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*Full Length Research Paper*

# Bioassay-based screening of myxobacteria producing antitumor secondary metabolites

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**Myxobacteria are gliding gram-negative bacteria and a class of prokaryote with complicated multicellular behaviors and morphogenesis. Reports show that myxobacteria generally produce large families of secondary metabolites with various bioactivities, such as antifungal and anti-tumor activities. In this paper, two strains producing metabolites with high anticancer bioactivity to 5 tumor cell lines were screened from over 370 isolated samples by adopting cell culturing technology and cell toxicity tests. The strains were identified as genus *polyangium* (*Polyangium vitellinum* and *Sorangium cellulosum*), named JSW103 and AHB125, based on the morphology of vegetative swarms, fruiting bodies, vegetative cells and myxospores. $IC_{50}$ values of JSW103 to tumor cell lines B16 and SGC7901 were 0.2715 and 4.1924 μg $ml^{-1}$, respectively, while for AHB125 metabolites to tumor cell lines B16 and SGC7901, the values were 0.0082 and 0.036 μg $ml^{-1}$, respectively.**

**Key words:** Antitumor activity, bioassay-based screening, $IC_{50}$, myxobacteria, secondary metabolites.

## INTRODUCTION

Myxobacteria are gram-negative unicellular rod shaped bacteria with suitable culture pH between 5.0 and 8.0. Myxobacteria can be frequently isolated from soil, dung of herbivorous animals, bark, rotting wood and other decaying organic material and are able to glide in swarms over solid surfaces and to exhibit “social behavior” because of their slime production, and easily carries contaminates, such as fungi and soil amoebae (Reichenbach and Dworkin, 1992).

Myxobacteria have been divided into two different groups based upon simple morphological examination of the cells, spores, and colonies. Many myxobacteria strains can produce secondary metabolites, most of which are bioactive substances, with antifungal and anti-tumor activities (Reichenbach and Hofle, 1993; Gerth et al., 2003). In the past decades, more and more laboratories have shown great interests in myxobacteria metabolites, especially the epothilones. The potential anti-tumor effects of epothilones had been proved much better than Taxol both *in vitro* and *in vivo* (Winkler and Axelsen 1966; Kowalski et al., 1997; Giannakakou et al., 1997; Kowalski et al. , 1997; Chou et al., 1998; Ojima et al. , 1999; Altmann et al., 2000; Lee et al., 2001; Yamaguchi et al., 2002; Nicolaou et al. , 2003). Hence, many research groups have tried to identify novel groups of natural product from myxobacteria. Up to 1993, about 50 basic structures and nearly 300 structural variants have been elucidated, and almost all of them were conformed to be new compounds (Reichenbach et al., 1993). Gerth et al. (1996) obtained the Epothilone A and B from the broth of *Sorangium cellulosum* strain So ce 90.

Although various strains of myxobacteria have been isolated, most of screening methods are traditional and laborious. Here, other than the traditional methods of isolation and purification, bioassay-directed screening method with specific tumor cell models was applied to screening out the target myxobacterium, which could produce some substances to inhibit specific tumor cell line. Further investigations were conducted to identify the genus of which the purified myxobacteria belong.

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## MATERIALS AND METHODS

### Preparation of samples

### Soil samples

Air-dried samples were collected from Yunnan, Guizhou, Jilin, Anhui, Jiangsu and Zhejiang of China, and stored at room temperature.

### Isolation and purification medium

Basal media for screening the myxobacteria in this study were WCX (Zhang et al., 2003), CNST (Li et al., 2000; Li et al., 1996), α-cellulose-CNST (0.1% α-cellulose was added into CNST medium) (Li et al., 1996), YV/4 (Li et al., 2000), CY (Zhang et al., 2003), α-cellulose-VY/4 (0.1% α-cellulose was added into VY/4 medium), and EBS (Reichenbach 1983). All media were adjusted to pH 7.4 with 10% KOH solution before autoclaving at 121°C for 20 min.

### Seed and fermentation medium

The seed medium was prepared according to Gerth et al. (1996). Its components are potato starch 1%; glucose 0.8%; defatted soybean meal 0.2%; yeast extract 0.2%; Fe(III)-Na-EDTA 0.008 mg/L; $CaCl_2 \cdot 2H_2O$ 0.1%; $MgSO_4 \cdot 7H_2O$ 0.1%; 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 1.15%, and the initial pH of the medium was adjusted to 7.5 with KOH solution. The fermentation medium for producing the anti-tumor secondary metabolites was a little different from the seed cultures, with 2% XAD-16 adsorbent resin used to replace the HEPES.

