CYTOTOXIC AND ANTI-METHICILLIN RESISTANT Staphylococcus aureus ACTIVITIES OF EXTRACTS FROM Streptomyces spp. ISOLATED FROM MINERAL ORES

Hurtado, J.1*; Vaisberg, A.2; Obando, P.1; Portales, R.3; Rojas, R.3

Email address: jasmin.hurtado@upch.pe

Abstract

Microorganisms living under stress conditions, such as acidophilic environments, usually respond by synthesizing compounds with important biological activities. The cytotoxic and antibacterial activities against methicillin-resistant Staphylococcus aureus (MRSA) of ten strains of Streptomyces isolated from mineral ores of Peru were evaluated.

Streptomyces variabilis F and S. variabilis X were more active than vancomycin against MSRA (MIC= 0.5, 0.5 and 1.0 µg/mL, respectively). Streptomyces sp. 6c, S. albido flavus Ya4, S. champavatii Frep13 and Streptomyces sp. F1 showed selective cytotoxic activity against human adenocarcinoma of the duodenum (HuTu80) and human lung large cell carcinoma (H460). The extracts of Streptomyces sp. F1 and S. champavatii Frep13 also showed good cytotoxic activities (IC50= 2.51 and 0.36 µg/mL, respectively) against human breast adenocarcinoma (MCF-7). Interestingly, the cytotoxic activities of some of the Streptomyces extracts were very similar to that of the positive control 5-fluorouracil.

Keywords: Actinobacteria, Methicillin-resistant Staphylococcus aureus, Streptomyces, tumor cells
Introduction

Streptomyces species are Gram-positive, chemoorganotrophic microorganisms that are widely distributed in different environments. They produce approximately 12,400 bioactive molecules (1), some of them are very well-known antibiotics and antitumorals (2). The natural habitat of Streptomyces is marine or terrestrial soil, but in the last years the search for new strains has been focused in places of high physical, chemical and/or biological stress that induce Streptomyces to produce secondary metabolites for their survival. For example, acidophilic and acid tolerant Streptomyces have been isolated from acid environments with high level of metals (3).

Staphylococcus aureus is a Gram-positive bacterium found as a commensal and pathogen in human population. It is by far the most clinically relevant pathogen because it can cause bacteremia and other important infections including infective endocarditis (4,5). Moreover, S. aureus has the capability to generate resistance to many antibiotics. The first Methicillin-resistant strain occurred in England in 1960 (6) and later on, the number of antibiotics resistant strains have been increasing. For the moment, Vancomycin is still recommended for the treatment of MRSA infection, and according to the Clinical and Laboratory Standards Institute the MIC against susceptible S. aureus should be less than 2 μg/ml (7).

There is an urgent need to find new bioactive compounds, especially against microorganisms resistant to the antibiotics of current use. The present work evaluated the anti-Staphylococcal and cytotoxic activity of extracts from ten Streptomyces strains isolated from acid Peruvian minerals and mineralized soils (3). We chose to study Streptomyces from these extreme environments since by growing under such stress conditions; they must have developed unique metabolic pathways and therefore the ability to produce novel bioactive compounds.

Methods

Materials and Reagents

Ethylacetate, acetic acid and magnesium sulfate were purchased from J.T. Baker Chem. Co. (Phillipsburg, NJ, USA). Vancomycin, sulfonamide, trichloroacetic acid and tris base were obtained from Sigma-Aldrich (St. Louis, MO). Cell lines were from American Type Culture Collection (Rockville, MD).

Strains

The Streptomyces strains used in this study were previously isolated from arsenopyrite, pyrite, polymetallic sulfides and magnetite mining zones in Peru. The characterization was carried out by morphological, physiological and molecular (16S rRNA gene amplification) studies (3).

Culture of Microorganisms

The growth medium was Glycerol-yeast extract (Xgal) broth that contains: 5 mL/L; yeast extract 2 g/L; K\textsubscript{2}HPO\textsubscript{4} 1 g/L (8,9). Bacteria strains were pre-cultured in Xgal broth for 7 days at 28 °C. Then, using a sterilized hyssop, the bacteria strain was inoculated in plates with Xgal agar medium (40 plates in total) and incubated at 28 °C until sporulation was observed. The agar was then cut with a scalpel to approximately 0.5 cm² pieces, which were afterwards placed in a 500 mL Erlenmeyer flask.

