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To cite this version:
Rima Ouchene, Laurent Intertaglia, Nawel Zaatout, Mouloud Kecha, Marcelino Suzuki. Selective isolation, antimicrobial screening, and phylogenetic diversity of marine actinomycetes derived from the Coast of Bejaia City, Algeria, a polluted and microbiologically unexplored environment. Journal of Applied Microbiology, 2022, 132 (4), pp.2870-2882. 10.1111/jam.15415. hal-03871265

HAL Id: hal-03871265
https://hal.science/hal-03871265
Submitted on 25 Nov 2022

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Selective isolation, antimicrobial screening, and phylogenetic diversity of marine actinomycetes derived from the Coast of Bejaia City, Algeria, a polluted and microbiologically unexplored environment

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Running title: Antimicrobial potential of marine Actinobacteria
Abstract

Aims: The aim of the current study was to evaluate the occurrence of actinomycetes in the Cost of Bejaia City using different isolation methods, as well as to evaluate their bioactivity.

Methods and Results: Different selective media and methods were used, leading to the isolation of 103 actinomycete strains. The number of strains was influenced by isolation procedures and their interactions based on a three-way ANOVA and a post-hoc Tukey test, which revealed that using M2 medium, dilution of samples followed by moderate heat treatment, and sampling sediment at 10-20 m yielded the highest numbers of actinomycetes. The isolates were screened for their antimicrobial activity against human pathogenic microorganisms using agar and well diffusion methods. Of all the isolates, ten displayed activity against at least one Gram-positive bacterium, of which P21 showed the highest activity against *Staphylococcus aureus*, Methicillin-resistant *S. aureus*, and *Bacillus subtilis*, with a diameter of 32, 28 and 25 mm, respectively. Subsequently, active isolates were assigned to *Streptomyces* spp. and *Nocardiopsis* spp. based on 16S rRNA gene sequencing, including a putative new *Streptomyces* species (S3). The phenotypic characteristics of the P21 strain were determined, and interesting enzymatic capacities were shown.

Conclusion: The recovery of actinomycetes along the coast of Bejaia City was influenced by the isolation procedure. Ten strains displayed interesting antibacterial activity against Gram-positive bacteria, of which the P21 strain was selected as the most active strain.

Significance and Impact of the Study: This work provides a new insight into the occurrence of actinobacteria in the Coast of Bejaia using selective isolation. It suggests also that polluted environments such as Bejaia Bay could provide access to interesting actinomycetes as sources of antibiotic leads.

Keywords: Marine actinomycetes, Polluted marine environment, Selective isolation, Antimicrobial potential, Phylogenetic diversity.
Introduction

For decades, severe infections caused by resistant bacteria have been responsible for a major global healthcare problem, requiring longer and more complex treatments that are significantly more expensive to diagnose and treat (Alanis 2005). Moreover, infections generated by Methicillin-resistant *Staphylococcus aureus* (MRSA) have emerged as a widespread cause of community infections, which is due to their ability to develop resistance (Chambers and DeLeo 2009; Pasberg-Gauhl 2014; Barbosa *et al.* 2020). This emerging problem has been observed in many countries, including Algeria (Mairi *et al.* 2020). Thus, the search for new antibiotics remains a worldwide research priority.

Marine environments present a vast source of new bioactive natural products, but they are still either unexplored or underexplored (Bhatnagar and Kim 2010, Gogineni and Hamann 2018). Those environments are characterized by particular conditions such as high salinity, pressure, low temperatures, and variable oxygen concentrations (Bullet *et al.* 2000). Hence, marine organisms require distinctive metabolic capabilities to adapt and thrive in these environments (Bose *et al.* 2015). An increasing number of studies have been conducted on marine actinobacteria as important producers of bioactive compounds in recent years (Maldonado *et al.* 2005; Bull and Stach 2007; Manivasagan *et al.* 2014; Kamjam *et al.* 2017; Barbosa *et al.* 2020; Ibrahimi *et al.* 2020; Goel *et al.* 2021). Interestingly, microorganisms living in unusual growth conditions, such as polluted environments, could be a significant source of interesting bacteria. Even though bacteria isolated from polluted environments have been instead investigated for their enzymatic activity their secondary metabolite production remains poorly understood.

