid and 0.25 mg of biotin ), pH7.2.

YIM 8

T/1 medium (for isolation of thermophiles) (Graveri and Pagani, 1962): Yeast ext. 2 g; soybean meal 5 g; crude maltose 20 g; agar 20-30 g; pH 7.2.

YIM 9

GG agar (for isolation of aquatic act): Glycerol 20 g; glycine 2.5 g; NaCl 1 g; K₂HPO₄ 1 g; FeSO₄ 10 mg; MgSO₄·7H₂O 0.1 g; CaCO₃ 0.1 g; agar 17 g; pH7.4.

YIM 10

GYEA 0 (for isolation of Actinomadura): Glucose 10 g; yeast ext. 5 g; CaCO₃ 2 g; k₂HPO₄ 1 g; MgSO₄·7H₂O 0.5 g; trace salt 1 ml; agar 20 g; pH7.2.

YIM 11

AV agar: Glycerol 5 g; arginin 0.5 g; glucose 1 g; K₂HPO₄ 0.3 g; MgSO₄·7H₂O 0.2 g; NaCl 0.3 g; B-vitamin (same as No.7 agar); agar 20 g, pH7.2.

YIM 12

Improvement casein agar: Casein 2 g; glucose 2 g; (NH₄)₂SO₄ 2.64 g; K₂HPO₄ 5.65 g; KH₂PO₄ 2.38 g; yeast ext. 0.1 g; CoCl₂ 0.01 g; Na₂B₄O₇·10H₂O 0.1 g; trace salt 1 ml; agar 20 g.

YIM 13

17+18 agar: Glycerol 5 g; asparagine 0.5 g; K₂HPO₄ 0.5 g; yeast ext. 0.5 g; malt ext. 2 g; glucose 1 g; trace salt 1 ml; agar 20 g; pH7.2.

YIM 14

Czapek's agar: Sucrose 30 g; NaNO₃ 2 g; K₂HPO₄ 1 g; MgSO₄·7H₂O 0.5 g; KCl 0.5 g; FeSO₄ 0.01 g; agar 20 g; pH 7.2-7.4; 8° 30 min.

YIM 15

Calcium Malate agar: Glucose 20 g; Cacium malate 10 g; NH₄Cl 0.5 g; agar 15 g; pH 7.2 - 7.4. 8° 30 min.

YIM 16.

Bennette agar: Glucose 10 g; yeast ext 1 g; beef ext 1 g; N-Z-Amine A (casein) 2 g; agar 20 g, pH 7.3.

YIM 17

Glycerol asparagines (ISP 5): L-asparagine 1g; glycerol 10g; K₂HPO₄ 1g; trace salt 1ml; agar 20g; PH 7.2-7.4.

YIM 18

Yeast ext-Malt ext agar (ISP 2): Yeast ext 4 g; glucose 4 g; malt ext 10 g; agar 20 g; pH 7.3.

YIM 19

Peptone-yeast ext-Fe agar (ISP 6): Peptone 15 g; Ferric ammonium citrate 0.5 g; sodium thiosulfate 0.09 g; K₂HPO₄ 1 g; yeast ext 1 g; agar 15 g; pH 7-7.2.

YIM 20

Tyrosine agar (ISP7): Glycerol 15 g; L-asparagine 1 g; L-tyrosine 0.5 g; K₂HPO₄ 0.5 g; NaCl 0.5 g; MgSO₄·7H₂O 0.5 g; FeSO₄·7H₂O 0.01 g; trace salt 1 ml; agar 20 g; pH 7.2.
YIM 24

Starch casein agar (Meiji Sheika 5#): Starch 5 g; casein 0.5 g; K₂HPO₄ 0.25 g; agar 20 g; pH 7.0-7.5.

