Detection of antibiotic-producing *Actinobacteria* in the sediment and water of Ma'in thermal springs (Jordan)

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Abstract

Introduction **Detection of new *Actinobacteria* is significant to discover new antibiotics because development of new antibiotics is connected to the characterization of novel bacterial taxa. This study has focused on the identification and isolation of antibiotic-producing *Actinobacteria* from the sediment and the water of Ma'in thermal springs (48-59°C) situated in the center area of Jordan.**

Methods **Samples of sediment and water were transferred to glucose yeast malt agar medium and *Actinobacteria* were cultivated, isolated and identified according to scanning electron microscopy and 16S rRNA gene analysis. Antibacterial activities of the isolates were then tested against different test bacteria by agar well diffusion method.**

Results **Three different species of *Actinobacteria* were isolated (M1-1, M2-2, M3-2) from sediment samples. Based on 16S rRNA gene analysis, isolate M1-1 was found to have only 90% identity percentage with *Nocardiopsis* sp., however, isolates M2-2 and M3-2 were found to be closely related *Streptomyces* sp. (97%) and *Nocardioides luteus* (99%), respectively. The antibacterial activity showed that strain M1-1 is active against *P. aeruginosa* ATCC 2785 (inhibition zone, 9 mm). Strain M2-2 was found to be active against *S. aureus* ATCC 29213 (12 mm), *B. cereus* ATCC 11778 (11 mm), and *E. coli* ATCC 25922 (9 mm). In respect to strain M3-2, it was found to be active against *S. aureus* ATCC 29213 (14 mm) and *B. cereus* ATCC 11778 (9 mm). There were no actinobacterial isolates obtained from water samples despite their significant diversity revealed by our previous metagenomic analysis, which showed the presence of 13 different species dominated by *Arthrobacter* (an Actinobacterium belonging to family *Actinomycetales*).**

Conclusion **There were 17 different *Actinobacteria* that could be detected in Ma'in thermal springs (13 unculturable species and 3 culturable species). The culturable *Actinobacteria* were found to have some antimicrobial activity. Further chemical analysis of the bioactive compounds is recommended.**

Keywords ***Actinobacteria*, antibiotics, Ma'in thermal springs, Jordan.**

Introduction

The phylum *Actinobacteria* represents one of the major taxonomic groups in the domain *Bacteria* with five subclasses, six orders, and fourteen suborders.[1] Most species of this phylum are Gram-positive with few exceptions.[2,3] Most

Received: 15 July 2018; revised: 05 October 2018 and 06 November 2018; accepted: 11 November 2018.

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Article downloaded from www.germs.ro
Published December 2018
© GERMS 2018
ISSN 2248 – 2997
ISSN – L = 2248 – 2997

species in this phylum are aerobic and chemoheterotrophic. In respect to morphology, various morphologies of *Actinobacteria* are present. However, the growth of some *Actinobacteria* is associated with the formation of hyphae of bacterial dimensions, less than half the size of fungal hyphae.[4,5]

Actinobacteria are a well recognized type of bacteria due to their ability to produce antibiotics.[4] Examples of antibiotics from *Actinobacteria* include streptomycin, streptothricin, and actinomycin.[4,6] In addition to their antimicrobial activity, previous research indicated that *Actinobacteria* are a good source of promising compounds with herbicidal, antitumor, antifungal and anthelminthic activities.[1]

Species of *Actinobacteria* are common inhabitants of soil.[4] However, it is now clear that they are more broadly dispersed in nature and they are not restricted to specific habitats. For instance, they can be found as common inhabitants of plant material,[4] aquatic ecosystems including freshwater and marine ecosystems,[1,7] and they are also found as harmless commensals in the human body, for example the oral cavity, gastrointestinal tract, and the genitourinary tract.[8] In some cases, *Actinobacteria* can be pathogenic to humans and the example here is *Mycobacterium tuberculosis*.[1]

Moreover, *Actinobacteria* are also known to exist under extreme environments such as those with high temperatures.[2] *Actinobacteria* surviving and growing at high temperature are referred to as thermophilic *Actinobacteria*. Thermophilic *Actinobacteria* are known to live at high temperatures between 40 and 80°C.[2] Several studies have focused on the microbial ecology of *Actinobacteria* in common environments; conversely, data about the microbial ecology of thermophilic *Actinobacteria* in thermal environments, especially those that are endemic to thermal springs, are still limited.[7]

Ma'in thermal springs (48-59°C),[9] located in central Jordan, represent an interesting habitat for different lineages of thermophilic organisms as revealed by earlier studies.[10-12] Recently, we have analyzed the microbial diversity in Ma'in thermal springs using culture-independent methods.[9] Our findings indicated that the microbial communities in Ma'in thermal springs are very diverse but dominated by *Bacteria* while *Archaea* represent just a minor fraction.