Actinomycetes have long been recognized as a major source of natural products, particularly antibiotics (Bérdy 2005; Kurtböke 2012; Jose and Jha 2016; Jose *et al.* 2021). Recently, rare actinobacteria have attracted more attention and are defined as strains other than the commonly known *Streptomyces* or strains with less frequency of isolation under conventional methods (Baltz
They have taken an increasingly important position in the production of compounds of biotechnological interest. They have yielded a wide range of molecules with medicinal, industrial, or agricultural uses (Dhakal et al. 2017; Ding et al. 2019; Mast and Stegmann 2019), notably those isolated from rare actinobacteria derived from extreme environments (Hui et al. 2021).

Although the isolation of rare actinomycetes in the marine environment is relatively low due to the need for appropriate isolation procedures and the application of different selection conditions, they are difficult to cultivate and maintain under conventional conditions (Subramani and Aalbersberg 2013; Azman et al. 2015). Therefore, a high number of them are "viable but non-cultur able" (VBNC) strains (Bernard et al. 2000). Recently, several strategies have been developed that have promoted the growth of some previously VBNC species, such as mimicking the natural environment in terms of pH, oxygen gradient, nutrient composition, etc. (Dhakal et al. 2017).

Microorganisms derived from extreme biomes have recently attracted scientists’ interest since they could house structurally unique natural products that are still unexploited. For that, the current study focuses on the selective isolation of actinomycetes from an unexplored marine environment along the coastline of Bejaia City (Algeria) and screening of their antimicrobial potential against a set of human pathogenic microorganisms.

Materials and methods

Sampling sites

The sampling was performed in the summer of 2017 from different sites (S1, S2 and S3) in the coastal area of Bejaia City, Algeria (Figure 1), and at different depths that ranged from 0.5 to 20 m with the help of a diver. S1 samples were collected at 10-20 m of depth, S2 at 1-2 m, and S3 samples were collected at 0-0.5 m of depth. The sampling area is polluted by different sources, mainly by the petroleum port (S1), industrial wastes (S2), and domestic discharges (S3). Sediment samples (S1) and water samples (S2 and S3) were taken from different points and were collected in sterile glass bottles, then transferred in a cool box to the laboratory for bacteria isolation.
Selective isolation of actinomycetes isolates

To isolate rare genera of actinomycetes, one should avoid the growth of non-actinomycetes bacteria that affect the isolation of actinomycetes known for their slow-growing. Therefore, different selective isolation media were used as previously recommended in several studies, such as: ISP2 (Claverías et al. 2015), Starch-Nitrate (Ghanem et al. 2000), A4 (Gontang et al. 2007), M2 (Jensen et al. 1991), and Chitin-agar (Pisano et al. 1986). In addition, the isolation media were combined with different isolation methods that are detailed in Table 1. The isolation media were inoculated with 100 µL aliquots of each sample previously pre-treated with methods 1, 2, and 3, as well as method 4, which consisted of the inoculation of untreated samples onto media with added antimicrobial compounds. All inoculated plates were incubated at 28 °C until up to 5 months (with wetted paper towels under the plates to avoid desiccation after 2 months) and examined daily, and all actinomycetes colonies were streaked into new plates containing the same media of isolation until a pure culture was obtained. It should be noted that all isolation media were adjusted to pH 7.2 ±0.2 and prepared with 100% of natural seawater.

Preliminary screening of antimicrobial activity

Preliminary screening for antimicrobial production was estimated by the conventional agar diffusion assay described by Shomura et al. (1979) and Engelhardt et al. (2010). Briefly, agar discs (8 mm in diameter) recovered from the actinomycetes culture (M2 medium, 14 days of incubation at 28 °C) were placed on the surface of Muller Hinton agar pre-seeded with X µl of the target microorganism (Table 2) at 10⁷ UFC/mL. Plates were kept for 2 h at 4 °C for good diffusion of the active compounds. Activity was evaluated by measuring the diameters of the inhibition zones around the agar disc after 24 h of incubation at 37 °C. The M2 medium was composed of 10 g/L of starch, 1 g/L of casein bovine milk, 18 g/L of microbiological agar (Jensen et al. 1991), and 1
mL (per liter of final medium) of trace salt solution prepared according to (Shirling and Gottlieb 1966). All experiments were performed three times independently.