### Isolation and purification

A simple method of isolation and purification of myxobacteria was introduced.Briefly, the soil samples were dried for about 1 ~ 2 h at 56°C in a blast oven. Then, the myxobacteria were induced by two methods: I) The soil samples were spread into dishes evenly and covered with with 25 μg $ml^{-1}$ cycloheximide for 6 h. After that, the rest solution was discarded. Then 10 hare dung was embedded into the soil and cultured at 30°C. II) A φ125 filter paper was covered on CNST medium, and a little soil dust was sprinkled evenly and cultured at 30°C. Myxobacteria were enriched and transferred to the WCX, VY/4, or α-VY/4 media. Colonies were transferred to α-VY/4 media immediately. The pure cultures were transferred to EBS medium, and cultured at 150 rpm at 30°C for 24 ~ 48 h. If the culture broth was lucid, the strain was confirmed to be the myxobacteria.

### Fermentation and secondary metabolites extraction

The pure strains were selected randomly and inoculated in seed medium in 250 ml Erlenmeyer flasks and incubated at 30°C, 150 r/min for three days, and then transferred to fermentation medium with 2% (w/v) adsorbent resin to adsorb the excreted secondary metabolites. When the fermentation was ended, the absorbent resin was removed from the broth by centrifugation, then washed with distilled water, and finally extracted with methanol (Reichenbach and Hofle, 1993). The methanol elution was concentrated to 0.5 ml and then was utilized for anti-tumor testing *in vitro*.

### Assay of the effect on tumor cell lines (MTT assay)

Various cell lines, such as B16, Bel7402, H446, MCF-7 and SGC7901, were used to evaluate the anti-tumor effects of the metabolites that myxobacteria produced. Tumor cells ($8 \times 10^3$ per well) in their exponential growth phase were transferred into 96 well plates containing RPMI-1640 with 10% FBS and incubated for 24 h at 37°C in a 5% $CO_2$ incubator before addition of the samples. Incubation was carried out for another 48 h. The viability of cells was assessed in the MTT assay as described elsewhere (Satyanarayana et al., 2004; Lu et al., 2005; Yamaguchi et al., 2002). Briefly, the medium was aspirated and MTT was added to cells at a concentration of 0.5 g $l^{-1}$. Cells were incubated at 37°C for 4 h and the formazan product was solubilized with dimethylsulfoxide (DMSO). The absorbance was detected in the microplate reader (Multiskan MK3, Labsystems, Finland) at 570 nm, and measured values were expressed as means ± SD.

**Table 1.** Identification of several myxobacteria isolated*.

| Strain genus | Strain amount |
|---|---|
| Genus Coralloccoccus | 2 |
| Genus Cystobacter | 1 |
| Genus Myxococcus | 11 |
| Genus Polyangium | 5 |

*According to the description of Bergey's Manual of Systematic Bacteriology (8th Ed. Chinese 1984).

### Morphology studies

Glutaraldehyde fixation of cells was done according to Wireman and Dworkin (1975), while electron microscopy was performed on colonies stained with uranyl acetate and viewed with a JEOL JEM-100S transmission electron microscope. Phase contrast microscope (Olympus BX41, Japan) was performed on colonies and vegetative cell after staining with crystal violet. Measurements were with vernier caliper.

## RESULTS AND DISCUSSION

Following the procedures elucidated in Figure 1, over 370 myxobacteria strains were isolated and identified by EBS preliminary and according to the Bergey's manual (8th Ed.), and 19 strains were identified as myxobacteria (Table 1). The colors of fruiting bodies were white slime, white, milk white, cheese white, light yellow, milk yellow, orange, yellow, brown, meet-red, light-red, light-green etc. Some could decompose dry yeast, *E. coli*, and several strains could decompose α-cellulose.

The MTT assay was applied to detect the anti-tumor activity of the secondary metabolites, and the results are shown in Table 3. 370 fermentation products were screened by B16 and SGC7901 *in vitro*, about 58% of them had effect on the two cell lines (inhibition ratio above 30% at the dose of 200 - 400 μg $ml^{-1}$), about 24% had inhibition ratio above 90% at the dose of 200 - 400 μg $ml^{-1}$.

