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Diversity and antimicrobial activities of indigenous *Streptomyces* isolates from Fetzara Lake, northeastern Algeria

Mabrouka Benhadj^{1,2,3,4}, Djamila Gacemi-Kirane², Maxime Toussaint^{3,4}, Laurence Hotel^{3,4}, Cyril Bontemps^{3,4}, Raphaël E. Duval^{5,6,7}, Bertrand Aigle^{3,4*#}, Pierre Leblond^{3,4*#}

¹Department of Biochemistry, Faculty of Science, University Badji Mokhtar Annaba, Annaba 23000, Algeria.

²Biomolecules and Application Laboratory, Faculty of Exact Sciences and Natural and Life Sciences, University of Tebessa, 12002 Tebessa, Algeria.

³Dynamique des Génomes et Adaptation Microbienne UMR1128, Université de Lorraine, Vandœuvre-lès-Nancy, F-54506, France.

⁴Dynamique des Génomes et Adaptation Microbienne UMR1128, INRA, Vandœuvre-lès-Nancy, F-54506, France.

⁵SRSMC, UMR 7565, CNRS, Vandœuvre-lès-Nancy, F-54506, France

⁶SRSMC, UMR 7565, Université de Lorraine, Nancy, F-54001, France

⁷ABC Platform®, Nancy, F-54001, France

#equally contributing authors

*corresponding authors: Bertrand Aigle, bertrand.aigle@univ-lorraine.fr, tel +33 (0)3 83 68 42 05,

Pierre Leblond, pierre.leblond@univ-lorraine.fr, tel +33 (0)3 83 68 42 07,

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Running title: Antimicrobial activities and diversity of *Streptomyces* isolates

ABSTRACT

Aims: To explore the diversity of actinomycetes from Fetzara lake and evaluate their antimicrobial potential activity.

Methods and results: A total of 125 *Streptomyces* strains were isolated from an Algerian wetland (Fetzara Lake) and characterized by growth on selective culture media. Phylogenetic analyses were carried out by 16S rDNA sequence comparison after PCR amplification using universal primers. Antibacterial bioassays performed by the agar diffusion method, enabled us to retain 33 *Streptomyces* isolates for their antimicrobial activity against two Gram-positive bacteria (*Bacillus subtilis* and *Micrococcus luteus*) and one Gram-negative bacteria (*Escherichia coli*). Among them, six isolates inhibited all three indicator strains. Antibacterial compounds were then extracted from the solid culture media with organic solvents (ethanol and ethyl acetate). The minimal inhibitory concentration (% v/v) of the extracts was evaluated by a standardized broth dilution method against different clinical-resistant bacterial isolates and a *Candida albicans* strain. The two most active extracts were selected for further characterization by chromatographic analysis (RP-HPLC).

Conclusion: Fetzara lake presents a great *Streptomyces* diversity including potentially and promising antagonist strains. Some of the strains show promising antimicrobial activities against clinical resistant strains. Isolation of some bioactive compounds has been initiated.

Significance and impact of this studies:

This work demonstrates that actinomycetes from this unexplored habitat present a prolific source for screening of newly antimicrobial metabolites against drug resistant microorganisms.

INTRODUCTION

Since the discovery of the first antibiotic, the emergence of resistant forms of treated microbial pathogens was reported soon after the introduction of the antibiotic as medical prescription. This phenomenon more than a concern became a real threat with the appearance of multidrug resistance and its spread among the bacterial populations. It is estimated that more than 4 million patients acquire a healthcare-associated infection in the European Union every year leading to 37,000 deaths (Source: European Centre for Disease Prevention and Control, URL: <http://ecdc.europa.eu>). In the same way, at least 2 million people are infected by antibiotic resistant pathogens each year in the United States, and at least 23,000 people die annually as a direct result of these infections (Source: Center for Disease Control and Prevention, USA. URL: <http://www.cdc.gov/>). The dissemination of the resistance is resulting from the misuse and overuse of antibiotics (inappropriate or delayed administration, use of broad-spectrum instead of narrow-spectrum antibiotics...). The appearance of antibiotic resistance, strengthened by the capacity of bacteria to gain and exchange resistance genes, results in a weapon race that urges us to renew the antibiotic arsenal and to look for renewed sources for antimicrobial drugs.

Microbial natural products (NPs) show an impressive diversity providing continuously renewed opportunities to discover bioactive compounds and to develop new therapeutic agents. Actinobacteria alone provide almost half of the biologically active compounds of microbial origin (Berdy 2005) and about 75% of bioactive metabolites effectively used in human therapy including those produced by the genus *Streptomyces*, well-known as a provider of a large range of bioactive compounds showing antibacterial, antiviral, antiproliferative or immunosuppressive activities (Challis and Hopwood 2003). Although, classical prospection of soil bacteria frequently results in the rediscovery of already known compounds (Baltz 2007; Fischbach and Walsh 2009), *Streptomyces* species still represent the most prolific source of antibiotic compounds. The

Streptomyces alone provide about the one third of the total of the bioactive microbial metabolites (Watve et al. 2001; Berdy 2005).

Streptomyces are ubiquitous and could represent up to 10% of the soil microflora (Janssen 2006). They colonize all terrestrial ecosystems and participate to biogeochemical cycles through their degradation properties of biopolymers such as cellulose, lignocellulose (resulting from wood decay) and chitin or diverse organic compounds. They contribute to plant health through plant growth promoter properties (*i.e.* mycorrhiza helper bacterium, nutrient uptake; siderophore production, volatiles providing disease suppressive soil) or phosphate solubilization. They are involved in biotic homeostasis for example by the development of symbiosis (*e.g.* fungi, plants and animals)(Schrey et al. 2005; Seipke et al. 2012; Battini et al. 2016). Since they produce spores, they are easily disseminated by water flows, by fungi hyphae in extension as well as by wind. They are also consequently encountered in aquatic environments and in marine ecosystems.

The access to genome sequences thanks to the development of new generation sequencing approaches revealed a new picture of the secondary metabolite biosynthesis capacity. Hence while each *Streptomyces* strain was known and exploited for the production of a limited number of bioactive compounds, the genome of each species includes several tens of genes or gene clusters responsible for NP biosynthesis (up to 30, sometimes more) (Bentley et al. 2002; Challis 2008; Nett et al. 2009). However, most of these biosynthetic genes remain silent under laboratory conditions (Aigle et al. 2014). Their expression can be revealed using many kinds of approaches including specific fermentation conditions, heterologous expression, genetic modifications (*e.g.* by playing with global or specific regulatory networks (Laureti et al. 2011), and culture conditions mimicking the ecological biotic or abiotic parameters (co-cultures, addition of metabolites...) (Liu et al. 2013). While diversity of soil *Streptomyces* has been widely studied and reported (Bontemps et al. 2013; Goljanian Tabrizi et al. 2013; Aouiche et al. 2014), diverse habitats and ecological niches as aquatic

environments (Yadav et al. 2013; Singh et al. 2014) are expected to offer a strong biological diversity associated to new secondary metabolite repertoires. The exploration of this diversity associated to the access to the genomic information will help to exploit the potential of the newly isolated biological diversity (Kurtboke 2012). The Fetzara Lake (northeast Algeria) is a representative of rare type of natural wetlands in the Mediterranean region (Habes 2011; Abdelkader 2012). This special ecosystem can be explored for the isolation of actinomycetes including *Streptomyces* with new metabolic capability. In the present study we report the first insight into (i) the biodiversity of actinomycetes in the water Lake using both cultural and molecular tools, and (ii) the determination of the antimicrobial potential of actinomycete isolates.