**Secondary screening of antimicrobial activity**

The previously selected isolates were screened a second time by the well diffusion method to improve the production of antibacterial substances in the broth medium using the supernatant of the culture prepared on M2 medium (Patil et al. 2010). Positive activity was estimated by the inhibition zone around the culture supernatant well (8 mm in diameter) of actinomycetes isolates. All experiments were performed three times independently and the results were shown as means with the standard error as error bars.

**Molecular identification and phylogenetic analyses of the active isolates**

All active isolates were cultured on M2 medium for 14 days at 28 °C. Genomic DNA was extracted from the isolates using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer’s instructions. PCR and sequencing were performed as previously described by (Fagervold et al. 2013), using universal primers recommended for bacteria 27F mod: 5’AGRGTTTGATCMTGGCTCAG 3’ and 1492R mod: 5’TACGGYTACCTTGTTAYGACTT 3’. PCR products were purified using a purification kit (Promega, USA) and sequenced using the Sanger-dideoxy-termination method on the Bio2Mar platform with an automated sequencer (3730XL). The 16S rRNA sequences have been deposited in the GenBank database under accession numbers that are given in parentheses in Figure 5.

The 16S rRNA gene sequences were submitted to the EZBiocloud database for identification (Yoon et al. 2017), available at: [https://www.ezbiocloud.net](https://www.ezbiocloud.net). The sequences were imported into the SILVA_138.1_SSUref_NR99_12_06_20_opt.arb database and aligned using the arb fast aligner tool. Sequence positions (662 total), excluding ambiguous and missing, and present in a majority of sequences, were exported and used in phylogenetic reconstruction. Subsequently,
a phylogenetic tree was constructed using MEGA X software (Kumar et al. 2018) by the Neighbor-Joining (NJ) method using the p-distance model (Felsenstein 1985), and the robustness of the tree was estimated by 1000 bootstrap random resampling of the original dataset.

**Phenotypic characterization of the selected strain (P21)**

The micro-morphology of the selected strain (P21) was established by light microscopy to visualize mycelia organization and sporulation. The strain was grown on M2 medium at 28 °C for two weeks before being observed under an optical microscope (Euromex). Furthermore, morphological and physiological characteristics were carried out as described in the International Streptomyces Project (ISP) recommendations by Shirling and Gottlieb in 1966, as well as on three "no ISP" media, including nutrient agar, Bennet medium (Yokota et al. 1993) and GYE medium (Athalye et al. 1981). Melanoid pigment production was determined on tyrosine agar medium (ISP7) recommended by (Shirling and Gottlieb 1966). In addition, the utilization of carbohydrates was tested on a minimal medium (ISP9) containing different carbon sources (D-glucose, sucrose, xylose, inositol, mannitol, D-fructose, rhamnose, and cellulose) at 1% concentration. Basal medium without a carbon source served as a control. Moreover, the conditions of growth, such as the effect of pH (4-14), temperature of growth (4-45 °C), and NaCl tolerance were evaluated (Aouiche et al. 2012). Resistance to different antibiotics was carried out using the disc diffusion test as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2016). The tested antibiotics were: Penicillin G (6 µg), Ciprofloxacin (5 µg), Clindamycin (2 µg), Tetracycline (30 µg), Gentamicin (10 µg), and Chloramphenicol (30 µg). Other tests on the selected strain were carried out according to (Gordon and Mihm 1956), such as the ability of the strain to decompose substrates including casein, starch, and gelatin, and resistance to lysozyme at 0.005% (W/V), while the tyrosine assimilation was performed according (Kurup and Fink 1975).

**Statistical analyses**
All experiments were carried out in triplicate. Firstly, the selective isolation data were analyzed using different statistical tests. The normality of the distribution in the studied groups was confirmed by the Shapiro-Wilk test. To find out the effect of the isolation methods, the used media, and the sampling depths on the number of actinomycetes isolated (note, we did not correct for the dilution in Method 1), isolation data were compared by three-way ANOVA (Analysis Of Variance) (3WA), followed by the post hoc Tukey test without correction for multiple comparisons. The test was considered statistically significant if ($p<0.05$) and was conducted using XLSTAT software (version 2018.1).

Secondly, the preliminary antimicrobial activity screening data were analyzed using principal component analysis (PCA) and hierarchical cluster analysis (HCA). The statistical analyses were performed using R software version 4.0.2 (https://www.r-project.org/), the RProject for Statistical Computing, with the plugin FactoMineR included in R. The PCA plot is used to reduce the dimensionality of such datasets, increasing interpretability while minimizing information loss.

