medical sciences forum

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Proceeding Paper

Antimicrobial Activities of Compounds Produced by Newly Isolated *Streptomyces* Strains from Mountain Caves †

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† Presented at the 2nd International Electronic Conference on Antibiotics—Drugs for Superbugs: Antibiotic Discovery, Modes of Action and Mechanisms of Resistance, 15–30 June 2022; Available online: https://eca2022.sciforum.net/.

Abstract: The "antibiotic crisis", defined as the appearance of microbial strains resistant to most, if not all, already known antibiotics, indicates that searching for previously unknown antimicrobial agents is crucial for further development of novel drugs that can be used to combat infections caused by bacteria and fungi. Bacteria living in untypical and extreme habitats appear to be a potentially reached source of such compounds. We recently reported an isolation of newly identified strains of Actinobacteria from the Szczelina Chochołowska cave (Tatra Mountains, Poland). Some of them produced molecules revealing antibacterial, antifungal and anticancer properties. Here, we describe further characterization of the selected strains. Their microbiological properties, ability to form biofilms and antimicrobial activities against various strains of bacteria and fungi are reported. The selected strains of newly isolated Actinobacteria belonging to the genus *Streptomyces* appear a promising source of previously unknown antimicrobial agents.

Keywords: cave Actinobacteria; *Streptomyces* spp.; antimicrobial activities; biofilm

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Citation: Jaroszewicz, W.; Bielańska, P.; Lubomska, D.; Kosznik-Kwaśnicka, K.; Golec, P.; Grabowski, Ł.; Wieczerzak, E.; Dróżdż, W.; Gaffke, L.; Pierzynowska, K.; et al. Antimicrobial Activities of Compounds Produced by Newly Isolated *Streptomyces* Strains from Mountain Caves. *Med. Sci. Forum* **2022**, *12*, 7. https://doi.org/10.3390/eca2022-12749

Academic Editor: Manuel Simões

Published: 16 June 2022

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1. Introduction

Antibiotics are compounds produced by microorganisms and acting to inhibit growth or kill other microbial cells [1,2]. They have played a crucial role in combating infectious diseases caused by bacteria and fungi. However, appearance of antibiotic-resistant strains, mainly due to the overuse of these compounds, has caused serious problems in medicine [3,4]. Currently, strains of pathogenic bacteria and fungi resistant to most, or even all, already known antibiotics have been identified, which makes tremendous difficulties in treating patients infected with such strains [5]. Therefore, searching for new antimicrobial drugs is mandatory, and this is an urgent need if effective therapeutic procedures for patients suffering from infectious diseases are considered in the near future. Without the discovery

of novel antibiotics, it is estimated that about 10 million death cases per year might be noted worldwide in the next several years [3].

Microorganisms occurring in extreme, high-to-rich environments can be potentially rich sources of newly isolated compounds revealing various and useful properties, as summarized and discussed recently [6]. Among them, strains producing previously unknown antimicrobial agents have been isolated, providing examples of an effective search for newly discovered antibiotics. One of the habitats especially rich in such strains are mountain caves. In fact, recent years have brought several reports on isolation of cave bacterial strains that are able to produce compounds revealing antimicrobial activities. These studies were reviewed recently, and indicated that Actinobacteria isolated from caves might be an especially rich source of newly discovered antibiotics [6–9]. In fact, very recent original reports confirmed that caves from very different geographical regions, from Asia [10] to Europe [11], are inhabited by microbes producing compounds strongly inhibiting growth of many bacterial and fungal strains.

In our previous work [11], we reported isolation of many bacterial strains from the Szczelina Chochołowska Cave (Tatra Mountains, Poland). Some of them, belonging to the genus *Streptomyces*, were found to produce compounds acting as antibacterial, antifungal and anticancer agents. The putative antimicrobial compounds were identified as isomers of dichloranthrabenzoxocinone and 4,10- or 10,12-dichloro-3-*O*-methylanthrabenzoxocinone; however, it is unknown if they are the only active molecules or if other chemicals of such activities are also produced by cells of these bacteria. In this paper, we report further microbiological characterization of the selected strains and indication of the reason of selection of particular strains for further analyses.

2. Materials and Methods

2.1. Bacetrial Strains and Grotwh Conditions

Actinobacterial strains, isolated previously from the Szczelina Chochołowska Cave (Tatra Mountains, Poland) and reported previously [11], are listed in Table 1. Strains of pathogenic or potentially pathogenic bacteria, tested for their sensitivity to the presence of the isolated Actinobacteria, were described previously [11].

Table 1. Actinobacterial strains isolated from the Szczelina Chochołowska cave [11].