Then the secondary screening with B16, Bel7402, H446, MCF-7 and SGC7901 cell lines were studied. Two samples were screened out finally until the dose was 0.78 μg $ml^{-1}$. To MCF-7, compared with epothilones,

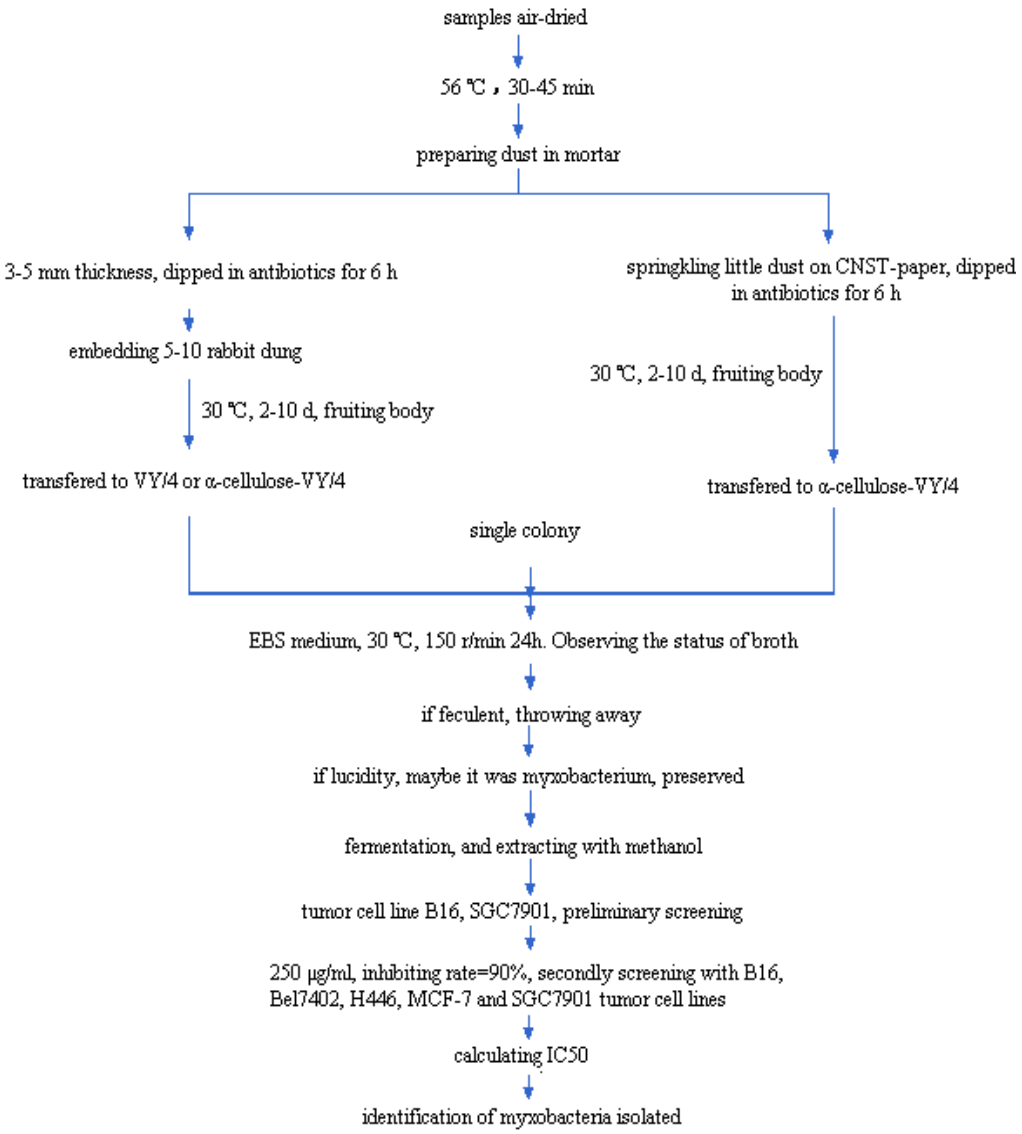


**Figure 1.** Flow chart of myxobacteria directed screening.

**Table 2.** *In vitro* growth inhibition potency of Ex1 and Ex2 to different tumor cell lines, $IC_{50}$ (μg $ml^{-1}$).

| Mixtures | Cell lines | | | | |
|---|---|---|---|---|---|
| | B16 | Bel7402 | H446 | MCF-7 | SGC7901 |
| Ex1 | 0.2715±0.1436 | 4.2016±2.4782 | 2.7728±0.6158 | 0.7201±0.1836 | 4.1924±1.2635 |
| EX2 | 0.0082±0.0034 | 2.7052±0.9697 | 1.5591±0.5273 | 1.0703±0.2658 | 0.0362±0.0238 |

*n = 3; Extraction 1 (Ex1), Extraction 2 (Ex2).