Preparation of extracts

Ethylacetate extracts of Streptomyces spp. cultivated in agar solid media were prepared according to Awla et al. (10). Ethyl acetate was added to an Erlenmeyer flask until the agar pieces were covered. The flask was sonicated for half an hour at room temperature and then kept under stirring (150 rpm at 28 °C) for 18 hours. Then, magnesium sulfate was added to trap remaining water molecules. The mixture was filtered to obtain the ethylacetate extract. The solvent was evaporated in a rotavapor at 40 °C under vacuum. The resulting solution was then placed in a vial and dried under a stream of nitrogen gas. The ethylacetate extract of each microorganism was kept at 4 °C until it was used.

Antibacterial activity of Streptomyces extracts

In order to estimate the antibiotic susceptibility of the bacterial extracts, a dilution method was used to measure the Minimum Inhibitory Concentration of 50% (MIC\textsubscript{50}) (11). In the MIC\textsubscript{50}
assays, Methicillin-resistant *S. aureus* ATCC 49330 served as the pathogenic strain. The concentration of *S. aureus* used for the trials was 0.5 on the McFarland scale and was taken as the positive control for growth. The concentrations of the extracts ranged between 0.025 and 25.6 µg/mL. The extracts plus the strain were placed in reaction tubes and incubated at 37 °C overnight, and then the OD was measured using optical spectrophotometry at a wavelength of 600 nm. The MIC<sub>50</sub> was calculated by taking the OD value that corresponded approximately to half the OD value of the positive control.

**Tumor cell cytotoxicity bioassays**

Cytotoxicity of the extracts was evaluated in various tumor cell lines and in a non tumorogenic cell line using the sulforhodamine B (SRB) assay method (12,13). Cell lines tested include BALB/3T3 (Non-tumorogenic, BALB/c mouse embryo cells), H460 (human lung large cell carcinoma), DU145 (human prostate carcinoma), HuTu 80 (human adenocarcinoma of the duodenum), M-14 (human amelanotic melanoma), K562 (human chronic myeloid leukemia cells), MCF-7 (human breast adenocarcinoma), and HT-29 (human colon adenocarcinoma).

SRB method is a whole cell assay used as a preliminary screening towards the search of bioactive anticancer extracts. Extracts or bioactive compounds may be then evaluated in more specific assays in order to investigate their mechanisms of action. The use of different cell lines will give more information on the selectivity of action of the crude extracts.

To determine the cytotoxicity of the extracts, cells were plated into 96-well tissue culture plates and in their corresponding growth medium at approximately 10% confluency and incubated at 37 °C in a 5% CO<sub>2</sub> and 95% air humidified atmosphere for 24 h to allow cells to attach. A plate containing each of these cells was fixed *in situ* with trichloroacetic acid (TCA) in order to obtain the cell values at zero time before adding the extracts. The rest of the plates containing the different cell lines received serial dilutions of the extract to be tested and further incubated at 37 °C in a 5% CO<sub>2</sub> and 95% air humidified atmosphere for 48 h. The assay was terminated by the addition of cold TCA. TCA treated plates were incubated at 4 °C for 1 hour and then washed five times with tap water to remove TCA; afterwards, plates were air dried. Background optical densities were measured in wells incubated with growth medium without cells. TCA-fixed cells were stained for 20 minutes with 0.4% (w/v) SRB dissolved in 1% acetic acid. After air drying the plates, bound dye was solubilized with 10 mM Tris base (pH 10.5) and the absorbance read on an automated plate reader at a wavelength of 510 nm. The GI<sub>50</sub> (Growth inhibition of 50% cells) value was defined as the concentration of test sample resulting in a 50% reduction of absorbance as compared with untreated controls that received a serial dilution of the solvent in which the test samples were dissolved, and was determined by linear regression analysis (14).