In this current study, we studied *Actinobacteria* from the sediments and the water of Ma'in thermal springs and examined their ability to produce antibacterial agents. Additionally, unpublished data from our previous metagenomic analysis that reflect the diversity of *Actinobacteria* in the studied habitat were presented.[9] The results of this study are expected to broaden our knowledge about thermophilic *Actinobacteria* present in the thermal springs in Jordan and to establish an initial record of the potential of this environment to yield novel antimicrobials.

Studying the diversity of *Actinobacteria* inhabiting thermal environments like thermal springs is important to detect new species that could be a promising source of new antibiotics. Taxonomy-based studies help us to reveal novel bacterial taxa and their capabilities of novel antibiotic production.[13] Analysis of the published literature shows very scarce information available about the antibiotic-producing *Actinobacteria* inhabiting Jordanian thermal springs and this study is expected to be a groundwork study on culturable and unculturable *Actinobacteria* inhabiting the thermal springs in Jordan.

Methods

Collection of sediment and water samples

This study was done on sediment and water obtained from Ma'in thermal springs (Jordan). Three samples of sediments were collected from three different sites at Ma'in Thermal Springs (M1, M2 and M3) that are close to each other. Sediment samples were collected in clean plastic bags. Alternatively, water samples were obtained from three different Ma'in thermal springs (HS1, HS2, HS3) in clean sterile glass bottles as described before.[9]

Chemical testing of sediment samples

Several chemical properties of the sediments used for the isolation of *Actinobacteria* were

examined. The chemical characteristics of sediment samples (pH, total dissolved solids (TDS), electrical conductivity (EC), salinity, concentration of NO_3^-, concentration of Cl^-, and concentration of F^-) were carried out as described previously.[9,12] The physical and chemical properties of water samples from HS1, HS2, and HS3 were tested in a previous study by our group.[9]

Isolation of *Actinobacteria*

Sediment samples were dried before enrichment by spreading the sediment in a sterile Petri dish and incubating at 50°C for three days. Water samples were not pre-treated. After that, serial dilutions were carried out using normal saline to dilute the samples to 10^{-1}, 10^{-2}, and 10^{-3}. A volume of 200 µL of both sample types was transferred to glucose yeast malt agar medium,[14] and incubation was done at 30°C for several days. The resulting bacterial colonies were then compared morphologically based on colonial properties such as shape, color, texture, height, and margins and transferred several times to fresh plates to obtain pure cultures.[15] Cultures were stored as glycerol stocks at -20°C.

Scanning electron microscopy

To examine the micro-morphology of the newly isolated *Actinobacteria*, the *Actinobacteria* were prepared as mentioned previously,[16] and viewed under scanning electron microscope (FEI quanta 200, Thermo Fisher Scientific, Oregon, USA).

DNA extraction

DNA extraction was done using fresh and axenic actinobacterial cultures for the purpose of identification using 16S rRNA gene analysis. Extraction was conducted as described previously.[12]

Molecular identification and phylogenetic tree construction

The extracted genomic DNA was subjected to 16S rRNA gene amplification and sequencing as described previously.[12] The generated sequences were compared to available sequences by BLASTn (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic analysis was done using maximum likelihood method based on the Tamura-Nei model.[17] The phylogenetic trees were built by using MEGA7 Software.[18]

Extraction of antibacterial agents and evaluating their activity

Extraction of antibacterial agents was done as described previously.[14] Briefly, isolates were cultured in medium 5294.[14] After 7 days of incubation at 30°C with shaking (160 rpm), twenty milliliters of cultures were mixed with equal volume of ethylacetate for 10 minutes and shaken by a rotary shaker (20 rpm). Then, samples were centrifuged and the upper phase was transferred to new flasks. Ethyl acetate was evaporated and the remaining was solved in ethylacetate:acetone:methanol (1:1:1, v:v:v:). Screening of the antimicrobial activity was carried out as mentioned previously.[12] Antibacterial activity of the extract was evaluated against four test bacteria: *Pseudomonas aeruginosa* ATCC 2785 and *Escherichia coli* ATCC 25922 (as representatives of Gram-negative bacteria) as well as *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* ATCC 11778 (as representatives of Gram-positive bacteria). The antibacterial activity tests were done by the agar well diffusion method.[12]

Detection of actinobacterial diversity in water samples

The data about actinobacterial diversity presented in this study are derived from our previous metagenomic analyses carried out by our group on Ma'in thermal springs (HS1, HS2, HS3).[9] However, the metagenomic data presented in the results of this study were not published before and they are presented and discussed for the first time.