Paclitaxe, Actinomycin D and Adriamycin, the $IC_{50}$ of extraction 1 (Ex1) produced by JW103 was 1 - 45 folds lower than other antibiotics except for Epothilone B; the $IC_{50}$ of Ex2 produced by AHB125 was equal to Actinomycin D or better than epothilone A, C, D, Paclitaxel, and Adriamycin except for epothilone B (Chou et al., 1998). The above results indicated that the two mixtures Ex1 and Ex2 could have broad-spectrum and high-effective anticancer abilities (Table 2).

Regrettably, the bioactive component of these mixed products seemed unstable in water solution. When the dried samples were dissolved with medium (10% FBS, 90% RPMI1640), and applied to evaluate their activities immediately, the $IC_{50}$ values of Ex2 were 0.0082 and

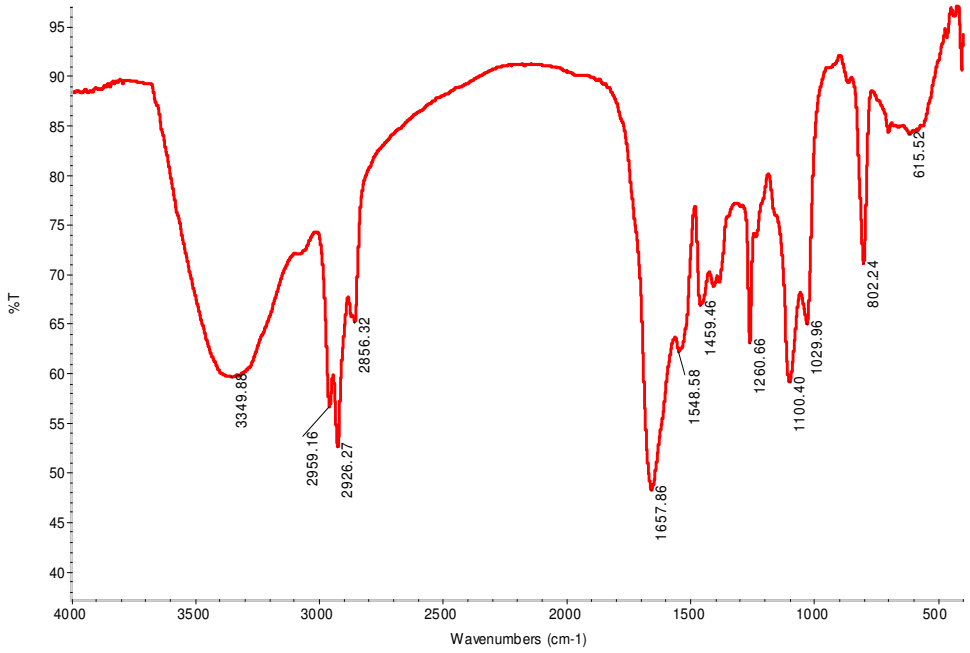


**Figure 2.** IR spectrum of eluted concentration.

**Table 3.** Primary screening results.

| Isolated strains | Fermentation strains | UV detection 190 - 500 nm | Inhibiting rate* (%) | |
|---|---|---|---|---|
| | | | ≥30 | ≥90 |
| 374 | 374 | 312 | 217 | 90 |

* Inhibiting rate to both B16 and SGC7901 of methanol extraction of fermentation.

0.036 μg $ml^{-1}$ to B16 and SGC7901, respectively. However, after four days, the $IC_{50}$ were 0.27 and 1.2847 μg $ml^{-1}$, which were about 40 times than that of the original samples. On the other hand, in order to maintain their anti-tumor effects, the mixtures should be prepared with higher concentration methanol solution, for example over 85% (v/v) for Ex1 and Ex2. This phenomenon indicated that the bioactive compounds possibly had unstable double or triple bonds, or had a lactone cycle or acylamide bond or other groups. The compounds could be hydrolyzed and their bioactivities gradually lost in water solution. This hypothesis was confirmed by IR spectrum. As shown in Figure 2, strong absorption at 3349 $cm^{-1}$ revealed that the compounds had free hydroxides. However, at 800 ~ 900 $cm^{-1}$ there were no obvious absorption indicating that the compounds did not have carboxyl group; mid-strong absorption at 3064 $cm^{-1}$ revealed that the bioactive substances had double bonds; strong absorption at 1657 $cm^{-1}$ revealed it had acylamide bond; combined with the mid-strong absorption at 1260 $cm^{-1}$ revealed a lactone cycle. Combined with HPLC spectrum (Figure 3) and IR spectrum, most of the compounds in the mixtures were identified as polar or mid-polar ones.

