



Bacterial growth in media mimicking the high salt and alkalinity of extreme kazakhstan environments results in production of antimicrobial compounds in soil actinomycetes isolated from these extremophile locations

Jill Roberts¹, Lyudmila Trenochnikova², Lylah Seaton³, Ami Patel³, Colton Faza³, Jacqueline Whitaker⁴, Christen Mayer⁴ and Azliyati Azizan^{3*}

*Correspondence: aazizan@health.usf.edu

¹Department of Environmental and Occupational Health, USF College of Public Health, Tampa, Florida, USA.

²Institute of Microbiology and Virology, Almaty, Kazakhstan.

³Department of Global Health, USF College of Public Health, Tampa, Florida, USA.

⁴Florida Hospital, Tampa, Florida, USA.

Abstract

Increasing antibiotic resistance among multidrug resistant pathogens necessitates the search for newer antimicrobials. *Streptomyces* historically produce the largest number of antibacterials and herein we describe isolation of antagonists from extremophiles using unusual culture media. Antagonists or antimicrobials produced under extremophile environmental conditions demonstrated activity against MRSA from Kazakhstan and the United States.

Keywords: Extremophiles, antagonists, antibacterial, antibiotics, HA-MRSA

Introduction

Staphylococcus aureus colonization of the nose is common in the United States with an estimated 30% of the population carrying the organism asymptotically [1]. However, *S. aureus* is an opportunistic pathogen accounting for as many as 478,000 hospitalizations as recently as 2005, more than half of which were due to methicillin-resistant *S. aureus* (MRSA) [2]. In fact, MRSA is the leading cause of multidrug resistant (MDR) hospital-associated infections in the United States [3]. MDR infections are associated with increased mortality, cost of care, and length of hospital stay [4,5,6,7]. Few antibiotic therapies are currently available to treat these multidrug-resistant pathogens and strains are increasingly insusceptible to new therapies.

Previously our laboratory has demonstrated that a compound produced by *Streptomyces* isolated from the soils of Kazakhstan possesses anti-Staphylococcal activity [8]. Historically, *Streptomyces* has been shown to be the largest antibiotic-producing genus [9] and consequently most clinically-used antibiotics are natural products or synthetic derivatives from the soil actinomycetes [10,11,12]. Mathematical modeling suggests that less than 10% of the antimicrobial compounds capable of being produced by *Streptomyces sp.* have been discovered [9,11]. Theories for the decline in soil actinomycetes-derived antibiotic discovery include decreased screening efforts and lack of focus on culture conditions, and these are the basis of the current study.

Because our earlier study suggested that antimicrobials may be produced by actinomycetes when grown under

stress conditions, we collected 5,936 strains from Kazakhstan soil and marine environments exhibiting high salinity and/or alkalinity (unpublished: Institute of Microbiology and Virology Report). From this, a total of 2,019 isolates which were morphologically consistent with actinomycetes were grown on variations of Bennett's agar, which differed by salt and pH concentration to approximate the extremophile's environmental conditions. The culture conditions used included three variants of Bennett's agar; a neutral medium of pH 7.2 (medium 1), a saline medium with 5% NaCl added (medium 2), and an alkaline medium with 0.5% Na₂CO₃ of pH 9.0 (medium 3). Potential antimicrobials were then isolated from the culture broths using extraction and chromatography methods as previously described [8]. A total of 424 of the extremophile strains produced antagonists, the majority of which exhibited inhibition against a Kazakhstan hospital-associated MRSA (HA-MRSA), when grown in extreme conditions (data not shown) using the standard disk diffusion assay [13]. We propose that actinomycetes may produce novel antimicrobials when cultivated under stress conditions and that these compounds have not previously been recognized due to the use of neutral culture conditions. The ability to produce antimicrobial compounds under stress conditions may provide the *Streptomyces* with an ecological advantage in extreme environments such as those found in Kazakhstan. We propose that anti-MDR antibiotics may be developed from these natural producers much like those currently in development from other unusual sources [10,11,13,14]

Table 1. Inhibition of Kazakhstan and US HA-MRSA by Actinomycetes Antagonists.

| Antagonist # and Source (pH) | Zone of Inhibition (Kazakhstan HA-MRSA) (mm) | | | United States HA-MRSA Zone of Inhibition ^e (mm) |
|------------------------------|--|----------------|----------------|--|
| | Growth | | Media | |
| | 1 ^a | 2 ^b | 3 ^c | |
| 2-2 mud (9.1) | 0 | 18 | NG | 0 |
| 6-12 rhizosphere (8.6) | 0 | 25 | NG | 0 |
| 18-7 sandy soil (10.0) | 13 | 44 | NG | 0 |
| 19-25 soil (9.3) | 0 | 35 | 0 | 11.5 |
| 33-1 mud (9.6) | NG ^d | 49 | 39 | 10.0 |
| 36-3 meadow soil (8.3) | 10 | 24 | 23 | 0 |
| 41-8 saline soil (10.0) | 11 | 29 | 15 | 7.0 |
| 48-29 sandy soil (10.0) | 0 | 32 | 22 | 20.5 |
| 51-9 rhizosphere (9.5) | 10 | 46 | 10 | 0 |
| 58-22 rhizosphere (8.9) | 0 | 18 | 14 | 22.5 |
| 72-1 soil (9.6) | 0 | 50 | NG | 0 |
| 96-1 soil (8.6) | 11 | 26 | 19 | 0 |
| Q4-39 soil (10.0) | 0 | 16 | 15 | 0 |
| Y-45 rhizosphere (9.8) | 0 | 20 | 16 | 0 |

^a Growth Medium 1 = Modified Bennett's pH=7.2.