**Results**

**Samples collection and isolation of actinomycetes**

Three polluted marine sites were sampled at different depths from the coastal areas of Bejaia City (Algeria), using different methods and media. A total of 103 colonies of presumptive actinomycetes were observed and isolated. The number of isolates varied with the used methods, media, and sampling depths. As a result of the selective methods that were used, Method 1 showed the highest number of isolates with 46, while 12, 20, and 25 isolates were obtained when Methods 2, 3, and 4 were applied (Figure 2A), respectively. Based on the isolation media used, we found that M2 medium, ISP2 and Chitin-agar medium gave the highest number of isolates with 32, 27 and 22 isolates, respectively. On the other hand, only eleven isolates were obtained with Starch-
Nitrate and A4 (Figure 2B). According to the results of sampling depths (Figure 2C), 56 isolates were obtained at D1 (10-20 m), 33 isolates at D2 (1-2 m), and only 14 isolates were obtained at D3 (0-0.5 m).

Furthermore, the isolation variables (methods, media, and depths) showed a significant effect on the number of actinomycetes isolated ($p<0.05$) based on the three-way ANOVA test. The M2 medium, Method 1 and D1 affected were the best combination for the recovery of Actinomycetes ($p<0.05$). Moreover, the post hoc Tukey test revealed a significant interaction between the different depths/isolation methods ($p<0.05$), but no significant interaction was found between the different depths/isolation media, or the isolation methods/isolation media.

**Preliminary screening assay**

The isolates were screened for their antimicrobial activity by the agar diffusion method against various pathogenic microorganisms (Gram-positive and Gram-negative bacteria, and a yeast). The results showed that 10 out of 103 isolates exhibited antibacterial activity against at least one of the pathogenic tested strains, particularly against Gram-positive bacteria including *S. aureus*, MRSA, and *B. subtilis*, while no activity was detected against the yeast and Gram-negative bacteria.

The results of preliminary antimicrobial screening were subjected to a PCA analysis, which was performed on the active isolates (10 isolates) using nine variables. Results showed that 100% of the total variation was distributed in three dimensions; the horizontal axis explained 65.64% of the total variance, and the vertical explained 32.65% (Figure 3A). The results demonstrated substantial differences between active strains. Moreover, it was found that only three variables contributed to the construction of this PCA, which are MRSA, *S. aureus*, and *B. subtilis*. Figure 3B presents the projection of individuals (actinomycetes isolates). Following that, the PCA data were used for Hierarchical Cluster Analysis (HCA). Therefore, the isolates were grouped into four clusters of active actinomycetes (Figure 3C) identified by their specific antimicrobial activity.
against targeted bacteria, according to the results of HCA analysis. The first cluster was presented by two isolates (S3 and S5) that showed activity only against *B. subtilis* with a 15 mm inhibition zone, while no activity was shown against MRSA and *S. aureus*. The isolates P19, P24, E, and C15 formed the second cluster, which showed significant activity against MRSA and *S. aureus* that varied between 11-22 mm, but no activity was observed against *B. subtilis*. The third cluster was presented by three isolates, S1, MP1m, and S4 that showed relatively similar activities against MRSA, *S. aureus*, and *B. subtilis*, with an inhibition zone that varied from 11-22 mm. The fourth cluster was presented by the strongest active isolate, P21, which showed 28, 32, and 25 mm of the inhibition zones against MRSA, *S. aureus*, and *B. subtilis*, respectively. Thus, cluster 4 is the most active cluster (*p*<0.05) based on inhibition diameter size with the highest antibacterial mean against MRSA and *S. aureus*.

**Secondary screening**

A secondary screening of the previously selected active actinomycetes was performed on M2 medium by the well diffusion method. Except for two isolates (S1 and S3), all active isolates showed positive activity (Figure 4). The P21 strain was found to be the most active isolate, with inhibition zones of 25, 33, and 27 mm against MRSA, *S. aureus*, and *B. subtilis*, respectively. The isolates C15, E, S4 and S5 showed activity against MRSA and *S. aureus* with inhibition zones that varied between 17-26 mm. The P24 isolate showed an inhibition zone of 9 mm against MRSA. The MP1m isolate showed 14, 22, and 8 mm of inhibition zones against MRSA, *S. aureus*, and *B. aureus*, respectively, and the P19 strain showed 25 and 17 mm against MRSA and *B. subtilis*. However, no activity was recorded against Gram-negative bacteria or yeast.