YIM 25

Inorganic salt-starch agar (ISP 4)
Sol starch 10 g; K₂HPO₄ 1 g; MgSO₄·7H₂O 1 g; NaCl 1 g; (NH₄)₂SO₄ 2 g; CaCO₃ 2 g; trace salt 1 ml; agar 20 g, pH 7-7.4.

Isolation of halophilic and alkalophilic actinobacteria

Media for isolation of halophilic and alkalophilic actinobacteria: 1, starch-casein medium; 2, YIM 171 medium; 3, Soil extract medium; 4, Horikoshi-1 medium.

pH of media were adjusted to 12 by NaCO₃ and NaOH for isolation alkalophilic actinobacteria; 10% to 25% of complex salt solution including NaCl₂, KCl, K₂SO₄, MgCl₂, MgSO₄·7H₂O, CaCO₃ and CaSO₄ were supplied for isolation of halophilic actinobacteria.

Plate dilution method was used for selective isolation of actinobacteria from the sample suspension. 0.2 ml of suspensions of each sample were inoculated on the agar plates, and cultivated for 7 to 35 days at 28°C, then take count of colonies, and pick up actinobacteria to slant with the same isolation medium.

Identification of pure cultivated actinobacteria

A total of 8159 pure strains were isolated from the 1068 soil samples. Representative 1064 strains were selected for further steps based on morphological and cultural characteristics. The genomic DNA of pure strains was extracted for 16S rDNA analysis (Orsini and Romano-Spica, 2001). PCR amplification of the 16S rDNA, purification and sequence of the PCR products were done as described in Cui et al. (2001). The forward primer F8 (8 ± 27), 5’-GAG AGT TTG ATC CTG GCT CAG-3’ and the reverse primer (1510 ± 1492), 5’-GGT TAC CTT GTT ACG ACT T-3’ were used. The sequences were manually aligned with available sequences from public databases. Phylogenetic trees were inferred by using the neighbour-joining (Saitou and Nei, 1987) and maximum-likelihood methods (Felsenstein, 1981). All pure cultivated strains were identified at a genus level.

RESULTS AND DISCUSSION

A brief introduction of sampling areas and results of actinomycete isolation are summarized in Table 1.

Kanasi National Nature Protect Region

Kanasi National Nature Protect Region is located in the northern edge of Xinjiang, is contiguous to Kazakhstan, Russia and Mongolia, and only belongs to the Arctic Ocean river system in China. Annual mean temperature at 1200 m is 0°C. The absolute protect region is 188.5 thousands hectare. Primitive vegetation has been kept best. There are about 1000 species of plants, 300 of insects, 100 of birds and 34 wild beasts. The main pick, Friendship, is 4374 m high. Zone over 3200 m is Permafrost. Zone between 3200 ~ 2500 m belongs to grassy marshland. Between 2500 - 2300 m is grassy marshland and bush, and between 2400 ~ 1200 m is coniferous forest. Fifty soil samples were collected from restrict between 1450 ~ 3200 m.

Thirteen genera of actinobacteria were identified. Actinomadura, Agroccoccus, Arthrobacter, Cellulomonas, Cryobacterium, Kocuria, Micrococcus, Micromonospora, Modestobacter, Nocardia, Rhodococcus, Saccharopolyspora and Streptomycyes were identified from samples of Kanasi National Nature Protect Region. Composition of actinobacteria was relative monotonous because of too cold weather conditions. Some strains of Kocuria, Micrococcus and Modestobacter were psychrophile, and can grow well at 4°C.