MATERIAL AND METHODS

Water sampling and isolation of actinomycetes

Five samples (1 L each) were collected from different sites of Fetzara lake, Annaba northeastern Algeria, a representative and rare type of natural wetland in the Mediterranean region (Coordinate 36°43' and 36°50'N, 7°24' and 7°39' E).

Isolation of actinomycetes was carried out using a dilution agar plating method (Zitouni et al. 2004; Busti et al. 2006). Samples from heated water (treatment at 50°C, 60 min to increase the ratio of sporulating actinomycetes versus other microorganisms) and untreated water were diluted three times and 0.1 ml aliquots were then spread separately on four different solid media, International *Streptomyces* Project n°2 (ISP2) (Shirling 1966), Starch Casein (SC), Emerson and Gauss agar (Larpen-Gourgaud 1997). To avoid the development of other bacteria and fungi, SC and ISP2 media were supplemented with antibiotics rifampicin (2.5 µg/ml) and amphotericin B (10 µg/ml) and fluconazole (75 µg/ml), respectively (Kitouni ; Peela S. 2005) while Emerson and Gauss agar media were supplemented with streptomycin sulphate (10 µg/ml)(Zitouni et al. 2004) and amphotericin B (10 µg/ml) and fluconazole (75 µg/ml). All the media for the isolation of actinomycetes were prepared with filtrated water from the Fetzara Lake.

The plates were incubated at 10°C, 28°C and 37°C for 30 days. Colonies of actinomycetes were recognized based on their microscopic aspects (filamentous growth, spore chains) and cultural characteristics (*e.g.* morphology of the colonies, color of substrate and aerial mycelia and presence of diffusible pigment). Isolated colonies were streaked on ISP2 medium for several sporulation rounds. Purified isolates were maintained on ISP2 slants at 4°C and on SFM medium (Kieser 2000). Spore suspensions were stored -20°C in glycerol 20% (v/v).

DNA extraction and 16SrRNA gene amplification

Genomic DNA was extracted as described by the method of Pospiech and Neumann (Pospiech and Neumann 1995) from cultures in HT liquid medium (Kieser 2000). The amplification of the gene encoding the 16S rRNA was carried out with the universal bacterial primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al. 1991) using the ThermoPol Kit (New England Biolabs). The following PCR conditions were used: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 90 s, and a final elongation step of 72°C for 5 min. The reaction mixture (50 µl) contained 5 µl 10x buffer, 5 µl dNTPs (2 mM/l), 2.5 µl DMSO, 0.5 µl FD1 forward primer (10 µmol/l, Sigma-Aldrich), 0.5 µl rP2 reverse primer (10 µmol/l; Sigma-Aldrich), 0.25 µl Taq-polymerase (DreamTaq-DNA polymerase) and 1 µl (~100 ng) of genomic DNA.

16S rRNA gene sequencing and phylogenetic analysis

The full length sequence of the 16S rRNA gene was determined on both strands using the primers FD1 and rP2. The sequences were aligned using MEGA 6.06 (Molecular Evolutionary Genetics Analysis, Version 3.1) software (Tamura et al. 2013) with the Clustal algorithm (Larkin et al. 2007) and the level of similarity was compared against corresponding nucleotide sequences of representative closely related strains available at the NCBI database (ncbi.nlm.nih.gov). Evolutionary distance matrices were generated using the Kimura 2-parameters (Kimura 1980). The phylogenetic tree were reconstructed with MEGA 6.06 based on a 970 pb alignment using the neighbor joining method (Saitou and Nei 1987). The topologies were evaluated by bootstrap analysis of the neighbor joining dataset based on 1000 resamplings (Felsenstein 1985). The 16S rRNA gene sequences were deposited in GenBank database (NCBI) under the accession numbers KX279522 to KX279646.

Antimicrobial activity assay

Indicator organisms

Eight indicator strains were used for assessing the antimicrobial properties, including the laboratory strains *Bacillus subtilis* ATCC6633, *Escherichia coli* DH5 α and *Micrococcus luteus* DSM1790 and the clinical bacterial strains *Bacillus cereus* ABC143 (isolated from patient's feces), *Staphylococcus aureus* ABC61, a *mecA* strain resistant to methicillin (MRSA strain, from blood specimen), *E. coli* ABC28, an extended spectrum β -Lactamase (ESBL) strain (from blood specimen), *Pseudomonas aeruginosa* ABC132 (resistance to antibiotic due to efflux pump; from skin and soft tissue infection) and the yeast *Candida albicans* AFC1 (from gynecological sample). *E. coli* ABC28, *S. aureus* ABC61 and *P. aeruginosa* ABC132 were isolated from nosocomial infections (Grare et al. 2007). All strains were maintained at 4°C on slants of Luria–Bertani (LB) or on Mueller-Hinton (MH) agar for bacteria and Sabouraud Dextrose (SD) agar for *C. albicans*.

Preliminary screening for antimicrobial activities

The actinomycete isolates were assessed for their ability to produce antibacterial activities against *B. subtilis* ATCC6633, *M. luteus* DSM1790 and *E. coli* DH5 α using the diffusion plate method (Busti et al. 2006). Actinomycetes were inoculated on two different media, ISP2 and Bennett agar plates, with 100 μ l of a spore suspension (10^8 spores/ml). Plugs from cultures of the different actinomycetes were then placed on the surface of a LB soft agar plate previously seeded with the indicator organisms (at an initial OD₆₀₀ of 0.04). Plates were incubated at 4°C for 2 h to allow diffusion of the antimicrobial compounds from the plugs, and then at 30°C (*B. subtilis*) or at 37°C (*M. luteus* and *E. coli*) for 24 h. Antibacterial activities were assessed over several days and inhibition activities were recorded. Plugs of non-inoculated ISP2 and Bennett culture media were used as negative controls.

Second round of screening and metabolite extraction procedure

The bioactive isolates (100 μ l at 10^8 spores/ml) were grown at 30°C on ISP2 or Bennett agar media. When necessary, plates were covered with a cellophane membrane before inoculation to allow separated extractions from mycelia and culture medium. Based on the results of the preliminary screening for each of the isolates, extractions were carried out at different days of growth (from 3 to 17 days). For the cellophane-covered plates, extractions were done separately on mycelia (scrapped from the cellulose membrane) and from the spent agar. Otherwise extractions were done from the whole plate (agar + mycelium). Two organic solvents (ethanol and ethyl acetate) were used for extraction. Compounds from mycelia and/or culture media were extracted with 40 ml of solvent per plate (~20 ml of culture medium) under shaking at room temperature for 2 h. Extracts were then centrifuged (3,600 x g for 15 min) and filtrated through a 0.45 μ m pore size Supor[®] polyether sulfone membrane (PALL Life Sciences). The extracts were then dried on a rotary evaporator at 40°C and dissolved in 400 μ l of dimethyl sulfoxide (DMSO) at 10% (v/v). Twenty microliters of extract were used for antimicrobial activity assays by the disc diffusion method using *B. subtilis* ATCC6633, *M. luteus* DSM1790 or *E. coli* DH5 α as indicator strains.