**Molecular identification and phylogenetic analyses of the active isolates**

The 16S rRNA sequences of active isolates were identified using the EZBiocloud database. Isolates were found to belong to the Actinobacteria phylum and were grouped into two genera:
Streptomyces (8 out of 10), and Nocardiopsis (2 out of 10), with similarity percentages to known stains ranging from 98 to 100% (Table S1). Isolates P21 and S5 belonging to the Nocardiopsis genus showed 100% similarity to Nocardiopsis halotolerans DSM 44410(T) and Nocardiopsis aegyptia DSM 44442(T), respectively. Two isolates, C15 and P19, were closely similar to Streptomyces xinghaiensis S187(T) with 99.78% and 99.71% of similarity, respectively. The P24 isolate had a 99.79% similarity with Streptomyces albido flavus DSM 40455(T). The S1 isolate showed the highest similarity (99.76%) to Streptomyces hydrogenans NBRC 13475(T). The S3 isolate was the most closely (98.44%) related strain to Streptomyces rimosus subsp. rimosus ATCC 10970(T), which could possibly indicate the presence of a new species within the Streptomyces genus. Moreover, the S4 isolate showed 99.79% similarity to Streptomyces albogriseolus NRRL B-1305(T). Both E and Mp1m isolates were similar to Streptomyces griseoflavus LMG 19344(T) with 99.62% and 99.61% of identity, respectively.

A phylogenetic tree based on the 16 rRNA gene sequences (Figure 5) was constructed using the neighbor-joining method (Saitou and Nei 1987) using Micrococcus luteus NCTC 26650 as an outgroup. The P21 strain formed a monophyletic line with the S5 strain supported by 100% of the bootstrap replicates. The following strains: C15, E, Mp1m, S4, P19, P24, S3, and S1 appeared in one cluster as closest neighbors.

**Phenotypic characterization of a selected strain (P21)**

The observation of the P21 strain under an optical microscope showed that it has a branching substrate mycelium and produces short chains of spores (Figure 6A). On the other hand, Figure 6B shows the aerial mycelium, which has the typical zig-zag form of Nocardiopsis strains that are fragmented in older cultures. The cultural characteristics of the selected strain are presented in Table S2. The P21 strain was growing well on all media except nutrient agar, where poor growth was observed. Differences in the growth rates were observed, and the color of the substrate mycelium varied from brown-red to white. The production of soluble pigments was observed in
some media (ISP2, ISP5, M2, and Bennet) but not in others (ISP3, ISP4, and Nutrient Agar). Generally, the colors of the aerial mycelium were not the same as those of the substrate mycelium. Table S3 lists the physiological and biochemical characteristics of the P21 isolate. The selected strain was not able to assimilate the carbon sources tested. However, it showed good growth when the medium was supplemented with 10% of sodium chloride, but poor growth was observed at 3-7% and no growth was found at 0% and 15-30% of NaCl. The optimum range of pH growth was found between 7 and 10. Moderate growth was observed at pH 3-5, while no growth was observed at pH 12-14. Moreover, the temperature ranges for growth were between 28 and 40 °C, with an optimum at 28 °C. The P21 strain was found to be able to degrade a variety of substrates, including starch, tyrosine, gelatin, and casein from bovine milk. Furthermore, based on the susceptibility test results, it was found that the P21 strain was resistant to Penicillin G and Clindamycin, but sensitive to Ciprofloxacin, Tetracycline, Gentamicin, and Chloramphenicol.

Discussion

Marine environments are unexplored in Algeria in terms of their microbiota; therefore, the aim of our study was to isolate actinomycetes from those environments. A total of 103 actinomycetes isolates were obtained from the Coast of Bejaia City using different methods and media. These isolates were screened for their antimicrobial activity, and ten isolates showed activity against at least one Gram-positive bacterium (S. aureus, MRSA, and B. subtilis), while no activity was found against Gram-negative bacteria or the yeast tested.