Results

Chemical properties of sediments

The examined chemical properties of sediments samples include pH, TDS, EC, salinity, concentration of nitrate, concentration of chloride ions, and concentration of fluoride

ions. The sediment samples were nearly neutral as indicated by their pH values. Other chemical characteristics of the sediment samples are shown in Table 1. In respect to water samples (HS1, HS2, and HS3), their measured temperature ranged from 48 to 59°C and the pH ranged from 7.44 to 7.76 as revealed previously.[9]

Table 1. The chemical properties of sediment samples from three Ma'in hot springs sites (M1, M2, and M3) used for the isolation of *Actinobacteria*

	M1	M2	M3
pH	7.6	6.96	6.84
Total dissolved solids (mg Kg^{-1})	2970	7005	6005
Electrical conductivity (mS cm^{-1})	0.66	16.15	13.05
Salinity (ppm)	3	7.5	6
Nitrate (mg Kg^{-1})	1.59	4.43	3.02
Chloride (mg Kg^{-1})	833.35	2488	2224.75
Fluoride (mg Kg^{-1})	3.45	2.30	2.86

Isolation and identification of *Actinobacteria* from sediments

Five different bacterial isolates were obtained from the sediment samples in this study. However, further analysis by scanning electron microscopy and 16S rRNA gene indicated that only 3 isolates belonged to the phylum *Actinobacteria* and the other two isolates belonged to the phylum *Firmicutes* (mainly to the genus *Bacillus*, data are not shown). The three actinobacterial isolates were assigned as M1-1, M2-2, and M3-2. On the basis of 16S rRNA gene, strain M1-1 was found to have only 90% identity percentage with *Nocardiopsis synnemataformans* (sequence length is 1072). Identity percentage may suggest that this strain represents a new species in the genus *Nocardiopsis* based on the similarity that is lower than 97%. Figure 1 shows the morphology of isolate M1-1and its relationship to the closest relatives.

Analysis was carried out by Maximum Likelihood method and the tree was constructed using MEGA7.

In respect to strain M2-2, it was found to be closely related to *Streptomyces* sp. (identity percentage = 97%; sequence length is 1136 nucleotides). Figure 2 shows the cellular morphology of strain M2-2 under scanning electron microscope and its phylogenetic relationship to the closest relatives.

Analysis was carried out by Maximum Likelihood method and the tree was constructed using MEGA7.

Moreover, strain M3-2 was found to be closely related to *Nocardioides luteus* (identity percentage = 99%; sequence length is 1105 nucleotides). Figure 3 shows cellular morphology of strain M3-2 under scanning electron microscope and its phylogenetic relationship to the closest relatives.

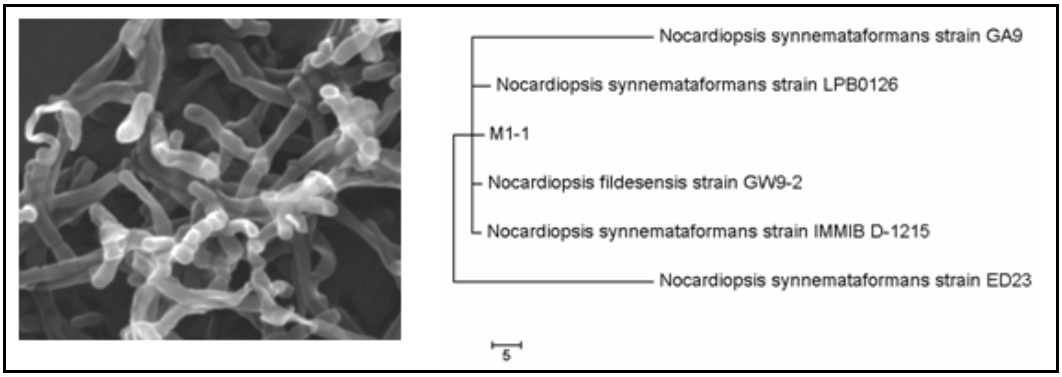
Analysis was carried out by Maximum Likelihood method and the tree was constructed using MEGA7.