Determination of the MICs

Extracts were assayed to determine the minimal inhibitory concentrations (MICs) against the clinical strains *B. cereus* ABC143, *E. coli* ABC28, *P. aeruginosa* ABC132, *S. aureus* ABC61 and *C. albicans* AFC1. MICs were determined by broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (2008, 2009) and the ‘Comité de l’Antibiogramme de la Société Française de Microbiologie’ (2014). Indicator strains were cultivated by inoculation of a cell suspension (dispersion of an isolated colony) into 5 ml of MH broth for the bacterial strains and 5 ml of SD broth for the yeast. After 24 h of growth, the cultures were diluted in sterile distilled water to obtain a final inoculum of 10^5 – 10^6 CFU/ml. Two-fold serial dilutions of each extract were

prepared in MH Broth in 96-wells plates (150 μ l per well) completed with an equal volume of bacterial indicator culture. After incubation for 24 h at 37°C, the MICs were determined as the lowest concentration of extract showing a 540 nm absorbance comparable to the negative controls (wells containing broth only or broth with extract without microbial inoculum). Two independent assays were done for each extract (except against the fungus *C. albicans*).

RP-HPLC analysis of the bioactive extracts

The metabolite extracts of two bioactive strains, E1N211 and E2N171, were analyzed by RP-HPLC. For each strain, extract was obtained from spent agar from four ISP2 culture plates (~20 ml per plate) covered with cellophane after three (E2N171) or seven (E1N211) days of growth at 30°C. Extractions were carried out with ethanol and after evaporation of the organic solvent; extracts were dissolved in 50 μ L of DMSO 10% and filtered on Phenex RC membrane (0.45 μ m; Phenomenex). Aliquots of 250 μ l or 500 μ l of extracts were analyzed by RP-HPLC (Alliance) unit equipped with a photodiode array detector 996 (Waters, Milford, USA) and a Lichrosphere RP18 column (150 x 2 mm, 5 μ m particle size and 10 nm porosity; Merck). A linear gradient from 5% to 75% acetonitrile in water was applied in the presence of 0.1% of trifluoroacetic acid for 70 min with a flow rate of 0.25 ml/min at a temperature of 30°C. Absorption was monitored at 232 nm and several fractions at different times were collected in microtubes, lyophilized and finally suspended in 50 μ l of DMSO 10%. The fractions (5 μ l) were tested in bioassays against *B. subtilis* ATCC6633, *M. luteus* DSM1790 and *E. coli* DH5 α by the disc diffusion method.

Some of the fractions (45 μ l) were submitted to a second round of RP-HPLC using the same experimental conditions for the elution. Sub-fractions were then collected at different times, lyophilized and dissolved in 60 μ l of DMSO 10%. Their biological activities were tested as above.

RESULTS

Isolation of actinomycetes from the lake of Fetzara (Algeria)

A collection of actinomycete strains was established from five water samples collected at different sites of the Fetzara lake (Annaba region, northeastern of Algeria, Figure S1), a representative type of natural wetland in the Mediterranean region. A total of 133 actinomycetes (76 and 57 clones from the heat-treated and untreated samples, respectively) were isolated, mostly from plates incubated at 28°C or 37°C and their morphological characteristics were assessed on ISP2 agar plate. The majority of the strains formed powdery colonies with vegetative and aerial mycelia (Figure S2). The mycelia were for the most colored with either gray, red or black pigments. In addition spore chains could be observed in some strains with either a linear or helix shape (data not shown). Sixteen isolates produced a diffusible pigment (brown, red or green-colored pigment). Altogether, these data suggested that most of the isolates (125/133) likely belonged to the *Streptomyces* genus based on their phenotypic traits.

Bacterial identification and phylogenetic analysis

The sequence of the 16S rRNA gene was determined in order to confirm the previous morphological analysis at the molecular level. PCR amplification using the universal 16S rRNA primers was obtained from the 133 isolates. Blastn analysis of the obtained DNA sequences showed that 125 isolates indeed belonged to the *Streptomyces* genus and eight isolates to other actinobacteria taxa (3 *Nocardia*, 3 *Micromospora*, 1 *Nonomuraea* and 1 *Actinomadura*). A great diversity was recorded among the *Streptomyces* since up to 40 different species could have been identified. The distribution of the percentage of sequence similarity with the closest type strains was as follow: 38 isolates showed a nucleotide identity of 100%, 81 between 99.0%-99.9% and 6 with an identity lower than 99.0% (Table 1). A polyphasic analysis would confirm this preliminary

result. For the non-streptomycete actinomycetes, which were not studied further, the similarities ranged from 99.8% to 100%.

A phylogenetic tree based on 16S rRNA gene sequences was constructed including the 125 newly isolated *Streptomyces* strains and their closest reference type strains using the neighbor-joining method in order to cluster the strains and to select representative isolates for further analyses. Analysis of the tree based on the percentage of bootstrap, on the evolutionary distance and on the position relative to reference type strains showed a wide distribution of the *Streptomyces* isolates (Figure 1). The isolates were grouped based on the structure of the phylogenetic tree into two major classes (Class I and Class II) and four subclasses referred as clusters 1 to 4 in Figure 1. The majority of isolates belonged to Class I including clusters 1 and 2 while comparatively few isolates belonged to Class II including clusters 3 and 4 (Figure 1).

Investigation of the antimicrobial activities from the *Streptomyces* isolates

Preliminary screening of the antibacterial activities

A sampling of 33 *Streptomyces* strains was retained for further antimicrobial activities assays. The strains were chosen to be representative of each main phylogenetic cluster (Figure 1) and to reflect the phenotypic diversity previously reported. These strains were evaluated for antibacterial activities after growth at 30°C on either ISP2 or Bennet agar medium by the agar plug diffusion method using *B. subtilis* ATCC6633, *M. luteus* DSM1790 and *E. coli* DH5a as indicator strains (Table 2).

Only four strains (E1N260, E1N302, E2N176 and E2N227) did not show any antimicrobial activities. The other 29 were active against *M. luteus* and 26 of them also prevented the growth of *B. subtilis*. Finally, only six isolates (E1N211, E2N171, E3N218, E3N312, E5N158, and E5N299) were found to be active against the Gram-negative bacterium *E. coli*. These strains were also able to

inhibit the growth of the two Gram-positive indicator strains. The inhibitory activity reached a maximum after varied times of growth (in days) and depended on the culture medium (Table 2).

The two strains exhibiting the highest inhibitory activities against the three indicator strains, *i.e.* strains E1N211 and E2N171 (Table 2), were analyzed for the production of antibacterial compound(s) during 23 days by the agar diffusion method from cultures on ISP2 at 30°C. The activity was assessed against the same indicator strains as above (Figure S3). Whatever the indicator strain, the highest activity was observed at day 3 and day 6 for E2N171 and E1N211, respectively. Then, the antibacterial activities decreased and became undetectable at day 10 with *E. coli* as indicator strain or at day 23 with the two other indicator strains (with the exception of *M. luteus* for the E1N211 strain). This suggested that the production of the compound(s) responsible for these activities is transient and that the compound(s) is (are) somehow unstable (it could be either degraded or inactivated).