Isolate/Strain	Organism
JHARAB1_N	*Arthrobacter* sp. strain VTT E-052904
JHARN2	*Rhodococcus* sp. strain UFZ-B528
JSZCO2	*Microbacterium* sp. strain JSZCO2
JSZCZL7	*Nocardia* sp. strain JSZCL7
M1_4	*Nocardia* sp. strain OAct 132
M1_7	*Arthrobacter* sp. strain 3S-5
M1_9	*Tomitella biformata* strain AHU 1821
M2_1	*Arthrobacter* sp. (uncultured clone)
M2_11	*Frigoribacterium* sp. strain FB3
M2_15	*Rhodococcus jialingiae* strain djl-6-2 16S
M2_4	*Arthrobacter* sp. strainRKS6-4
M2_9	*Streptomyces* sp. strain MM56
M3_10	*Streptomyces* sp. strain MM56
M3_8	*Arthrobacter* sp. strain 3S-5
M3_9	*Arthrobacter* sp. strain MNPB6
M4_18	*Rhodococcus maanshanensis* strain GMC121
M4_21	*Arthrobacter* sp. strain EM0174
M4_24	*Streptomyces* sp. strain MM56
M4_9	*Nocardiopsis umidischolae* strain NBRC 100349
M5_2	*Nocardia* sp. strain OAct 132
M5_6	*Nocardia* sp. strain OAct 132
M5_8	*Streptomyces* sp. strain MM56
M5_9	*Streptomyces* sp. strain MM56
W2_1	*Microbacterium phyllosphaerae* IHBB 11136

Bacteria were cultured in R2A or Oatmeal media (Merck) or on corresponding solid plates with agar at room temperature (18–22 °C).

2.2. Antimicrobial Activities of Actinobacterial Strains

To determine the effects of the tested Actinobacteria on the growth of strains of various bacteria and fungi, the streak-test was performed as described previously [11]. Briefly, Actinobacterial strains were streaked perpendicularly on plates with the R2A agar, and after 48 h of incubation, other bacterial and fungal strains were streaked diagonally onto the same plates. Following 24 h of incubation, growth inhibition zones were determined by measuring growth-free areas at the crossing regions of the streaks.

2.3. Biofilm Analysis

The formation of biofilms by Actinobacteria was analyzed as described previously [12], in 12-well polystyrene microtiter plates filled with R2A medium adjusted to various pH values. The biofilm was visualized via staining with crystal violet (Sigma-Aldrich). This compound (at the concentration of 0.1%) was added to each well for 30 min, and then the biofilm (if formed) was rinsed 5 times with 1 mL of PBS. Samples were photographed for documentation.

3. Results

To test antimicrobial activities of the isolated Actinobacteria, the streak test was performed as described in Section 2.2. Zones of growth inhibition of various bacterial and fungal strains were measured, and the results are depicted in Figure 1 as a heatmap. From this analysis, it is clear that significant antimicrobial activities are presented by the *Streptomyces* strains named M2_9, M4_24 and M5_8. Since it was demonstrated previously that the 16S rDNA sequences of the M2_9 and M5_8 strains are identical [11], only the latter one was tested further. Nevertheless, the patterns of antimicrobial activities of M2_9 and M5_8 are different (Figure 1); thus, it is likely that they are not genetically identical. When comparing fractions of strains belonging to different bacterial and fungal species that were inhibited by *Streptomyces* M2_9 and M5_8, it appeared evident that the former isolate is more potent in its antimicrobial properties (Table 2).

Further microbiological characterization of the *Streptomyces* M4_24 and M5_8 strains indicated that they formed colonies of different morphologies on R2A and Oatmeal agar plates (Figure 2).

Table 2. Sensitivity of strains of various species of bacteria and fungi to contact with isolated *Streptomyces* strains. Sensitivity was determined as appearance of the growth inhibition zone equal or above 3 mm in the streak-test.

Species [1]	**Fraction of Strains Sensitive to Contact with Isolated *Streptomyces* Strains (%)** [2]	
	M4_24	**M5_8**
Candida spp.	76	35
Escherichia coli	100	100
Pseudomonas aeruginosa	100	100
Salmonella enterica	81	48
Staphylococcus aureus	94	72

[1]: Following number of strains of particular microbial species were tested: *Candida* spp, 17; *E. coli*, 5; *P. aeruginosa*, 4; *S. enterica*, 21; *S. aureus*, 18. [2]: Sensitivity was determined as appearance of the growth inhibition zone equal or above 3 mm in the streak-test.