Antimicrobial activity of the new isolates

The antibacterial activity of the newly isolated *Actinobacteria* was examined against *P. aeruginosa* ATCC 2785, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *B. cereus* ATCC 11778. Strain M1-1 was found to be active against *P. aeruginosa* ATCC 2785 (inhibition zone, 9 mm). Strain M2-2 was found to be active against *S. aureus* ATCC 29213 (12 mm), *B. cereus* ATCC 11778 (11 mm), and *E. coli* ATCC 25922 (9 mm). In respect to strain M3-2, it was found to be active against *S. aureus* ATCC 29213 (14 mm) and *B. cereus* ATCC 11778 (9 mm) – Table 2.

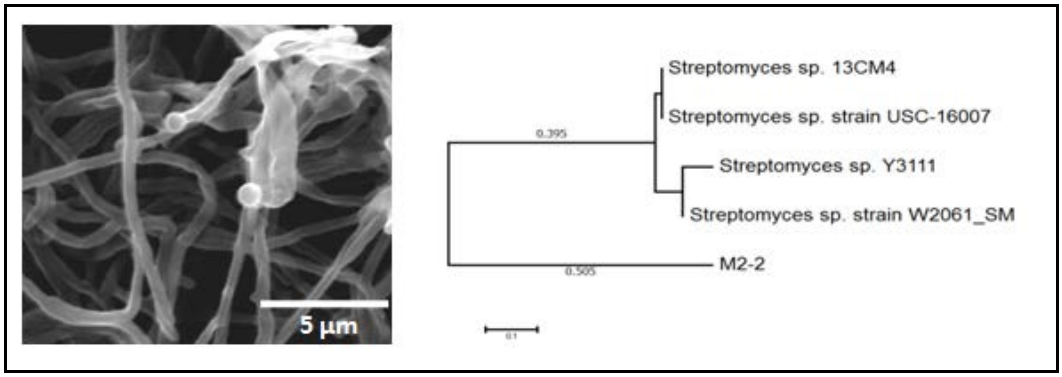
Actinobacterial diversity in water samples

Actinobacteria from water samples could not be isolated by applying the same methods used for sediment samples. However, our unpublished data from a previous metagenomic analysis of Ma'in thermal springs water have shown that the water of Ma'in thermal springs includes a variety of actinobacterial species (13 different species) that are dominated by the



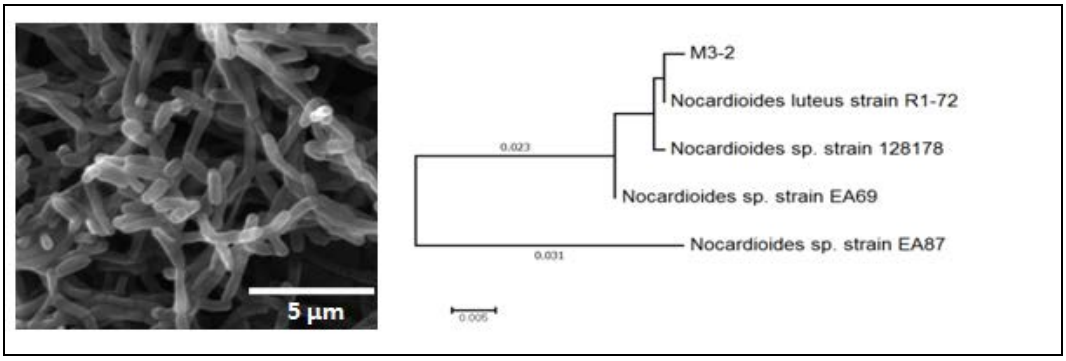
Analysis was carried out by Maximum Likelihood method and the tree was constructed using MEGA7.

Figure 1. Scanning electron microscopic image of strain M1-1 and its phylogenetic relationship to the related relatives



Analysis was carried out by Maximum Likelihood method and the tree was constructed using MEGA7.

Figure 2. Scanning electron microscopic image of strain M2-2 and its phylogenetic relationship to the mostly related relatives



Analysis was carried out by Maximum Likelihood method and the tree was constructed using MEGA7.