Extraction of the antibacterial compounds

We then tested the efficiency of two organic solvents (ethanol and ethyl acetate) to extract the antibacterial compounds produced on ISP2 or Bennet agar by the six *Streptomyces* isolates showing high inhibitory activities against the three indicator strains. The compounds were extracted from the whole plates (mycelium and agar) and, after evaporation of the solvent, were dissolved in 400 µl of DMSO 10% (see Material and Methods). A 20 µl fraction of each extract was then tested for antibacterial activities (Table 3). Based on the diameter of the inhibition zones, ethanol appears to be more efficient than ethyl acetate to extract the antibacterial compounds produced by the six isolates, thus revealing that the bioactive molecules show polar characteristics.

The six isolates were then grown on the same media (ISP2 or Bennett agar) but covered with a cellophane membrane to allow separated extraction of the secreted compounds from mycelium and spent agar. The extractions were then done with ethanol (see Materials and Methods). We observed

that, with the notable exception of the E1N211 strain, most microbial activities originated from the culture media (Table 4). In contrast to the preliminary screening, no activity against *E. coli* was observed for the E3N312 extracts whatever their origin, mycelium or spent agar (Tables 3 and 4). This could be due to the low efficiency of ethanol to extract the active compound against *E. coli*. This compound could indeed be distinct from the compound(s) active against the Gram-positive indicator strains. Nevertheless, one cannot exclude that the same compound is active against the three strains but with different efficacy and is at too low concentration in the extract to prevent the growth of *E. coli*.

The ethanol extracts from agar culture media were then tested for their inhibitory activities against different clinical strains characterized for their resistance to antibiotics: *E. coli* ABC28, an ESBL strain, *P. aeruginosa* ABC132 which shows resistance thanks to an efflux pump system and *S. aureus* ABC68 which carried the *mecA* gene conferring resistance to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics (Chambers 1997). Additionally, we also included in the test *B. cereus* ABC143 and the yeast *C. albicans*. MICs were determined by dilution method following guidelines of the CLSI (Institute 2008; Institute 2009) and of the CA-SFM (Microbiologie 2014) as described in the Material and Methods section. All extracts but the one from strain E5N158 inhibited the growth of the four pathogenic bacteria and the yeast (Table 5). Indeed, even if the MIC could not be determined for some of the extracts, a clear dose-dependent growth inhibition was observed. Interestingly, all the extracts show a strong activities against *P. aeruginosa* with a MIC of 0.4 (% vol/vol) or below (see discussion). Furthermore, the results revealed that the extract from the E1N211 isolate was the most efficient considering the inhibitory effect on the five indicator strains all together.

RP-HPLC analysis of spent agar ethanol extracts

The antimicrobial compounds produced by the E2N171 and E1N211 strains were then extracted with ethanol from spent agar of four ISP2 plates covered with cellophane after three and seven days of growth, respectively (see Materials and Methods). RP-HPLC was then carried out on each of the extracts. For the extract of the E2N171 strain, four fractions were collected over a length of time of 80 min (Figure 2A). Each fraction was tested against three indicator strains as described previously, *i.e.* *B. subtilis*, *M. luteus* and *E. coli*. The FI fraction eluted from t_0 to t_{20} presented the highest activity against the three indicator strains. The second fraction (t_{20} to t_{43}) was also active but to a lesser extent (Table 6). The two other fractions did not show any antibacterial activity. The FI fraction was then submitted to a second round of RP-HPLC (Figure 2B) and thirty sub-fractions were collected and analyzed for the presence of the antagonist activity. The sub-fraction FI-5 (elution between 6 and 6.75 min) showed the strongest inhibitory activity against the three indicator strains (Figure 3A). For the E1N211 extract, 40 fractions were directly over a length of time of 80 min (Figure 2C) and tested for their antibacterial activities (Figure 3B). The F11 fraction proved to be the only fraction active against the three indicator strains and corresponded to an elution time between 10.22 to 15.60 min. Nevertheless, several fractions were active against the two Gram-positive test strains suggesting that E1N211 produces different bioactive metabolites regarding these indicator strains. Same observations can be done with E2N171 at least concerning *B. subtilis* (Figure 3A).

DISCUSSION

Our study assessed the taxonomic diversity and biosynthetic potential of an environmental strain collection isolated from water samples from the yet non explored Fetzara lake, northeast Algeria. This location constitutes a type of natural wetlands which is rare in this Mediterranean region (Habes 2011; Abdelkader 2012) and thus provides a good opportunity for ecological studies as well as for the identification of new bacterial strains of biotechnological interest. Actinomycetes are predominant in lake and marine environments (Cross 1981), *Streptomyces* and *Rhodococcus* are among the most representative groups. A previous survey of water lakes in Yunnan (China) showed that while *Micromonospora* was the dominant genus, *Streptomyces* was the second most abundant (Jiang and Xu 1996). Water and sediments of lakes revealed the presence of a huge bacterial diversity closely in correlation with ecological and physicochemical parameters (Mesbah et al. 2007; Paul et al. 2015; Baatar et al. 2016). In most cases, actinomycetes are an abundant bacterial group although non-streptomycetes predominated. If non-streptomycetes and rare actinomycetes are meant to provide a new source of natural products since their products account for up to 25% of the total actinomycetes metabolites (Subramani and Aalbersberg 2013), *Streptomyces* remain a serious provider for new drugs. Hence, the high rate of rediscovery of known compounds by classical screenings is indicative of the failure of screening methods to detect new activities and their related compounds but not of the exhaustion of the biodiversity reservoir of the *Streptomyces* genus. In fact, genomic approaches and the development of new screening methods strongly renewed the interest for *Streptomyces*, even if not isolated from rare environments. Each *Streptomyces* genome project reveals its part of new secondary metabolite gene clusters. Among them, one can distinguish those constituting the ‘core’ secondary metabolism potentially involved in biosynthesis of metabolites needed in the environment (siderophores, carotenoids...) and those which are unique to strains or even to isolates and constitute the ‘contingent secondary metabolism’. The latter are mostly involved in biosynthesis of antibiotic activities (antibacterial, antifungal...) and may be

directly involved in the establishment and dynamics of *Streptomyces* populations in their environments and trigger competitive interactions (Kinkel et al. 2014; Abrudan et al. 2015). However, these gene clusters stayed unsuspected since most of them are silent and need to be activated to deliver their related compounds (Liu et al. 2013; Aigle et al. 2014).

The phylogenetic reconstruction of the 133 newly isolated actinomycetes from the Fetzara lake allowed to assign 125 of them to the *Streptomyces* genus, the remaining being assigned to *Nocardia*, *Micromospora*, *Nonomureae* and *Actinomadura* genera. Hence, the 125 isolates could be assigned to 40 different species. For this purpose, we considered a 16S rRNA similarity threshold of 99% to assign an isolate to an already recorded species. Stach and colleagues suggested this threshold value to describe actinobacterial diversity as a relevant value based on statistical correlations (Stach et al. 2003), although the classical 16S rRNA similarity threshold considered for most prokaryotes is 98.2 to 99.0% (Kim et al. 2014). Hence, isolates whose sequence identity ranged from 98.78% to 98.99% with the closest reference species (*i.e.*, E2N107, E5N137, E5N356, E5N341, E3N86, E5N422) may lead to define new species. This result will be confirmed using a polyphasic approach.