**Results**

**Anti-Staphylococcal activity of microbial extracts**

Based on the screening results (Figure 1), the extracts of two strains of *Streptomyces* (*S. thermocarboxylycus* K1B and *Streptomyces* sp. F1) had similar activity as vancomycin against MSRA (MIC = 1 µg/mL). Moreover, the extracts of *S. variabilis* F and *S. variabilis* X were more potent (MIC<sub>50</sub> = 0.5 µg/mL).

**Cytotoxic activity of microbial extracts**

Five of the 10 extracts of *Streptomyces* isolated from mineral ores of Peru showed interesting cytotoxic activity against one or more of the tumor cell lines tested (Table 1). For example, strains *S. sp. 6c*, *S. albidoflavus* ya4, *S. champavatii* Frep13 and *S. sp. F1* showed selective cytotoxic activity against human adenocarcinoma of the duodenum (HuTu80) and human lung large cell carcinoma (H460). The activity of *S. champavatii* Frep13 extract against these tumor cell lines (GI<sub>50</sub> < 0.24 µg/mL) was more than 100 times greater than the activity against non-tumorogenic BALB/c mouse embryo cells (IC<sub>50</sub> = 26.95 µg/mL).

The extracts of *S. sp. F1* and *S. champavatii* Frep13 also showed good cytotoxic activities (GI<sub>50</sub> = 2.51 and 0.36 µg/mL, respectively) against human breast...
adenocarcinoma (MCF-7) with good selective indexes (10 and 74, respectively).

**Discussion**

It has been reported that the genus *Streptomyces* is able to produce different metabolites with diverse molecular structures depending on ecological factors. Kemung et al. (15) reported that from 86 *Streptomyces* species isolated from terrestrial soils, 37 demonstrated moderate to potent anti-MRSA action. One of them, *Streptomyces* sp. HW-003, isolated from the soil of the primary mountain forest Gyebangsan (1,577 m), yielded the active compound AMRSA1 which showed potent anti-MRSA activity at 0.01–0.1 µg/mL and is by far the most potent anti-MRSA compound isolated from terrestrial soil samples (16). Its structure however, remains unknown to date. *Streptomyces* sp. C34 isolated from the Chilean hyper-arid Atacama Desert soil was shown to produce chaxamycins that exhibit potent anti-MRSA activity with MIC values of 0.13–0.25 µg/mL (17).

Recently a new antibiotic called Dalbavancin has been approved for use against MRSA, it is a modified natural antibiotic produced by actinobacteria *Nonomuria* spp. Its structure has been altered to enhance activity against *S. aureus* and to extend its half-life. The MIC breakpoint was defined as 0.0125 µg/mL by the FDA, against MRSA (18). Comparatively, the crude extracts of our strains *Streptomyces* sp. F and *Streptomyces variabilis* X have exhibited MIC values 40 times greater (MIC= 0.5 µg/mL), and since we do not know yet the concentration of the active principle in these extracts, it is possible that we could find an antibiotic as potent as Dalbavancin.

The antitumoral activity found for *S. sp. F1* and *S. champavatii* Frep13 was greater than the one reported by Cartuche et al. (19) for a marine *Streptomyces* sp. (IC<sub>50</sub>= 83 µg/mL) isolated from the Ecuadorian coast. Similarly, their extracts were more active against MCF-7 cells compared to an extract of *Streptomyces pluripotens* MUSC 137 isolated from mangrove soil in Malaysia (IC<sub>50</sub>= 61 µg/mL; Selective index: 4.22) (20).

The cytotoxic activities shown by *Streptomyces* sp F1 and *S. champavatii* Frep13 against MCF-7 cells were more interesting than that exhibited by some plant extracts reported by other researchers. For example, Akter et al. (21) evaluated the cytotoxic activity of 23 plant extracts from Bangladesh, only 3 of them showed IC<sub>50</sub> values lower than 50 µg/mL. The IC<sub>50</sub> value of the two most active plant extracts (*Diospyros peregrina* and *Jasminum sambac*) was 7 µg/mL.

There are few studies on the bioactive compounds of *S. champavatii*; Pesic et al. (22) isolated this microorganism from marine sediments of the Baltic sea and found that it produces the octapeptide champacyclin, which exhibits moderate activity against *Erwinia amylovora*.