Different selective methods and media were used in this study to promote the growth of rare genera of actinobacteria and avoid the growth of terrestrial actinomycetes while reducing undesirable flora occurring on the isolation plates, as have been reported by others (Pisano et al. 1986; Undabarrena et al. 2016; Kamjam et al. 2017). The application of pretreatments or the presence of antimicrobial substances influences the number of actinomycetes isolates (Bull and Stach 2007). In the present study, Method 1 proved to be among the most effective methods for
isolating actinomycetes, which is consistent with the observation of Pisano et al. (1987) that recorded that heat pretreatment of either 55 °C or 100 °C is a suitable alternative to the higher temperature for actinomycetes isolation, the fact that we did not correct the numbers for the initial dilution makes this result more notable and could also suggest that dilution of inhibiting substances in this highly polluted samples could have also be in play. The low (103) number of total strains in 540 petri dishes supports the possibility of negative effects of the pollution on the actinomycete community.

Using a filter membrane (Method 3) or adding antimicrobial substances to the isolation media (Method 4) have been previously reported to be the best methods for actinomycetes isolation from water and sediments according to (Hirsch and Christensen 1983) and (Bredholdt et al. 2007), but in our case, these methods (3 and 4) gave fewer isolates compared to Method 1. Even though Bredholt et al. (2008) found that heating and exposure of samples to phenol increased the number of mycelium-forming actinomycetes, this method (2) yielded the lowest number of isolates. Based on the isolation media used, the M2, ISP2 and Chitin-agar seem to be the best media for isolating actinomycetes from marine environments. Nevertheless, other studies revealed that low-nutrient media were more efficient than rich-nutrient media for the isolation of rare marine actinobacteria (Jensen et al. 2005; Maldonado et al. 2005). Furthermore, the sampling depth influences the number of actinomycetes isolated. It was therefore found that the number of isolates increased proportionally with the depth, and the highest number of isolates was obtained at D1 (10-20 m). A similar observation was reported previously by (Jensen et al. 1991). Nonetheless, based on the statistical significance of 3WA and Tukey’s post hoc test, the best procedure that gave the highest number of actinomycetes isolates involves using the M2 medium, Method 1, and sampling at 10-20 m (D1) of depth. According to these findings, we suggest that the differences in the responses to the isolation conditions between our study and other studies are very likely due to the level of pollution in the isolation area that affects the abundance of actinobacteria strains. Moreover, the
designated methods to isolate actinomycetes from polluted marine environments including sediment and water samples are not optimal for such biotopes, and more effects are required to establish specific isolation methods for such environments.

The obtained isolates were screened for their antimicrobial activity against nine human pathogenic bacteria. Targeting more than one pathogenic microorganism to obtain diverse bioactive bacteria has previously been supported by (Genilloud et al. 2011). Ten isolates were selected among 103 that showed activity against at least one targeted Gram-positive bacteria. However, this percentage is relatively low in comparison with the results of Peela et al. (2005), who reported that the majority of marine actinomycetes isolated showed antagonistic potential, including antibacterial and antifungal activities. The low rate of active strains could have several possible reasons: 1). Is probably due to the fact that the screening of isolates was based on a specific activity that could be inappropriate for the isolates’ potential, and thus we missed other biological activities. 2). The isolates might possess biosynthesis gene clusters that encode for bioactive compounds but are, "silent" or "cryptic", which means they are not expressed under laboratory conditions. 3). The method or medium used is inappropriate for the production of metabolites. For these reasons, further investigations are being envisaged. Interestingly, the active isolates only showed activity against Gram-positive bacteria, suggesting that the isolates have developed specific marine adaptation mechanisms that likely affect secondary metabolite biosynthetic pathways.

The P21 isolate was shown to be the most active strain against Gram-positive bacteria and belongs to the *Nocardiopsis* genus, which has been described as a producer of a wide range of bioactive compounds (Fu et al. 2013; Hatem Ibrahim et al. 2018; Tatar 2020). Although this genus has received less attention than *Streptomyces*, which has been extensively investigated (Lazzarini et al. 2000; Takahashi et al. 2018). Previous studies have shown the anti-MRSA activity of the *Nocardiopsis* genus, such as the activity of the angucyclines (nocardiopsistins A-C) isolated from
a marine sponge-derived *Nocardiopsis* sp. (Xu et al. 2018). The P21 strain does not show any activity against Gram-negative bacteria even though previous studies have provided the antagonistic potential of *Nocardiopsis* strains against Gram-negative bacteria, such as *Pseudomonas aeruginosa, Klebsiella pneumoniae* (Rajivgandhi et al. 2019), *Enterococcus faecium* and *Candida albicans* (Engelhardt et al. 2010). Hence, this difference is likely due to the specific activity of the secreted molecules by the P21 strain against Gram-positive bacteria, especially MRSA (Figure 7).