Inhibitory activities of newly isolated *Streptomyces* were screened on classical representative of bacterial Gram positive/negative groups (*E. coli*, *M. luteus*, *B. subtilis*), and further tested, for the most active isolates, on clinical bacterial strains. These bacterial (*E. coli*, *S. aureus*, *B. cereus*, *P. aeruginosa*) species were selected for two main criteria. First, they are prevalent in hospital acquired infections. Thus, *E. coli*, *S. aureus* and *P. aeruginosa* are the most encountered pathogens in nosocomial infection events. For example, they were responsible respectively for 26%, 15.9% and 8.4% of these infections in French hospitals in 2012 (source: RAISIN network, Report 2012, <http://www.invs.sante.fr/>). *E. coli* alone is involved in 49.8% of the urinary tract infections while *P. aeruginosa* and *S. aureus* are associated to pneumonia in 18.1% and 14.7% of the cases, respectively. Similar situations were observed in other European countries (source: European

Centre for Disease Prevention and Control, 2015). Secondly, they are also relevant indicator strains because they are drug-resistant through a unique major mechanism (β -lactamase, efflux pump, penicillin-binding protein). For example, the efflux pump is a classical resistance mechanism in the *P. aeruginosa* strains. It confers a wide resistance to the antibiotics of the β -lactamine family and some efflux pump systems may accommodate multiple drugs, and thus contribute to bacterial multidrug resistance (MDR) (Hirsch and Tam 2010). It is therefore noteworthy and promising that all the six tested bioactive strains were able to inhibit efficiently the *P. aeruginosa* ABC132 strain used in our study. Indeed, the bacterium *P. aeruginosa* is naturally highly resistant to classically available antibiotics and can also acquire very easily new antibiotic resistance mechanisms; this can lead to limited therapeutic treatment options, and even to therapeutic dead ends. Hence, it is urgent to discover new valuable antibacterial compounds.

Interestingly, five of the *Streptomyces* strains were also shown to be active against the major opportunistic fungal pathogen, *Candida albicans* which is present in 2.3% of the nosocomial infections (source: RAISIN network, Report 2012, <http://www.invs.sante.fr/>). This fungus is both a member of the healthy human microbiome and a major pathogen in immuno-compromised individuals and when the fungus is able to spread, which can lead to serious diseases that are fatal in 42% of cases.

The two *Streptomyces* strains, E2N171 and E1N211, showing the strongest inhibitor activity against the reference and resistant bacterial and fungus targets were subjected to a run of whole genome sequencing (not shown). The preliminary draft assembly revealed a genome of approximately 7 Mb and 7.3 Mb for the strains EN171 and EN221, respectively. A preliminary search for gene clusters involved in biosynthesis of secondary metabolites using the antiSMASH software (Medema et al. 2011; Weber et al. 2015) allowed to detect 25 and 29 putative gene clusters in the genomes of E2N171 and E1N221, respectively. These data constitute another example showing that *Streptomyces* genomes are enriched in biosynthetic gene clusters.

From the antiSMASH predictions, both strains include a gene cluster likely responsible for the biosynthesis of antimycins (Seipke et al. 2012) which are a potent fungicidal antibiotic (Dunshee BR 1949). The presence of this cluster could be related to the activity observed against *C. albicans*. Even if the activity was relatively weak with the extract of the E2N171 strain, it was nevertheless significant (Table 5). Several reasons could explain these distinct inhibitory activities such as growth differences between the two strains or difference of the expression of the antimycin structural genes. The genome of the E2N171 and E1N211 isolates also encodes clusters which may be involved in the antibacterial activities against the Gram-negative and Gram-positive indicator and clinical strains. Indeed, antiSMASH analysis revealed the presence of bacteriocin, lantipeptide or lasso peptide biosynthetic gene clusters as well as those of type II or type I PKS gene clusters and NRPS gene clusters. Both strains share a type III PKS gene cluster likely responsible for the production of a compound from the phenolic lipid family that may also be involved in antibacterial activities of the isolates as well as in their antifungal activities since phenolic lipids display both activities (Ross et al. 2004).

Nevertheless establishing a correlation between the inhibitory compounds and the identified biosynthetic gene clusters will request more work and is far most the more time-consuming and uncertain step of this approach. However, this is an issue that deserves our greatest attention to renew our antibiotic arsenal.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest

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Figure legends

Figure 1: Neighbor-joining phylogenetic tree based on a full-length 16S rRNA gene sequence alignment of 125 *Streptomyces* isolates and their related type-strains.

Bootstrap values based on 1000 resampled data sets are shown at the nodes; only values above 75% are given. The scale bar indicates 0.005 nucleotide substitution per nucleotide position. The selected strains for antimicrobial tests are highlighted in grey. The topology of the tree enabled to distinguish two major classes (Class I and Class II) and four subclasses referred as clusters 1 to 4.

Figure 2: RP-HPLC chromatograms of the extracts from the *Streptomyces* isolates E2N171 and E1N211.

A. Analysis of the E2N171 ethanol extract from ISP2 spent agar after three days of growth at 30°C. The area delimited with the dashed lines corresponds to the FI fraction (elution between 0 and 20 min). B. RP-HPLC analysis of the FI fraction. The area delimited with the dashed lines corresponds to the FI-5 fraction (elution between 6 and 6.5 min). C. Analysis of the E1N211 ethanol extract from ISP2 spent agar after seven days of growth at 30°C. The area delimited with the dashed lines corresponds to the fraction F11 (elution between 10.22 and 15.60 min). A linear gradient from 5% to 75% acetonitrile in water was applied in the presence of 0.1% of trifluoroacetic acid for 70 min with a flow rate of 0.25 ml/min at a temperature of 30°C.

Figure 3: Antimicrobial activity of the subfractions from the E2N171 extract (A) and of the fractions from the E1N211 extract (B) against different indicator strains.

The FI-5 subfraction from E2N171 and the F11 fraction from E1N211, the only fractions active against the three indicator strains, are highlighted by an arrow. 1: *M. luteus*; 2: *E. coli*; 3: *B. subtilis*.

Table legends

Table 1: Identification of the closest reference *Streptomyces* species for the 125 isolates based on their 16S rRNA gene sequence identity. The range of nucleotide identity is given for the assigned group of isolates.

Table 2: Antibacterial activity of the 33 selected *Streptomyces* isolates.

The strains were grown at 30°C on ISP2 agar medium or Bennet agar medium during 24 days. Activity (diameter of inhibition, in mm) is given as the largest inhibition zone observed during the 24 days period of culture (D: day at which the activity was the most important). -: no activity whatever the growth medium of the isolate.

Table 3: Antimicrobial activities of whole plate extracts (agar + mycelia) of active isolates. Extractions were done either with ethanol or ethyl acetate. -: no activity; +: activity <8mm; ++: 9mm<activity<15mm, +++: 16mm<activity<25mm, ++++:activity>26mm.

Table 4: Antimicrobial activities of ethanol extracts from bioactive isolates obtained from either mycelium or spent agar. -: no activity; +: activity <8mm; ++: 9mm<activity<15mm; +++: 16mm<activity<25mm; ++++: activity>26mm.

Table 5: Minimum inhibitory concentrations (MICs) of the antimicrobial extracts of the selected bioactive *Streptomyces* isolates against clinical microorganisms.

Repetition: R=2 for antibacterial activity and R=1 for antifungal activity; #clear dose-dependent inhibition growth of microorganisms but insufficient for MIC determination; *MIC confirmed after re-culture on solid media. ISP2 and Bennet extracts were used as negative controls.

Table 6: Antimicrobial activity of different RP-HPLC fractions of ethanol extracts from the E2N171 isolate.