Results of morphological studies showed that swarms of strain JSW103 was milk-white semispherical bulge slime, colorless to light-white spreads and its bar was 0.1 ~ 0.5 cm after culturing for 5 ~ 6 days (Figure 4A). Under electron microscopy and microscope, the spore capsules combined with colorless slime were easy to find (Figure 4B); its vegetative cells similar to the spores, they were rod-shaped with blunt ends, length between 1.162 ~ 1.265 μm (Figure 4C) and 2 ~ 6 μm (Figure 4D), and width between 0.3 ~ 0.5 μm. At young spore capsules term, the spore was just like vegetative cells, and formatting bundles shapes. According to the description of Bergey's Manual of Systematic Bacteriology (McCurdy's 8th Ed. Chinese 1984), the strain was *Polyangium vitellinum*. The genus had only been cultured on original basic substance, no pure culturing has been obtained on artificial medium.

The morphologies of AHB125 spores and vegetative cells were rod-shape with blunt ends similar to those of strain JSW103. However, a little difference existed. Its swarms was brown and irregular slime. Its bar was 0.5 ~ 1 cm (Figure 4E), under phase-contrast microscope, the

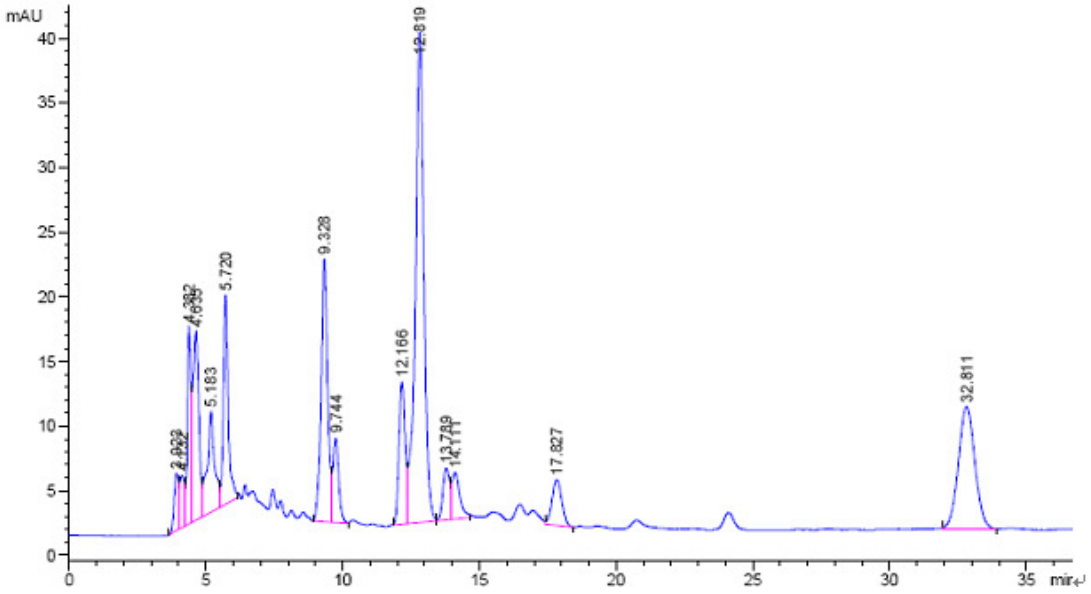


**Figure 3.** Methanol elution analysis by HPLC. HP1100 (Agilent Ltd. USA),Methanol:water = 65:35, XDB C-18, 0.46×250 mm, flow 0.5 ml min, diode-array detector, 254 nm.