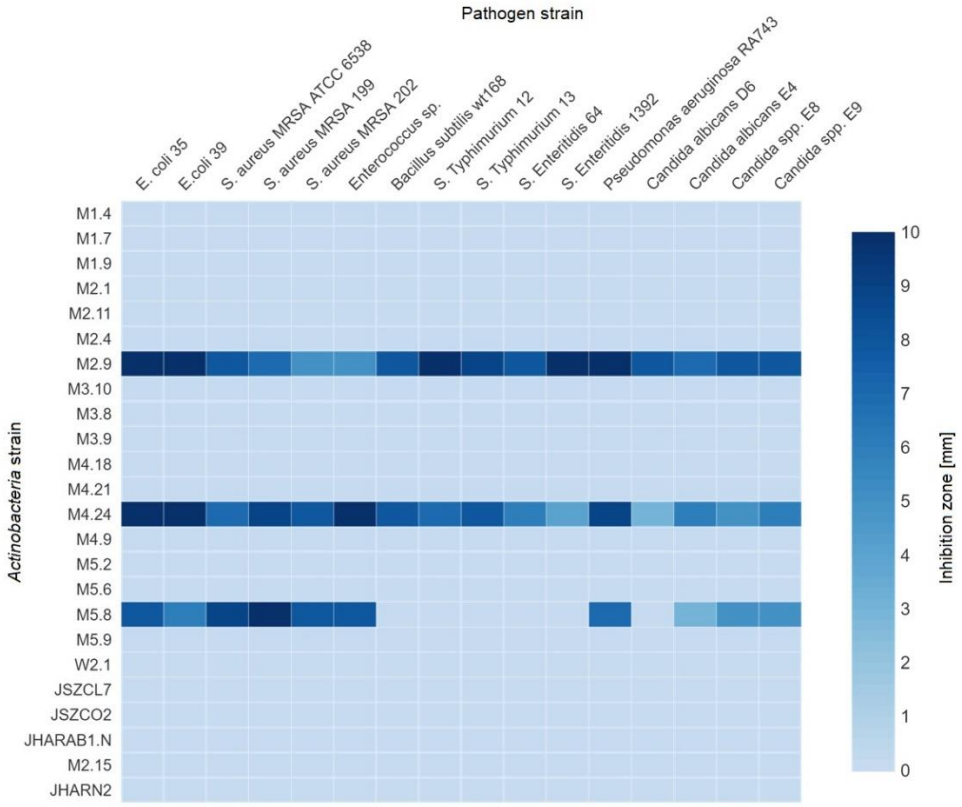

Figure 1. Inhibition of growth of various pathogenic and potentially pathogenic strains of bacteria and fungi by cave Actinobacterial strains isolated previously [11]. The heatmap was constructed considering mean values from 3 independent experiments. The image was created with Displayr software (www.displayr.com (accessed on 14 May 2021)).

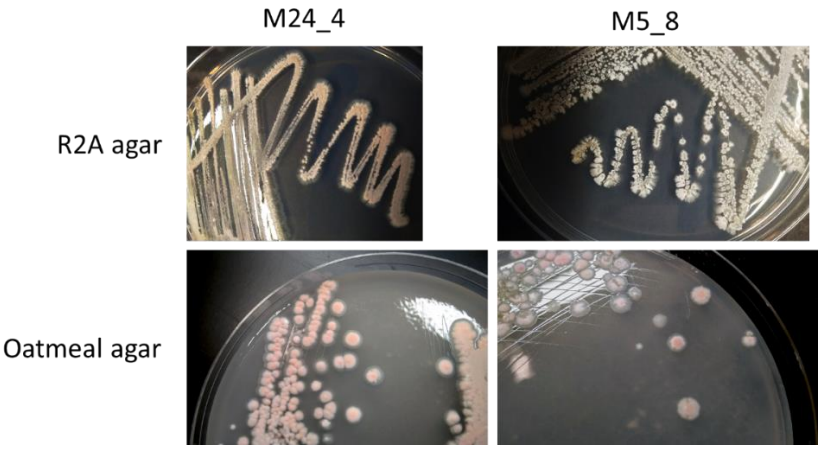

Figure 2. Morphology of colonies of *Streptomyces* M24_4 and M5_8 strains grown on R2A and Oatmeal agars for 7 and 14 days, respectively.

We tested the ability of the investigated strains to form biofilms. Actinobacteria were grown in the R2A medium adjusted to pH 7.2 or 8.5 and the presence of biofilm was assessed via staining with crystal violet. The results are presented in Figure 3. It is evident that no biofilm could be formed by the M4_24 strain, and the M5_8 strain produced only negligible biofilm at pH 7.2 after incubation for 14 days. However, at pH 8.5, the *Streptomyces* M5_8 formed a well-visible biofilm, while the M4_24 strain produced only a weak biofilm. These results indicated that both tested *Streptomyces* strains could form

biofilm, but this property is significantly more pronounced in M5_8 than in M4_24. Elevated pH facilitated this biological activity.