-: no activity; +: activity <8mm; ++: 9mm< activity<15mm; +++: 16mm<activity<25mm; ++++: activity>26mm.

Additional data

Figure S1: Geographic location of the sampled sites (solid circles S1-S5) of Fetzara lake, Annaba, northeastern Algeria

Figure S2: Phenotypic traits of colonies of different *Streptomyces* isolates from the Fetzara lake. The set of the six photographs at the bottom of the figure correspond to the most active isolates that inhibit the growth of Gram-negative and Gram-positive bacteria (see Results). The strains were grown on ISP2 medium or SFM medium (for the last six strains) at 28°C.

Figure S3: Time-course of the antimicrobial production of the strains E1N211 (A) and E2N171 (B). The antimicrobial production is given as the diameter of the inhibition zone observed around the plug. Activity was tested against three indicator strains, *E. coli* DH5 α , *M. luteus* DSM1790 and *B. subtilis* ATCC633.

Table 1

<i>Streptomyces</i> species (accession number)	Identity (%)	Number of isolates
<i>S. tendae</i> ATCC 19812 (D63873)	99.87–100	11
<i>S. rubrogriseus</i> LMG 20318 (AJ781373)	99.04–100	10
<i>S. violaceorubidus</i> LMG 20319 (AJ781374)	99.73–99.93	8
<i>S. griseorubens</i> NBRC 12780 (AB184139)	99.45–100	7
<i>S. mutabilis</i> NBRC 12800 (AB184156)	99.57–99.65	7
<i>S. variabilis</i> NBRC 12825 (AB184884)	100	7
<i>S. albogriseolus</i> NRRL B-1305 (AJ494865)	99.97–100	6
<i>S. anulatus</i> NRRL B-2000 (DQ026637)	99.93–100	6
<i>S. ambofaciens</i> ATCC 23877 (M27245)	99.51–99.60	4
<i>S. anthocyanicus</i> NBRC 14892 (AB184631)	100	4
<i>S. xiamenensis</i> MCCC 1A01550 (EF012099)	99.26–99.58	4
<i>S. diastaticus</i> NRRL B-1773 (DQ026631)	99.32–99.45	3
<i>S. griseoflavus</i> LMG 19344 (AJ781322)	99.72–100	3
<i>S. marokkonensis</i> Ap1 (AJ965470)	99.88–99.36	3
<i>S. viridochromogenes</i> NBRC 3113 (AB184728)	99.86–100	3
<i>S. viridodiastaticus</i> NBRC 13106 (AB184317)	100	3
<i>S. althioticus</i> NRRL B-3981 (AY999791)	98.83–98.91	2
<i>S. coelescens</i> DSM 40421 (AF503496)	99.80	2
<i>S. flaveolus</i> NBRC 3715 (AB184786)	100	2
<i>S. ghanaensis</i> KCTC 9882 (AY999851)	99.58	2
<i>S. griseoincarnatus</i> LMG 19316 (AJ781321)	99.80–99.93	2
<i>S. griseorubiginosus</i> NBRC 13047 (AB184276)	100	2
<i>S. heliomycini</i> NBRC 15899 (AB184712)	99.93–100	2
<i>S. intermedius</i> NBRC 13049 (AB184277)	99.37–99.44	2
<i>S. malachitospinus</i> NBRC 101004 (AB249954)	99.93	2
<i>S. qinglanensis</i> 172205 (HQ660227)	99.80–99.91	2
<i>S. rubiginosohelvolus</i> NBRC 12912 (AB184240)	100	2
<i>S. smyrnaeus</i> SM3501 (KF006349)	99.52–99.78	2
<i>S. atrovirens</i> NRRL B-16357 (DQ026672)	99.30	1
<i>S. axinellae</i> Po1001 (EU683612)	99.86	1
<i>S. badius</i> NRRL B-2567(AY999783)	99.37	1
<i>S. carpaticus</i> NBRC 15390(AB184641)	99.86	1
<i>S. cavourensis</i> NBRC 13026 (AB184264)	100	1
<i>S. cyaneofuscatus</i> JCM 4364 (AY999770)	99.93	1
<i>S. eurocidicus</i> NRRL B-1676 (AY999790)	99.12	1
<i>S. hyderabadensis</i> OU-40 (FM998652)	99.86	1
<i>S. lienomycini</i> LMG 20091 (AJ781353)	99.93	1
<i>S. malaysiensis</i> NBRC 16446 (AB249918)	99.86	1
<i>S. sporoclivatus</i> NBRC 100767 (AB249934)	99.80	1
<i>S. yerevanensis</i> NBRC 12517 (AB184099)	99.52	1

Table 2

Isolates	<i>B. subtilis</i> ATCC6633		<i>M.luteus</i> DSM1790		<i>E. coli</i> DH5 α	
	Activity (mm)	Medium*	Activity (mm)	Medium*	Activity	Medium*
E2N171	32 (D3)	A	29 (D3)	A	19 (D3)	A
E1N211	32 (D7)	A	26 (D7)	A	16 (D7)	A
E5N450	30 (D17)	B	28 (D24)	B	-	A or B
E3N218	26 (D17)	B	24 (D3)	B	13 (D3)	B
E3N312	25 (D17)	A	21 (D7)	A	11 (D17)	A
E1N264	25 (D10)	A	21 (D10)	B	-	A or B
E5N158	21 (D10)	A	21 (D7)	B	18 (D3)	A
E3N368	21 (D3)	A	18 (D7)	A	-	A or B
E5N299'	20 (D3)	A	24 (D3)	A	17 (D13)	A
E1N316	20 (D3)	A	20 (D3)	B	-	A or B
E5N237	20 (D3)	A	18 (D3)	A	-	A or B
E5N356	20 (D10)	B	13 (D3)	A	-	A or B
E5N235	20 (D17)	B	12 (D7)	B	-	A or B
E4N257	16 (D3)	B	14 (D3)	B	-	A or B
E5N280	15 (D10)	B	23 (D10)	A	-	A or B
E4N288	14 (D17)	A	16 (D7)	B	-	A or B
E4N74	14 (D3)	B	16 (D10)	A	-	A or B
E5N292	13 (D7)	A	15 (D3)	A	-	A or B
E2N459	13 (D7)	A	13 (D7)	A	-	A or B
E2N282	13 (D3)	A	10 (D3)	A	-	A or B
E1N263	12 (D17)	B	24 (D17)	B	-	A or B
E2N98	11 (D7)	A	21 (D7)	A	-	A or B
E2N167	11 (D1)	B	10 (D7)	B	-	A or B
E4N275g	10 (D7)	B	18 (D7)	B	-	A or B
E1N370	10 (D10)	B	10 (D7)	B	-	A or B
E2N287	9 (D3)	A	8 (D24)	B	-	A or B
E3N82	-	A or B	19 (D1)	B	-	A or B
E3N311	-	A or B	18 (D1)	B	-	A or B
E2N284	-	A or B	14 (D1)	B	-	A or B
E1N260	-	A or B	-	A or B	-	A or B
E1N302	-	A or B	-	A or B	-	A or B
E2N176	-	A or B	-	A or B	-	A or B
E2N227	-	A or B	-	A or B	-	A or B
Control (ISP2)	-	-	-	-	-	-
Control (Bennet)	-	-	-	-	-	-