Grand Shangri-La

Grand Shangri-La covers a big triangle zone in edge of three provinces, Yunnan, Tibetan (Xizang) and Sichuan. It belongs to Hengduan Mountain chain. There are Meili, Baiman, Haba, Yulong, Gongga, Nianbaoyuze, Nanjiabawa snow mountains, and Lu, Nancang, Jinsha, Yalong, Dadu and Min rivers are flowing among them. Altitude difference is up to 6000 m. Original vegetation is very perfectly protected, and it situated away from human settlement. Main plants found are Castanopsis delavayi, Lithocarpus craibianus, L. Pachyphyl/us, Schima argentea, Pinus yunnanensis and Alnus nepalensis in the zone of 1950m-3000m: Abies georgei, Pinus liiangensis var. balfuriana, Taxus mairei, Quercus pannosa, Rhododendron rubiginosum, Iris bulleyana and Clinelymus nutans at 3000 - 4000 m; Juniperus wallachiana, Quercus guayavaefolia, Primula serrantifolia, Kobresia stiebritziana, Eremopogon delavayi, Festuca ovina, Salix calyculata, Rhododendron roxieanum and Rh. Traillianum at 4000 ~ 5250 m. 220 soil samples were collected from the zone from 1950 to 5250 m. 2442 strains of actinomycetes were isolated with four media from 220 soil samples collected at different altitudes (Table 2). In these places, 1463 strains were isolated in 80 samples collected at the zone at 1950 ~ 3000 m, and average of 18 strains a sample. 697 strains were isolated from 80 samples at 3000 ~ 4000 m, 8 strains a sample. 282 strains were isolated from 60 samples at 4000 ~ 5250 m, 5
Table 1. Sampling site and numbers of isolated and identified strains in the study.

<table>
<thead>
<tr>
<th>Number of sampling areas</th>
<th>Latitude (N)</th>
<th>Longitude (E)</th>
<th>Type of vegetation</th>
<th>Number of soil samples</th>
<th>Number of isolated strains</th>
<th>Number of identified strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>48°23'~49°11'</td>
<td>86°45'~88°11'</td>
<td>Original meadow and coniferous forest of frigid</td>
<td>50</td>
<td>566</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>26°~34'</td>
<td>94°~102°</td>
<td>Original meadow, Coniferous forest and every green broadleaved forest</td>
<td>220</td>
<td>2442</td>
<td>128</td>
</tr>
<tr>
<td>3</td>
<td>33°05'</td>
<td>104°</td>
<td>Original coniferous forest</td>
<td>50</td>
<td>386</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>29°59'~30°54'</td>
<td>103°4'~103°35'</td>
<td>Secondary mingled sub-tropical every green broadleaved forest</td>
<td>100</td>
<td>782</td>
<td>86</td>
</tr>
<tr>
<td>5</td>
<td>28°05'~28°20'</td>
<td>105°39'~105°52'</td>
<td>Original sub-tropical every green broadleaved forest</td>
<td>50</td>
<td>662</td>
<td>98</td>
</tr>
<tr>
<td>6</td>
<td>27°83'~29°3'</td>
<td>108°82'~110°5'</td>
<td>Original sub-tropical every green broadleaved forest</td>
<td>280</td>
<td>1134</td>
<td>112</td>
</tr>
<tr>
<td>7</td>
<td>21°41'~22°10'</td>
<td>100°7'~101°5'</td>
<td>Original tropical rain forest, and tropical season forest</td>
<td>100</td>
<td>1653</td>
<td>388</td>
</tr>
<tr>
<td>8</td>
<td>36°5'~37°5'</td>
<td>96°4'~100°8'</td>
<td>Saline and alkaline soil in Qinghai</td>
<td>115</td>
<td>534</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>41°24'~42°51'</td>
<td>83°37'~89°12'</td>
<td>Saline and alkaline soil in Xinjiang</td>
<td>100</td>
<td>514</td>
<td>2442</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>1065</td>
<td>8159</td>
<td>1064</td>
</tr>
</tbody>
</table>

* = Figure 1

Table 2. Number of isolated actinomycetes in samples collected from different altitude.