Marine sediments harbor a large diversity of culturable actinobacteria (Bredholdt et al. 2007; Gontang et al. 2007; Claverías et al. 2015). In the current work, the phylogenetic diversity analysis of the active strains showed that the isolates belonged to two genera (*Streptomyces* and *Nocardiopsis*), which are widely distributed in marine environments worldwide (Claverías et al. 2015; Jose and Jha 2016). The low recovery observed both in number and diversity could be due to the influence of the contaminants that are present in the isolation region, which can reduce the biological diversity of actinobacteria. In addition, the results of the phylogenetic analysis showed that the homology of the sequences with the closely related species ranged from 98 to 100%. In particular, the S3 isolate showed 98.44% similarity to the closest strain, which is below the 98.65% threshold for this genus (Kim et al. 2014). This isolate can thus be considered a presumptive new *Streptomyces* species. Moreover, several studies in Algeria have recorded the *Nocardiopsis* genera from soil samples, such as the desert soil (Zitouni et al. 2005; Meklat et al. 2011), the salt pan of the Kenadsa region (located in southwest Algeria) (Messaoudi et al. 2019), a salt lake in the Algerian Sahara (Hadj Rabia-Boukhalfa et al. 2017), and from the rhizosphere-associated with semi-arid soil in the North-East of Algeria (Aouar et al. 2012). However, *Nocardiopsis* stains had never been isolated from marine environments in Algeria.

Morphological characteristics, mainly based on the substrate mycelium, the aerial hyphae, pigment production, spore appearance, and chaining, have been proven to be an effective and
widely used reference for the preliminary identification of actinobacteria (Taddei et al. 2006). The P21 isolate showed the typical microscopic appearance of most species of the Nocardiopsis genus. It grew well on several media and showed various cultural characteristics that are related to the available substrate and growth conditions. The P21 strain was susceptible to four antibiotics and displayed resistance to two antibiotics tested (Penicillin G and Clindamycin). According to previous reports, antibiotic resistance may reveal an isolate's ability to produce the antibiotic(s) to which it is resistant (Basu and Paul 1992; Jain et al. 2012). Furthermore, biochemical characteristics showed the potential of the P21 strain to degrade several substrates, which is in line with previous reports that described the Nocardiopsis genus as a potential source of extracellular enzymes (Bennur et al. 2014). Further experiments should be carried out to confirm the enzymatic abilities of this strain.

In conclusion, the Coast of Bejaia City was an interesting source of bioactive actinobacteria strains with the possibility of the presence of new Streptomyces species. Nocardiopsis sp. P21 was found to be the most active strain, with potential for new antimicrobial therapies to fight MRSA infections. Further studies are required to understand the biosynthetic pathways involved in the antibacterial activity of the active strains, including genome mining and metabolomics analyses.

Acknowledgements: We thank the Bio2Mar platform (http://bio2mar.obsbanyuls.fr) for providing technical support and access to instrumentation. This work benefited from access to the Observatoire Oceanologique de Banyuls-sur-Mer, an EMBRC-France and EMBRC-ERIC site.

Authors Contributions: R.O; Methodology, performing the experiments, conceptualization, formal analysis, data collection, and writing-original draft. L.I and R.O; Molecular identification of strains. R.O and M.S; phylogenetic analyses. R.O, L.I, N.Z, K.M and M.S; review & editing. All authors have given approval to the final version of the manuscript.

Conflict of interest: The authors declare no conflict of interest.
Data Availability Statement: The data are not yet available on line.

References


Figure legends

**Figure 1** Map showing the sampling sites of actinomycetes isolates. Sampling spots (S1: 36°45'29.6"N5°05'50.3"E, S2: 36°44'12.1"N5°04'50.7"E, and S3: 36°43'55.2"N5°04'37.9"E) were indicated. The map was generated with the R package ggmap (Kahle and Wickham 2013) under the development environment Rstudio 1.4.1 (https://www.R-project.org/) coupled with R 4.0.2, then it was polished with the image-editing tool Paint.net 4.2.14.