*A: ISP2 agar medium, B: Bennet gar medium

Table 3

Strain	Medium	Extraction solvent	Antibacterial activity		
			<i>B. subtilis</i>	<i>M. luteus</i>	<i>E. coli</i>
E2N171	ISP2	ethyl acetate	+++	+++	-
		ethanol	++++	++++	++++
E1N211	ISP2	ethyl acetate	++++	++++	++
		ethanol	++++	+++	++
E3N312	ISP2	ethyl acetate	-	+	-
		ethanol	++	+++	-
E3N218	Bennet	ethyl acetate	+++	++	++
		ethanol	+++	++++	+++
E5N158	ISP2	ethyl acetate	-	+	++
		ethanol	++	++	+++
E5N299'	ISP2	ethyl acetate	+++	+++	+
		ethanol	++++	++++	+++
Non inoculated medium	ISP2	ethanol	-	-	-
Non inoculated medium	Bennet	ethanol	-	-	-
Control DMSO 10%	/	/	-	-	-

Table 4

Strain (medium, time of growth)	Origin of the extract	Antibacterial activity		
		<i>B. subtilis</i>	<i>M. luteus</i>	<i>E. coli</i>
Control ISP2	-	-	-	-
Control Bennet	-	-	-	-
Control DMSO 10%	-	-	-	-
E5N158 (ISP2, D4)	mycelium	-	+	-
	agar	++	++	+++
E2N171 (ISP2, D4)	mycelium	+++	+++	-
	agar	++++	++++	+++
E1N211 (ISP2, D9)	mycelium	++++	++++	++++
	agar	++++	++++	++++
E3N218 (Bennet, D17)	mycelium	+++	++	-
	agar	+++	++++	+++
E5N299' (ISP2, D3)	mycelium	+	+++	+
	agar	+++	++++	+++
E3N312 (ISP2, D17)	mycelium	+	++	-
	agar	++	++++	-

Table 5

Test organisms	MIC (% v/v)							
	E5N15 8 (ISP2)	E2N171 (ISP2)	E1N211 (ISP2)	E3N218 (Bennet)	E5N299' (ISP2)	E3N312 (ISP2)	ISP2	Bennet
<i>E. coli</i> ABC 28	>25 >25	>25# >25#	6.25 12.5	6.25 6.25	12.5 25	>25# >25#*	-	-
<i>S. aureus</i> ABC61	12.5 12.5	3 1.5	<0.1 3	3 6	6.25 >25#	>25# >25#*	-	-
<i>B. cereus</i> ABC143	>25 >25	ND 3	1.5 6	>25# >25#	>25# >25#	>25# >25#*	-	-
<i>P. aeruginosa</i> ABC132	0.4 0.4	0.2 <0.1	<0.1 <0.1	<0.1 <0.1	<0.1 0.4	0.2 <0.1*	-	-
<i>C. albicans</i> AFC1	>25	>25#	3	>25#	>25#	<0.1	-	-

Table 6

Isolate	Fractions	<i>Antibacterial activity</i>		
		<i>B. subtilis</i>	<i>M. luteus</i>	<i>E. coli</i>
E2N171	F1 (0-20 min)	++++	++++	+++
	F2 (20-43 min)	+++	+++	++
	F3 (44-49 min)	-	-	-
	F4 (50-76 min)	-	-	-