Figure 3. Scanning electron microscopic image of strain M3-2 and its phylogenetic relationship to the mostly related relatives

genus *Arthrobacter*. The diversity of *Actinobacteria* in Ma'in thermal springs is shown in Figure 4.

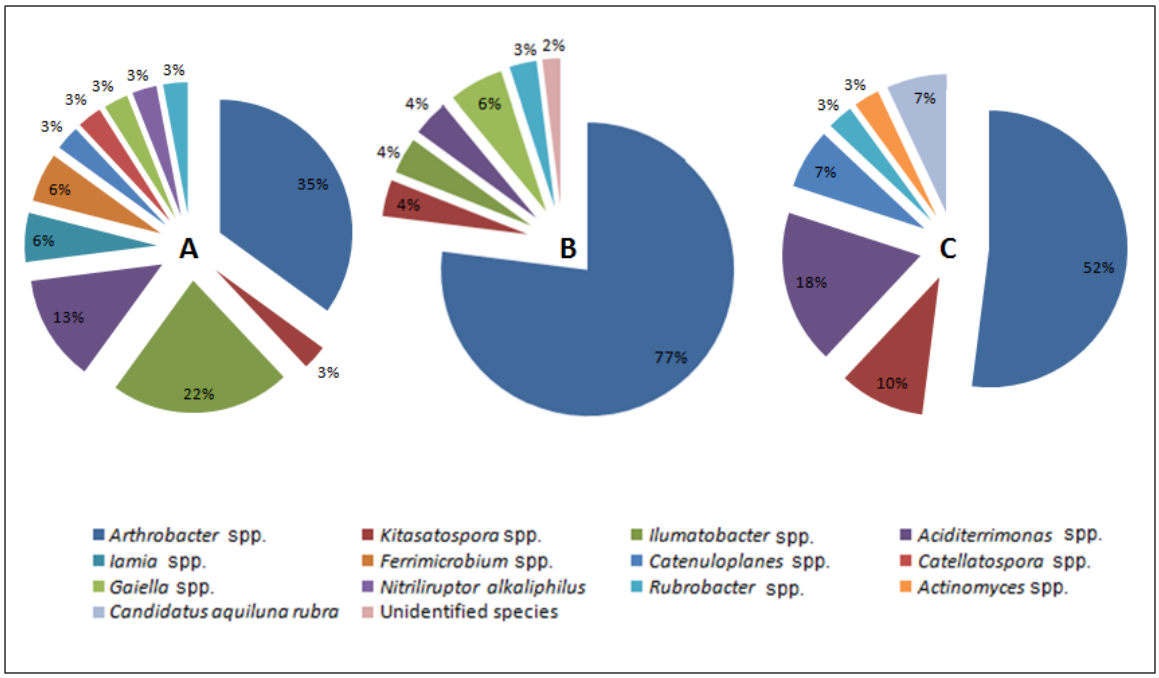
Discussion

Exploring the diversity of *Actinobacteria* inhabiting thermal environments like thermal springs is important to detect new isolates that could be a promising supply of novel antibiotics.[13] The need for new antibiotics is important due to the emergence of pandrug-resistant bacteria and new pathogens.[19] Reports indicate that microbial diseases remain the

Table 2. Diameter of inhibition zones (mm) ± SD formed around the crude extract obtained from antibacterial cultures

	M1-1	M2-2	M3-2
P. aeruginosa ATCC 2785	9 ± 0.58	NA	NA
E. coli ATCC 25922	NA	9 ± 2.3	NA
S. aureus ATCC 29213	NA	12 ± 2	14 ± 2
B. cereus ATCC 11778	NA	11 ± 1.5	9 ± 1

NA – no activity; **SD** – standard deviation.