<table>
<thead>
<tr>
<th>Altitude (m)</th>
<th>YIM 7 Streptomyces</th>
<th>Other</th>
<th>YIM 171 Streptomyces</th>
<th>Other</th>
<th>YIM 37 Streptomyces</th>
<th>Other</th>
<th>YIM 212 Streptomyces</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1950 ~ 3000</td>
<td>164</td>
<td>187</td>
<td>172</td>
<td>71</td>
<td>178</td>
<td>105</td>
<td>228</td>
<td>358</td>
<td>1463</td>
</tr>
<tr>
<td>3000 ~ 4000</td>
<td>94</td>
<td>90</td>
<td>67</td>
<td>55</td>
<td>98</td>
<td>60</td>
<td>105</td>
<td>128</td>
<td>697</td>
</tr>
<tr>
<td>4000 ~ 5250</td>
<td>41</td>
<td>59</td>
<td>54</td>
<td>19</td>
<td>29</td>
<td>30</td>
<td>22</td>
<td>28</td>
<td>282</td>
</tr>
<tr>
<td>Total</td>
<td>299</td>
<td>336</td>
<td>293</td>
<td>145</td>
<td>305</td>
<td>195</td>
<td>355</td>
<td>514</td>
<td>2442</td>
</tr>
<tr>
<td>%</td>
<td>47</td>
<td>53</td>
<td>67</td>
<td>33</td>
<td>60</td>
<td>40</td>
<td>41</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>
strains a sample. Quantity of actinomycetes gradually decreased with heighten of altitude. YIM 171 and YIM 37 were 33% and 40% (Table 2).

128 selected strains from the 2442 were identified. Members of 20 genera, Actinomadura, Actinoplanes, Agromyces, Arthrobacter, Clavibacter, Dactylosporangium, Kocuria, Lentzea, Micrococcus, Micromonospora, Myco- cotoxum, Nocardia, Nocardiodites, Oerskovia, Promicromonospora, Pseudonocardia, Rhodococcus, Streptomyces, Streptosporangium and Tsukamurella were identified with phylogenetic analysis based on 16S rDNA sequencing. They belong to 7 Orders, 14 Families. Except Streptomyces, the members of Order Micrococcaceae occupied a larger ratio. Members of Clavibacter, Lentzea, Myco- cophila and Tsukamurella were not easy to find in common soil. 212 strains of the 2442 (9%) belonging to Actinomadura, Arthrobacter, Kocuria, Micrococcus, Micromonospora, No- cardia, Pseudonocardia and Streptomyces grew well at 4°C, and should be psychrophiles.

Jiuzhaigou National Nature Protect Region

Jiuzhaigou National Nature Protect Region is located in the edge of Sichuan and Gansu, a transition zone from Sichuan basin to Qinghai-Tibetan Plateau, and the altitude is from 2000 to 4000 m. Area of the protect region is about 20,000 hectare. Perpendicular distribution of vegetation is distinct. Main species of plants are Korean pine, Picea asperata, P. schrenkiana, P. likiangensis var. montigena, Abies fabric, Betula albo-sinensis, Eupetlea pleiospermum and Cedri- phylum japonicum, etc. Original vegetation is protected very well. Fifty soil samples were collected from the central zone of the protect region.

386 actinomycete strains were isolated from the 50 samples. 66 strains of them were selected and identified. 18 genera of Actinobacteria, Actinomadura, Actinoplanes, Agromyces, Arthrobacter, Corynebacterium, Jiangella, Kribbelia, Micro- bispora, Micrococcus, Micromonospora, Mycobacterium, Nocardia, Nonomuraea, Promicromonospora, Pseudonocardia, Rhodococcus, Streptomyces and Streptosporangium were identified, and 12 strains remained unidentified. Members of the genus Streptomyces were 78% and Promicromonospora were 13%. Figure 2 shows the phylogenetic analysis of part strains from Jiuzhaigou.

Emei and Qingcheng Mountains

Emei Mountain is located in the southwestern suburb of Chengdu city, Sichuan, covers about 154 km², and has been set as a national scenic spot for a long time. The highest peak is 3099 m. About 3200 species of higher plants, main families that grow there are Fabaceae, Pinaceae and Cupressaceae, and the vegetation belongs to secondary mingled sub-tropical every green broadleaved forest.