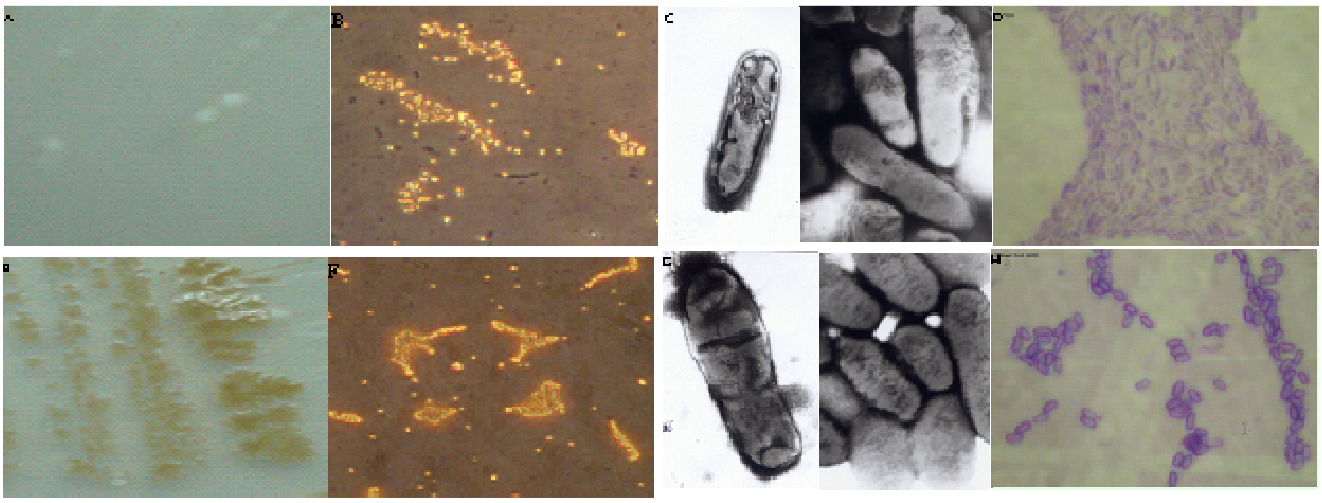

**Figure 4.** Typical morphological structure of JSW103: A. Swarms, Bar = 0.2~0.5 cm; B. Fruiting bodies, Bar = 10 µm; C. myxospore Bar =1.162~1.265 µm; D. Vegetative cells Bar = 2~6 µm. ABH105: E. Swarms, Ba r = 0.2~1 cm; F. fruiting bodies, Bar = 5~10 µm; G. myxospore bar =1.324~2.171 µm; H. Vegetative cells Bar = 2.5~7 µm.

morphologies were capsules (Figure 4F), spores length of AHB125 was 1.3 ~ 2.2 µm, width was 0.5 ~ 0.7 µm, and its length of vegetative cells were about 5 ~ 7 µm (Figure 4G and Figure 4H). At young term, its spores resembled vegetative cells, and the vegetative cells usually formed piled capsule (Figure 4G). In the beginning, color of fruiting bodies were colorless or light white, and about three days later, the colonies became light yellow, orange, yellow-brown, up to deep brown (Figure 4E). According to the description of Bergey's Manual of Systematic Bacteriology (8th Ed. Chinese 1984), the strain was *Sorangium cellulosum*.

As is well known, isolation and purification of myxobacteria is not easy. In most cases, swarm colonies emerged within 1 - 5 days; maybe the swarms and fruiting bodies could be discovered after 2 to 20 days. Hence purification procedure did become tedious, and the myxobacteria were easily polluted by *Mucor*, *Rhizopus* and *Aspergillus*. As myxobacteria fruiting bodies do not germinate in nutrient rich media, they can be treated in such media with high concentrations of otherwise toxic antibiotics such as cycloheximide, terramycin and kanamycin. The contaminants would grow and be killed by the inhibitors. If there were surviving contaminants, they usually ap-

peared as separate colonies. Some colonies look pure in plates or tubes, but after transferring to liquor medium, culturing for 1 - 2 days and transferring to plates again, other colonies could be found. A simple method to eliminate them was to suspend in water at 56°C for about 45 min, then cool to room temperature, and keep at –80°C overnight (Reichenbach 1983).

## Conclusion

In summary, anti-tumor bioactivities assay-directed screening method was applied to isolate and purify the myxobacteria strains that effectively produced high bioactive secondary metabolites. And from the results of morphological studies, the two strains which produced the secondary metabolites with high anti-tumor activity were identified as genus *Polyangium vitellinum* (JSW103) and *Sorangium cellulosum* (AHB125). This is the first reported to describe the phylogenic feature *P. vitellinum*. Further investigations are necessary to purify and identify the bioactive components and to elucidate their mechanism of its anti-tumor effects.

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