These unpublished data were generated from our previous metagenomic study.[9]

Figure 4. The major actinobacterial species detected in the three studied Ma'in thermal springs: HS1 (A), HS2 (B) and HS3 (C).

second main cause of mortality around the world despite the vast number of utilized antibiotics. In numbers, microbial infections cause about 17 million deaths per annum.[20]

Actinobacteria represent a significant source of antibiotics and continue to be a promising target for innovation and discovery. Today, most antibiotics are derived from *Actinobacteria* and most importantly the Actinomycetes.[20] Moreover, extremophilic *Actinobacteria* represent an important antibiotic source due to several reasons related to their high growth rate and the tendency of their mycelium for rapid breakage and lysis.[21]

This study revealed the diversity of culturable and unculturable *Actinobacteria* in the sediments and the water of Ma'in thermal springs (located in Jordan) and reported isolates that are able to produce antimicrobial compounds. Previous studies have documented the isolation of a range of antibiotic-producing species of *Actinobacteria* originating from thermal springs that are different from our studied springs either in geographical location or the physical and chemical aspects such as temperature and pH. Local and regional studies have frequently documented the isolation of species from thermal habitats belonging to *Actinobacteria*, like the

genus *Streptomyces*. For instance, Abussaud et al.[22] have isolated 30 actinobacterial strains from water and soil of four Jordanian thermal springs (Alshouneh, Waggas, Al-Mansheyah, and Deirallah thermal springs). All isolated strains were identified as *Streptomyces* species. In the study, the capability of novel isolates to make antimicrobial compounds against *E. coli* and *S. aureus* was noticed among 20% and 26% of isolates, respectively.[22] At the regional level, Al-Dhabi et al. have recently isolated new strain of *Streptomyces* (*Streptomyces* sp. Al-Dhabi-1) originating from Tharban thermal springs soil located in the southwestern part of Saudi Arabia.[23] The strain has also shown a good antimicrobial activity against *Streptococcus agalactiae* and *Klebsiella pneumoniae* in addition to some fungal species.[23]

Thermophilic actinobacterial species with autolytic characteristics were also isolated from the thermal springs in Yunnan, China.[21] More diverse actinobacterial populations were also described somewhere else. For instance, Chaudhary and Prabhu (2016) have described the presence of five different genera of *Actinobacteria* in sediment samples obtained from water springs in Mumbai, India.[24] These isolates belong to genera *Streptomyces*, *Micromonospora*, *Actinomadura*, *Saccharomonospora*, and *Thermoactinomyces*.[24] The isolated strains have a prominent enzymatic activity.[24]

Based on these studies, it can be concluded that the number and the type of *Actinobacteria* differ according to the sampling site, physical and chemical properties of the sampling sites, and the isolation strategy. In our study, we were able to detect the frequently isolated genus *Streptomyces* with a clear antibacterial activity. This agrees with the findings of previous Jordanian and some regional studies that showed *Streptomyces* as an important genus in such habitats.[22-24] Nevertheless, we report in this study the isolation of two additional actinobacterial species: *Nocardiopsis* sp. and *Nocardioides luteus*. Both species possess an apparent antibacterial activity.

In respect to water samples, *Actinobacteria* from water samples could not be isolated even though our metagenomic analysis provided us with the evidence that there is a great diversity of *Actinobacteria* in Ma'in thermal springs. This can be explained by the isolation method utilized (the medium and the set of incubation conditions), which was not suitable for isolating the detected species. Therefore, we need to apply new isolation strategies to obtain the native *Actinobacteria* from water samples because this and earlier studies have already indicated that the isolated bacteria obtained in the laboratory represent a minor fraction of what is existing in nature.[25] The limitations in this study are variable. As in any isolation study, the success in isolation is dependent on providing the suitable nutritional requirements and how to replicate the in situ physicochemical conditions in the lab and how to suppress the competing groups during isolation.

Conclusion

The following antibiotic-producing *Actinobacteria* were isolated from the sediment of Ma'in thermal springs (Jordan): *Nocardiopsis* sp., *Streptomyces* sp. and *Nocardioides luteus*. The isolates showed a clear inhibitory activity against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 2785, *B. cereus* ATCC 11778, and *S. aureus* ATCC 29213. Nevertheless, no actinobacterial isolates could be obtained from water samples despite their significant diversity as revealed by our previous metagenomic analysis, which also showed the dominance of *Arthrobacter*. New isolation strategies and further chemical analysis of the bioactive compounds is recommended.

Authors' contributions statement: All authors contributed equally to this study.

Conflicts of interest: All authors – none to disclose.

Funding: None to declare.

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Please cite this article as:

Hussein EI, Jacob JH, Shakhatreh MAK, Al-Razaq MAA, Juhmani ASF, Cornelison CT. Detection of antibiotic-producing *Actinobacteria* in the sediment and water of Ma'in thermal springs (Jordan). GERMS 2018;8(4):191-198. doi: 10.18683/germs.2018.1146.