Alsophilus spinulosa, Cephalotaxus oliveri, Ailanthus altissima, Manglietia szechuana and Michelia wilsonii finetet etc. are national protected plants. Qingcheng Mountain is in the northwestern border of Sichuan basin, scenic area is 1522 m², and is a secondary sub-tropical every green broadleaved forest. 100 soil samples were collected from the two scenic areas.

782 of actinomycete strains were isolated. 86 selected strains of them were identified and belong to 9 genera, Actinomadura, Dactylosporangium, Micrococcus, Micromonospora, Mycobacterium, Nocardia, Nonomuraea, Promicromonospora and Streptomyces. 18 strains were unidentified. This is the least in the number of genera, and actinomycete community was relative monotonous, and all genera are common in the most of soil.

Huangjing National Nature Protect Region

Huangjing forest is located in the north of Gulin country and the south of Sichuan Province, southern edge of Sichuan basin, and near Guizhou Province. The geomorphology belongs to Danxia landform. The protect region is about 430 km², mean altitude is about 1300 m, and is scarcely disturbed by human. Main rare plants are Liriodendron chinensis, Fokienia hodgensii, Cinnamomum camphora, Phoebe zhennan, Alsophila spinulosa etc., and rare animals are Bos gaurus, Panthera pardus, Ursus thibetanus, Sus scrofa, Vivera zibetha etc. Type of vegetation belongs to sub-tropical every green broadleaved forest, and the original vegetation has been protected perfectly. 50 soil samples were collected. 662 strains of actinomycete were isolated from the 50 soil samples, and 98 selected strains were identified. 22 genera of actino-bacteria, Actinomadura, Actinoplanes, Actinopolyomorpha, Arthrobacter, Cellulomonas, Cellulosimicrobium, Clitiroccus, Kitasatospora, Micrococcus, Micromonospora, Mycobacterium, Nocardia, Nocardiodites, Nonomuraea, Promicromonospora, Pseudonocardia, Rhodococcus, Saccharomonospora, Saccharopolyspora, Streptomyces, Streptosporangium and Verrucosispora, were identified. Two genera, Actinopolyomorpha and Verrucosispora, are rare in the soil. Figure 3 is phylogenetic analysis of a part of strains from Huangjing.

Wulin Mountain Chain National Nature Protect Region

Wulin Mountain Chain is located in the edge of Chongqing, Hubei, Hunan and Guizhou, altitude is over 1000 m, and the area is 10,000 km². Fanjing Mountain Protect Region is the main body of the chain, and the top peak, Fenghuang Mountain, is 2570 m. Other two sampling Protect Region are Tianzi Mountain and Suoxigu in Zhangjiajie, northwest of Hunan. The geomorphology belongs to karst. Original vegetations of the three National Nature Protect Regions have been protected very well. The vegetation type is sub-
tropical evergreen broadleaved forest, and some similar as 
Huangjing. 280 soil 
samples were collected. 1134 pure 
strains of actinomycetes were isolated. 112 selected strains 
were identified. 19 genera of actinobacteria, 
Actinomadura, 
Actinoplanes, Arthrobacter, Catellatospora, Curtobacterium, 
Dactylosporangium, Kocuria, Microbacterium, Micromonospora, 
Promicromonospora, Mycobacterium, Nocardia, Nonomuraea, 
Pseudonocardia, Rhodococcus, Sphaerosporangium, Streptomyces, Streptosporangium and Williamsia were identified. 
Catellatospora, Curtobacterium, Sphaerosporangium and Williamsia were rare and not found in the above areas.