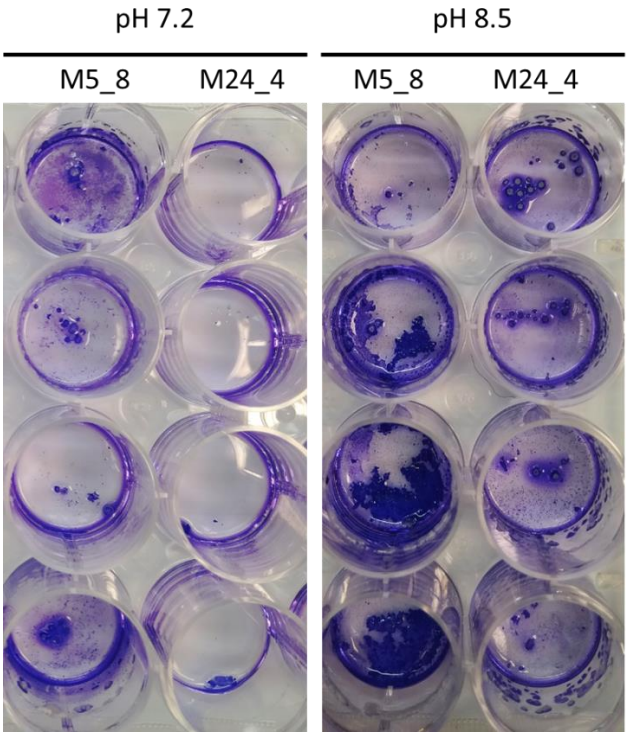

Figure 3. Biofilm formation by *Streptomyces* M24_4 and M5_8 strains grown in R2A medium, adjusted to pH value of 7.2 or 8.5, for 14 days.

4. Discussion

A search for previously unknown antimicrobial compounds is one of the necessary strategies to develop novel therapies against infectious diseases [5]. This is due to the appearance of more and more highly pathogenic bacterial and fungal strains resistant to many antibiotics that are currently in clinical use [3,4]. Importantly, mountain caves were demonstrated previously to be sources of many bacterial strains, mostly classified as Actinobacteria, which are able to produce antimicrobial molecules that have not been described to date [6–9]. Recently, we described the isolation of many strains of Actinobacteria from the Szczelina Chochołowska Cave (Tatra Mountains, Poland) that produce compounds inhibiting growth of various bacteria and fungi and are able to kill cancer cells [11]. Here, we report microbiological characterization of selected strains and present a summary of their antimicrobial activities.

Among the tested isolates, only three revealed significant inhibition of growth of several pathogenic (or potentially pathogenic) strains of bacteria and fungi (Figure 1). However, since the 16S rDNA sequences of two of them were previously demonstrated to be identical, only M4_24 and M5_8 strains were tested in further assays. Nevertheless, different patterns of antimicrobial effects between M2_9 and M5_8 strains suggest that despite full identity of the 16S rDNA sequence, these isolates are not identical. Among the two strains tested in more detail, M4_24 was more effective in inhibiting growth of other bacteria and fungi than M5_8 (Table 2). These two strains differ significantly in the morphology of colonies (Figure 2) and ability to form biofilm (Figure 3). Whether more pronounced biofilm formation by M4_24 is correlated with higher antimicrobial activity remains to be elucidated.

In summary, the newly isolated *Streptomyces* strains M4_24 and M5_8 reveal significant antimicrobial activities. Further studies are needed to substantiate and characterize

chemical compounds produced by these bacteria that might be the basis for developing novel antimicrobial drugs.

Author Contributions: Conceptualization, D.L., K.K.-K., Ł.G., P.G., E.W., L.G., K.P., Z.C., A.W. and G.W.; methodology, W.J., D.L., K.K.-K., P.G., Ł.G., E.W., L.G. and K.P.; investigation, W.J., P.B., D.L., K.K.-K., Ł.G., E.W., W.D., L.G. and K.P.; writing—original draft preparation, G.W.; writing—review and editing, W.J., K.K.-K., Ł.G., P.G., L.G., K.P., Z.C., A.W. and G.W; visualization, W.J., K.K.-K., Ł.G. and G.W.; supervision, P.G., K.P., A.W. and G.W.; funding acquisition, G.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by University of Gdansk (grant no. 531-D020-D242-21).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Original data are available from the authors at request.

Conflicts of Interest: The authors declare no conflict of interest.

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