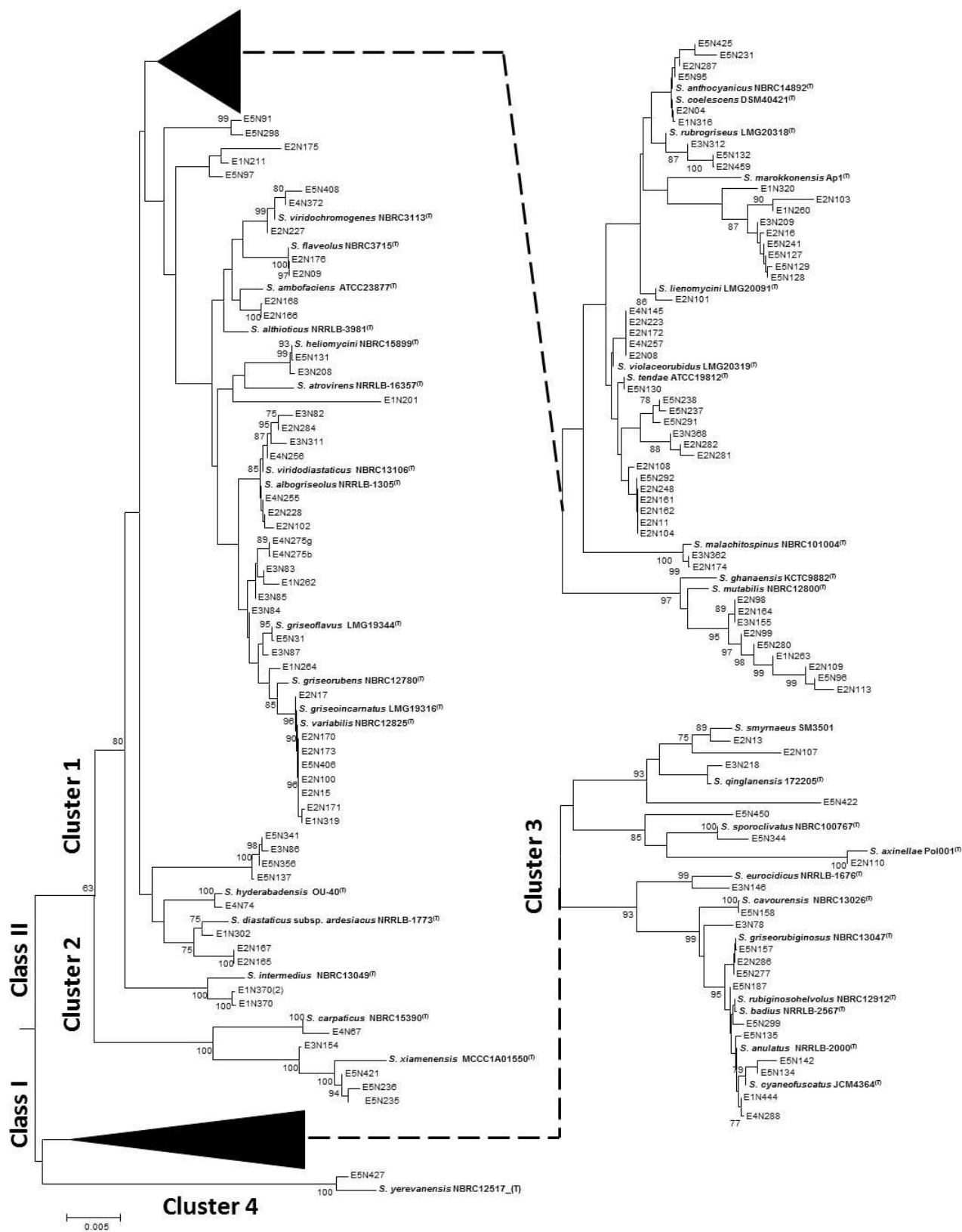


Figure 1

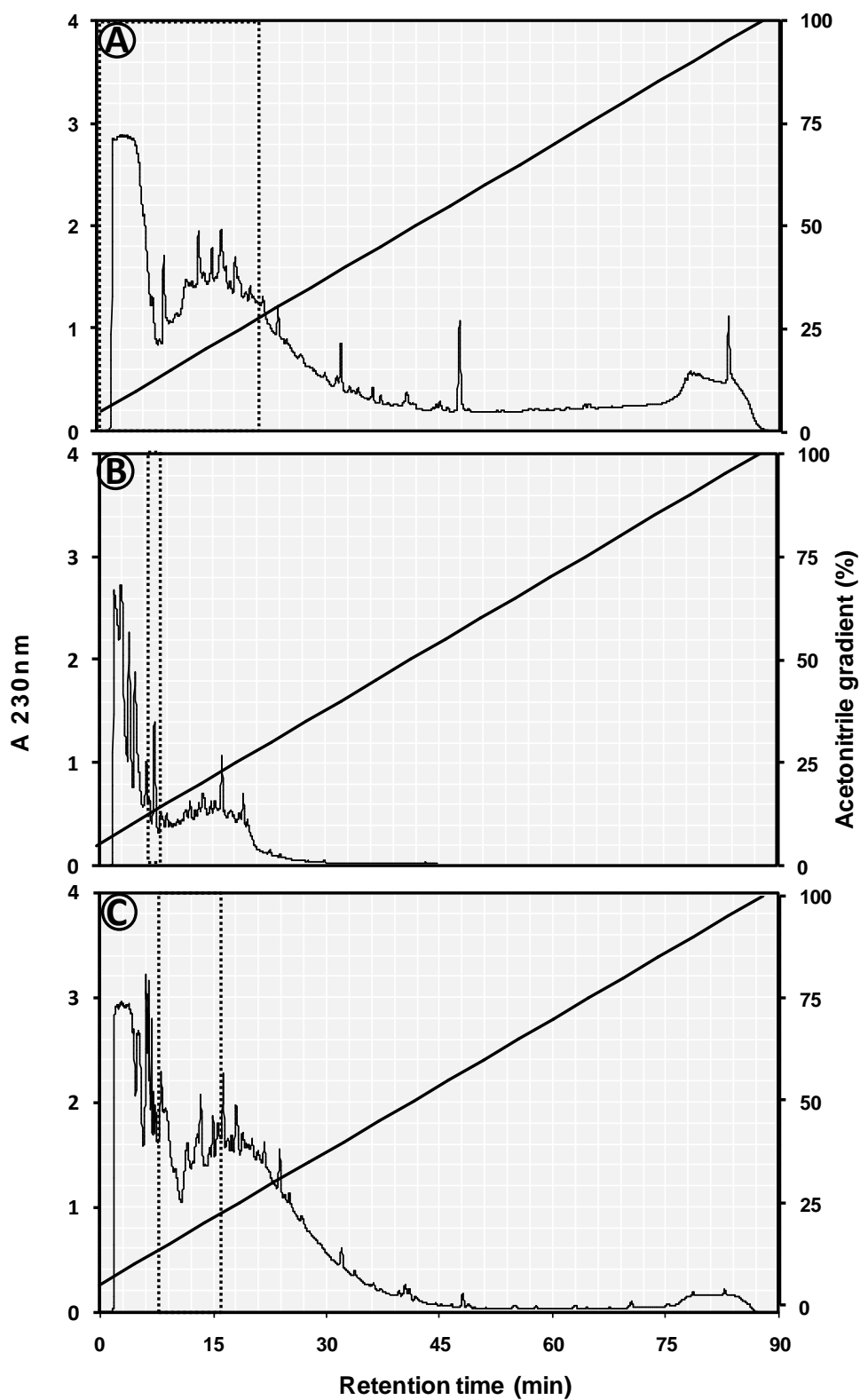


Figure 2

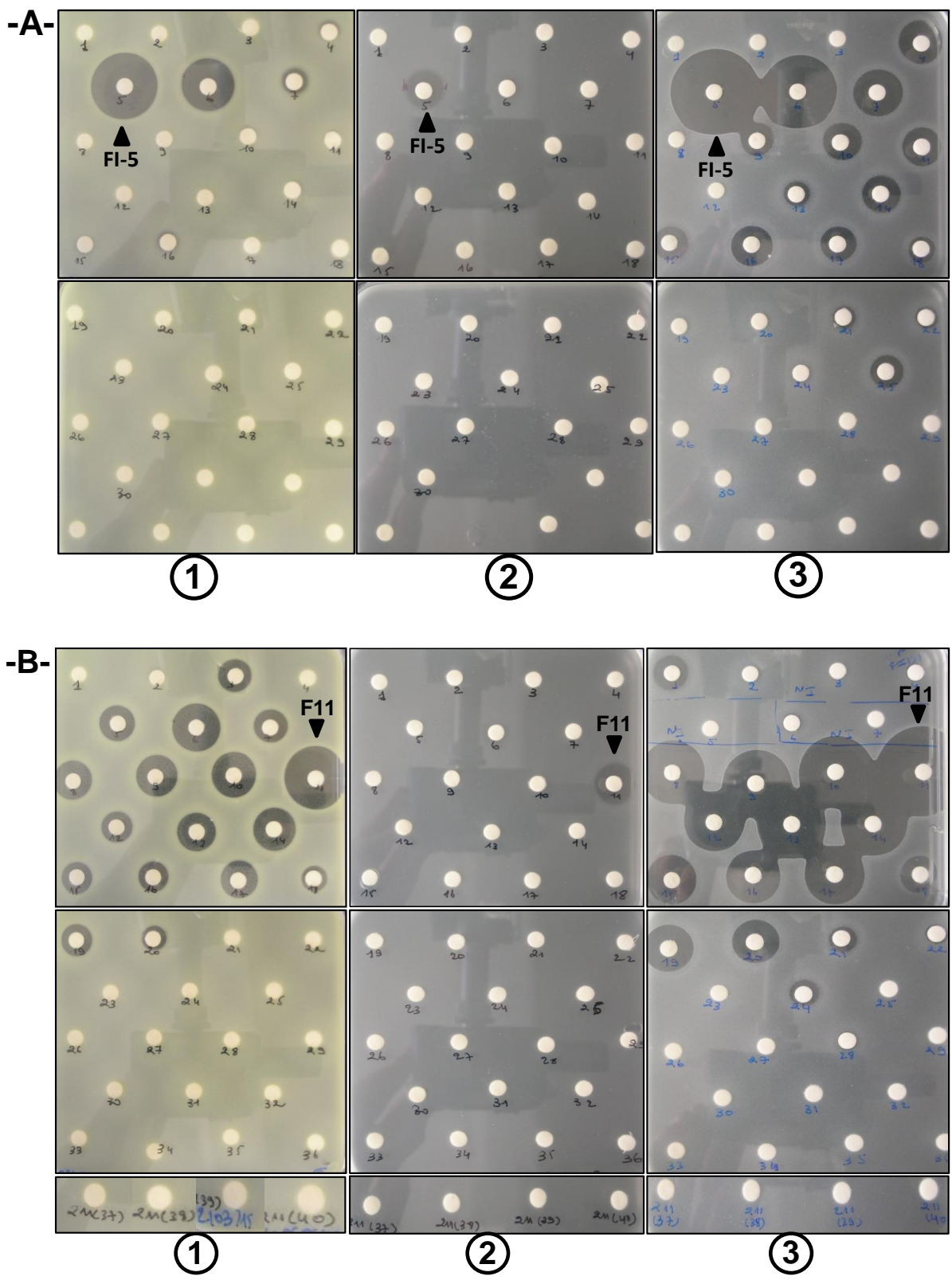


Figure 3