Xishuangbanna National Nature Protect Region

Xishuangbanna is located in the south of Yunnan, borders 
on Laos and Burma, and belongs to the Indo-Burma 
biodiversity hotspots and contains over 5000 species of 
vascular plants, comprising 16% of China's total plant 
diversity (Cao and Zhang, 1997; Li et al., 1996; Myers et al., 
2000). The biodiversity of Xishuangbanna forests is 
important both globally and nationally. Typical vegetation 
is mainly consisted of Family Sapindaceae, Annonaceae, 
Meliaeae, Euphobiaceae, Moraceae, Lauracear, Datiscaee,
Rubiaceae, Binnoniacae, and Orchidaceae etc. The fauna of Xishuangbanna are no less diverse, as 36.2, 21.7, and 14.6% of China’s birds, mammals, and reptiles and amphibians occur in the region, respectively (Kou and Zhang, 1987; Wang and Jin, 1987; Yang et al., 1987). Five sampling protect regions, Mengla, Menglun, Mandian, Xiaomengyang and Guanping are located within a radius of N 21° and E 101°, covering about 350 km², altitude is 450 to 900 m. Mean annual temperature is 21°C, and accumulated temperature is over 18°C for more than 200 days. Annual precipitation is about 1500 mm. Annual sunshine is about 2000 h. Soil belongs to brick-red soil, and pH 5.3. 100 soil samples were collected from the five protect regions in Xishuangbanna.

**Effect of the isolation media on the diversity of cultivable actinobacteria**

It is showed from Table 3 and Figure 4 that a total of 1652 pure cultural actinomycete strains were isolated, and 238 (60%) of the 1652 strains were rare actinomycetes with...
Table 3. Amount of actinomycetes isolated with five media.

<table>
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<tr>
<th>Medium</th>
<th>YIM 171</th>
<th>YIM 212</th>
<th>YIM 37</th>
<th>YIM 7</th>
<th>HVG</th>
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<td>189</td>
<td>159</td>
<td>238</td>
<td>93</td>
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</table>

%  

| Sample source | 55 | 45 | 39 | 61 | 45 | 55 | 44 | 56 | 56 |

S = Streptomyces; R = rare actinomycetes; YIM 171 = Improvement Glycerol-Asparagine medium; YIM 212 = Mycose-Proline medium; YIM 213 = Raffinose-Histidine medium; 7 = HV medium; HVG = Improvement HVG; *1 = Mengla; 2 = Menglun; 3 = Mandian; 4 = Xiaomengyang; 5 = Guanping.

Figure 4. Amount of pure strains isolated with five media.

YIM 212 medium; 220 strains were isolated, 124 (56%) of them were rare actinomycetes with YIM 7 (HV); 205 strains were isolated, and 112 (55%) were rare actinomycetes with YIM 213. 459 pure strains were isolated, and 270 strains (55%) of them were streptomycetes with YIM 171; 371 pure strains were isolated, and 205 strains (56%) of them were streptomycetes with HVG. Therefore, we propose that the former three media can be used for selective isolation of rare actinomycetes, and the last two media were used to isolate streptomycetes from forest soil samples.

Type and concentration of inhibitors for isolation of actinobacteria from forest soil were tested many times in our laboratories, the optimum composition of K$_2$Cr$_2$O$_7$ was 50 mg/L, for mixture solution of nystatin 100 mg/L, cycloheximide 50 mg/L and nalidixic acid 20 mg/L, most part of Gram negative bacteria were inhibited, and no fungi grew on all five medium plates.