**Figure 2** Actinomycetes counts in sampling sites collected from the Coast of Bejaia (Alegria) depending; (A) isolation method (1, 2, 3 and 4), (B) isolation media (M2, ISP2, Starch-Nitrate, and Chitin-agar), (C) sampling depth (D1: 10-20 m, D2: 1-2 m and D3: 0-0.5 m).

**Figure 3** Principal component analysis (PCA) and hierarchical clustering analysis of active actinomycetes based on antimicrobial activity against 9 human pathogenic bacteria. (A) Projection of the evaluated variables into the two-dimensional space, Dim1 and Dim2. (B) Projection of the active strains into the two-dimensional space, Dim1 and Dim2. (C) Hierarchical clustering of active isolates based on antimicrobial activity. (D) Actinomycetes isolates hierarchical grouping in a three-dimensional space.

**Figure 4** Average inhibition zones (mm) of antimicrobial activity of active actinomycetes isolates against targeted microorganisms using the well diffusion method. Error bars indicate ± s.e. averaged over three measurements.

**Figure 5** Neighbor Joining-phylogenetic tree showing the evolutionary relationship of bioactive strains (marked with a triangle) and closely related sequences based on partial 16S rRNA gene
sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the $p$-distance method and are in the units of the number of base differences per site. The accession number is shown in parentheses following the strain name. The scale bar corresponds to 0.01 substitutions per nucleotide position.

**Figure 6** Morphological appearance of P21 strain grown on M2 medium after 2 weeks at 28 °C. (A): Micro-morphology of Filamentous vegetative mycelia under light microscopy (x40), (B): macroscopic observation.

**Figure 7** Antibacterial activity of the P21 strain against MRSA. Using the agar diffusion method (A) and well diffusion method (B). The delimited zones on red present the inhibition zones.

**Legends for the supporting information**

**Table S1** Molecular identification of actives isolates by EZBiocloud database

**Table S2** Cultural characteristics of the P21 isolate

**Table S3** Physiological and biochemical characteristics of the P21 isolate
Table 1 Descriptions of the applied isolation methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Method 1</strong></td>
<td>Samples were diluted at 1:4 (v/v) with sterile sea water, then vigorously shaken. They were then heated for 6 min at 55 °C (Gontang <em>et al</em>. 2007). A 100 µL volume of the treated sample was inoculated into each media.</td>
</tr>
<tr>
<td><strong>Method 2</strong></td>
<td>Samples were heated at 120 °C for 1 h, then exposed to 1.5% phenol for 30 min (Bredholt <em>et al</em>. 2008). A 100 µL volume of the treated sample was inoculated into each media.</td>
</tr>
<tr>
<td><strong>Method 3</strong></td>
<td>The agar plates of all media were overlaid with a cellulose ester membrane filter (0.45 µm pore size) and then inoculated with a 100 µL volume of the untreated sample. After 2 weeks, the filters were removed and the plates were reincubated (Hirsch and Christensen 1983).</td>
</tr>
<tr>
<td><strong>Method 4</strong></td>
<td>The isolation media were supplemented with filtered (0.2-µm pore size) Nalidixic acid (10 µg/mL), Nystatin (50 µg/mL) (Bredholdt <em>et al</em>. 2007), and Cycloheximide at (50 µg/mL) as recommended by (Bredholt <em>et al</em>. 2008). A 100 µL volume of the untreated sample was inoculated into each media.</td>
</tr>
</tbody>
</table>

Table 2 Targeted organisms tested to select bioactive Actinomycetes
<table>
<thead>
<tr>
<th>Microorganisms tested</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram + Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Methicillin-resistant <em>Staphylococcus aureus (MRSA)</em></td>
<td>ATCC 43300</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 25923</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 10541</td>
</tr>
<tr>
<td><strong>Gram – Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 25922</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ATCC 27853</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>ATCC 6539</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca (Flugge)</em></td>
<td>ATCC 13182</td>
</tr>
<tr>
<td><strong>Yeast</strong></td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>ATCC 10231</td>
</tr>
</tbody>
</table>

ATCC: American Type Culture Collection.
Figure 4
Figure 6