^b Growth Medium 2 = Modified Bennett's pH=7.2, 5% NaCl.

^c Growth Medium 3 = Modified Bennett's pH=9.0, 0.5% Na₂CO₃.

^d NG = No growth of the Actinomycetes producer.

^e Zone of Inhibition for US HA-MRSA reported is an average of multiple disk diffusion assays (DDA's).

Notes on Growth Medium:

Modified Bennett's Growth Media 1^a, 2^b and 3^c comprised of glucose (0.2%), peptone (0.2%), yeast extract (0.1%), and agar (2%), were used to grow actinomycete strains and incubated at 28°C, and growth was checked after 1-2 weeks incubation.

Purified isolates were investigated in antibacterial tests against Kazakhstan HA-MRSA using the standard disk diffusion assay (DDA); strains with positive anti-MRSA activities were chosen for preparation of Kazakhstan (KZ) extracts.

Notes on Antagonistic KZ extract antibacterial testing against Kazakhstan and United States HA-MRSA:

Fourteen KZ extracts (first column of Table 1 labeled as "Antagonistic #") containing components with antibacterial activities shipped from Kazakhstan were tested in antagonistic tests against United States HA-MRSA grown on Mueller-Hinton agar plates, using standard Kirby-Bauer disk diffusion assay (DDA). Dilution of KZ extracts and antibacterial testing were performed using similar conditions for HA-MRSA isolates from Kazakhstan and United States, using our previously reported methods [8]. Sterile disks containing 10 µg/mL of crude powder of KZ extracts were placed on fresh plates of the Mueller-Hinton agar seeded with bacterial suspensions at a cell density of 5×10⁵ CFU/ml of overnight cultures of the test.

HA-MRSA strains. The diameters of the zones of inhibition of growth (mm) around the disks measured after incubation periods of 18 h at 37°C presented are average values from triplicate experiments.

and that further screening of these compounds is warranted.

Geographical studies of the distribution of epidemic MRSA have demonstrated that the prominent strains in the United States differ from those found in the Kazakhstan region [15,16,17] suggesting that antibiotic efficacy is not equivalent worldwide. Therefore we selected fourteen of the purified anti-staphylococcal compounds produced by the Kazakhstan extremophiles for disk diffusion susceptibility testing using HA-MRSA in the United States. Table 1 lists the fourteen antimicrobial products tested, the stress conditions used to produce the antagonists, the environment from which the actinomycetes producer was isolated, and the observed zone of inhibition against the Kazakhstan HA-MRSA isolate. Sterile disks containing the Kazakhstan antimicrobials were prepared as previously described [8] and disk diffusion susceptibility assay against HA-MRSA was performed using the standard Kirby-Bauer protocol [18]. While the fourteen antagonists tested displayed varied degrees of inhibition against Kazakhstan HA-MRSA, less than half (5 of 14) inhibited US HA-MRSA (Table 1). These data are consistent with the known geographical differences in antibiotic resistance profiles namely in that MRSA strains from the United States are resistant to more antibiotics than those in the Kazakhstan region. These data suggest that the screening method used here in is a valuable tool in identifying potential antibiotics of worldwide utility and future work will include the continued susceptibility testing of the compounds against a wider variety of *Staphylococcus* isolates.

Studies suggest that the classical methodology of screening natural products, particularly those produced by bacterial sources for antibacterial properties is far from reaching its potential [10] despite a decline in the use of these methods. Antibiotic production from natural products may see resurgence as few new synthetic compounds are available and genomic approaches have been largely disappointing. Our data suggest that antagonists produced by actinomycetes when cultivated under stress conditions inhibit MDR strains of *Staphylococcus aureus*. We therefore suggest that high throughput screening of additional Kazakhstan extremophile products incorporating inhibition assays against global MDR isolates is warranted.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JR analyzed the data and wrote the manuscript. LT performed screening of antibiotic producers, prepared and provided extracts from Kazakhstan. LS optimized laboratory experimental protocols and provided technical supervision in the laboratory. AP and CF conducted laboratory experiments, analyzed data and contributed to manuscript preparation. JW and CM provided MRSA clinical isolate and oversight regarding culture and growth conditions. AA as the Principal Investigator provided oversight for the project including laboratory experiments, data analyses and maintained communications with members of the collaborating team. All authors provided intellectual input, read and approved the final manuscript.

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