16S rDNA sequences of 388 selected pure strains from 1653 were determined. The phylogenetic analysis was carried out. Strains were identified at a genus level. Total 36 genera of actinobacteria, Actinomadura, Actinomycetospora, Actinoplanes, Actinopolymorpha, Agrococcus, Agromyces, Arthrobacter, Cellulomonas, Cellulosimicrobium, Citricoccus, Curtobacterium, Dactylorporangium, Friedmanniella, Intrasporangium, Isoptericola, Kitasatospora, Kineicoccus, Kribella, Lentzea, Microbacterium, Micromonospora, Mycobacterium, Nocardia, Nocardioides, Nonomuraea, Oerskovia, Planosporangium, Polymorphospora, Promicromonospora, Pseudonocardia, Rhodococcus, Saccharomonospora, Saccharopolyspora, Sphaerisporangium, Streptomyces and Streptosporangium, were identified. These genera belong to 8 orders, and 14 families. Members of four genera, Streptomyces, Micromonospora, and Nocardia were most in number and widely distributed in all 6 regions above. However Actinomycetospora, Actinopolymorpha, Curtobacterium, Isoptericola, Kineicoccus, Lentzea, Planosporangium, Polymorphospora and Sphaerisporangium which are very rare in nature, were isolated from soil samples collected from Xishuangbanna. Actinomycetospora chiangmaensis gen. nov., sp. nov. (Jiang et al., 2008), Planosporangium flaviriseum gen. nov., sp. nov. (Wiese et al., 2008), Planosporangium mesophilum sp. nov. (Cao et al., 2011), Actinopolymorpha alba sp. nov. (Cao et al., 2009) and Agromyces auranticus sp. nov. (Li et al., 2003) were characterized and published in IJSEM. Based on general regularization in taxonomic world, the similarity of 16S rRNA gene sequence of a strain with the closest valid species...
Saline and alkaline soil in Qinghai and Xinjiang

Qinghai and Xinjiang is the dry area in China. There are large area of saline and alkaline soil, lakes and desert. Salt content of the earth's surface is up to 50% in some high saline soil. A total of 215 of soil samples were collected around Daqing, Ganjia, Xiaoyanhu, Bosqi and Kucheoi salt lakes and salt and alkaline soil in Yanqi, Buerjin, Huangshuiqi, in Xinjiang, and around Chaka, Keke salt lakes in Qinghai. 534 of pure cultured actinomycetes were isolates, and 118 of them were identified.

24 genera of actinobacteria, Citrococcus, Corynebacterium, Isoptericola, Kitasatospora, Marinococcus, Nesterenkonia, Nocardia, Nocardiosis, Prauserella, Rhodococcus, Saccharomonospora, Saccharopolyspora, Salinimicrobium, Streptomyces: Amycolicicoccus, Halotimporolyspora, Halotimcamera, Halotimcamera, Myceligererans, Sinacurtobacterium, Streptomonospora, Yaniella, Zhihengliuella, were identified. The last ten genera are novel genus. The 10 novel genera and over 60 novel species were published validly by our laboratories in International Journal of Systematic and Evolutionary Microbiology, Systematic and Applied Microbiology and Antonie van Leeuwenhoek. Nocardiosis was a typical representative group of alkalophilic actinobacteria in saline environments. Four novel genera of bacteria, Alkalibacillus, Aidingimonas, Salinicoccus and Sinococcus, were found from Qinghai and Xinjiang, and published validly. Therefore, it should be indicate that a large number of novel (or un-known) actinomycetes and other microbes exist in heavy saline and alkaline soil and lakes in Qinghai and Xinjiang, and the actinomycete communities were completely different from all forest soils in this study. But growth of halophilic and alkalophilic actinomycetes were very slow, and cultivation of them is very difficult. These features are a big hindrance for studying and utilizing them. So, the research works on physiology of halophiles and alkalophiles should be enhanced.

CONCLUSION

Special and different microbial communities were formed in different region, climate zone and natural environments in the course of organic co-evolution. Table 4 and Figure 5 summarized the results of this study. From the results, it is shown that composition of actinobacteria in each region were different from each other; actinomycete community of original forest (even expand to “original environments”) which is has never been troubled by action of human, was more complex than secondary forest; in the latter, only 9 genera of actinomycetes were isolated from Emei and Qingcheng mountains. The diversity was increased gradually along with climate warming in the all the original forests, 13 genera of actinobacteria were identified in soil samples collected from original meadow and coniferous forest of frigid in Kanasi, 19 to 22 genera were identified in Grand Shangri-La, Jiuzhaigou, Huangjianging and Wulin Mountain Chain, and 36 genera were obtained from original tropical rain forest and tropical season forest in Xishuangbanna. Therefore, Xishuangbanna was one of the areas with highest diversity of actinomycetes in the western of China.