The extract of *S. albidoflavus* ya4, isolated from mineral ores of Peru, was the most active (IC<sub>50</sub>&lt; 0.24 µg/mL, Selective index=6) against human prostate carcinoma cell line (DU145). Compared to other species of *Streptomyces* with activity against this tumor cell line, *S. albidoflavus* ya4 extract was more potent than the one obtained from *Streptomyces pluripotens* (IC<sub>50</sub>&gt; 400 µg/mL) investigated by Ser et al. (20). *S. albidoflavus* isolate from a mangrove plant from China produces antimycin A18, a nonpolyenic lactone with activity against several plant pathogenic fungi (23).

The extract of *S. heilongjiangensis* Frep14 (Table 1) was the only one that showed an interesting activity (IC<sub>50</sub>&lt; 0.24 µg/mL, Selective index=12) against human chronic myeloid leukemia cells (K562). *S. heilongjiangensis* isolated from the root surface of soybean produces the polyketide macrolide Treponemycin (borrelidin), which has a broad spectrum activity against Gram-positive and Gram negative bacteria; as well as against *Candida albicans* and *Mycobacterium tuberculosis* (24,25).

Interestingly, the activities of some of the *Streptomyces* extracts were very similar to that of the positive control 5-fluorouracil (Table 1). For example, *S. champavatii* Frep13 extract has a very good activity against H460, HuTu80 and MCF-7 cells and better selectivity indexes than those shown by 5-fluorouracil. Similar results were observed for the extracts of *S. albidoflavus* ya4 and *S. heilongjiangensis* Frep14; they are more selective, compared to the positive control, against the tumor cells DU145 and K562, respectively.
Streptomyces species isolated from mineral ores in Peru showed good in vitro activities against methicillin-resistant Staphylococcus aureus, as well as selective cytotoxic activities against the tumor cell lines MCF-7, HUTU80, H460, DU145 and K562. Streptomyces variabilis F and S. variabilis X were more active than vancomycin against MSRA. Strains of S. sp. 6c, S. albidoflavus ya4, S. champavatii Frep13 and S. sp. F1 showed good and selective cytotoxic activity HuTu80 and H460 tumor cells. Further research is needed in order to isolate and characterize the compounds responsible for these activities.

Acknowledgments

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References


Table 1. Cytotoxic activities of *Streptomyces* extracts

<table>
<thead>
<tr>
<th><em>Streptomyces</em> strains</th>
<th>3T3</th>
<th>H460</th>
<th>HuTu80</th>
<th>DU145</th>
<th>MCF-7</th>
<th>M14</th>
<th>HT-29</th>
<th>K562</th>
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<tr>
<td><em>Streptomyces</em> sp. 6c</td>
<td>14.75</td>
<td>&lt;0.24</td>
<td>0.56</td>
<td>29.94</td>
<td>3.42</td>
<td>52.64</td>
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<td>1.53</td>
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<td>&lt;0.24</td>
<td>&lt;0.24</td>
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<td>0.66</td>
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<td><em>S. champavatil</em> Frep13</td>
<td>26.95</td>
<td>&lt;0.24</td>
<td>&lt;0.24</td>
<td>65.46</td>
<td>0.36</td>
<td>81.32</td>
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<td><em>S. heilongjiangensis</em> Frep14</td>
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<tr>
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<td><em>S. thermocarboxydus</em> K1B</td>
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<td><em>S. variabilis</em> AB5</td>
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<td>0.35</td>
<td>0.16</td>
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*G_{50}, Concentration required for 50% growth inhibition. 3T3, non-tumorogenic, BALB/c mouse embryo cells; H460, human lung large cell carcinoma; HuTu 80, human adenocarcinoma of the duodenum; DU145, human prostate carcinoma; MCF-7, human breast adenocarcinoma; M-14, human amelanotic melanoma; HT-29, human colon adenocarcinoma; K562, human chronic myeloid leukemia cells.*

**Figure 1.** Anti-MRSA activities of *Streptomyces* extracts

![Graph](http://pharmacologyonline.silae.it)