Huangjianging and Wulin belong to sub-tropical evergreen broadleaved forest, and are located in the same latitude. But Huangjianging belongs to Danxia landform, and Wulin belongs to karst. A total of 41 genera of actinobacteria were isolated from these two regions. However, only 9 genera (22%) of them are mutual, and the rest 32 genera are different from each other.

Bergery's Manual of Systematic Bacteriology (Volume 5) collected 16 Orders, 44 Families and 216 genera of the Class Actinobacteria (Whitman et al., 2012). A total of 64 genera of actinobacteria were identified from 8 regions of western China, and occupy nearly 40% of the 216 genera. It was shown that a large number of unknown actinomycete resources existed in original forests and saline and alkaline soil in the west of China.

In our view, in order to develop new drugs, only knowing the existence of a large of uncultivable actinomycetes and other microbes in natural world by using “culture-independent analysis approach”, including 16S rDNA sequence analysis, Restriction Fragment Length Polymorphism (PCR-RFLP), Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), metagenome analysis, 454 Pyrosequencing of V3 hypervariable region of 16S rDNA gene etc., is not enough, and it is necessary to use every means to make the uncultured to cultured or pure cultured actinomycetes and other microbes. So, we still recognize that obtaining new or unknown actinomycetes is a principle premise for discovery of new drug leads, although it is showed that new species of actinomycetes do not always produce new compounds with high frequency (Takagi and Kazuo, 2011).

Key of selective isolating actinobacteria from soil samples

Selective isolation methods for actinobacteria, especially unknown actinomycetes, are very important, and as a research project, it should be studied, improved and replaced constantly. Existence of Gram negative bacteria, fungi and even known actinomycetes, in a large number in
Table 4. Distribution of different genus in 8 sampling areas.

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Total 64                          13  20  18  9  22  19  36  24

1* = Figure 1; ✓ = existence

soil samples is a main problem for selective isolation of unknown actinobacteria. In order to eliminate these troubles, the key points are obtaining much more unknown actinobacteria for discovering novel drug lead and other products, sampling and isolation methods.

Based on many years of research in our laboratory: i) it is best to collect test samples from original habitats which have never been disturbed by action of humans, such as original forests and other original (or extreme) environments; ii) soil samples have to been dried at 25-28°C for 7 to 10 days; iii) pre-treatment of dried samples at 80 to 100°C for 60 min has to be carried out before isolation; iv) to isolate special group of actinobacteria, potassium dichromate 50mg/L, or mixture solution of nystatin 100mg, cycloheximide 50 mg and nalidixic acid 20 mg for 1000 ml medium, or other selective inhibitors, have to be added in the isolation medium for inhibiting fungi, Gram negative bacteria and even common actinomycetes; v) improvement Glycerol-Asparagine medium (YIM 171), Mycose-Proline medium (YIM 212) and HV medium (9) were better for isolation of actinobacteria from soil samples.

We have to emphasize that adding mixture of salt including Na+, K+, Mg2+, Ca2+, SO42-, CO32- and HCO3−; total concentration at 15 to 25%, and suitable inhibitor into isolation media, are necessary for selective isolation of halophilic and alkalophilic actinomycetes, based on our experiments for many years. Furthermore, cultivation of actinomycetes from extreme environments including saline and alkaline habitats is very difficult than common environments. They grow very slowly. So, special attention should be paid to research on physiology and cultural condition producing bioactive metabolites of these microbes.

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REFERENCES


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