Letters in Applied Microbiology
February 2014, Volume 58, Issue 2, pages 102–108
http://dx.doi.org/10.1111/lam.12162

Isolation and partial characterization of bacteria (*Pseudoalteromonas* sp.) with potential antibacterial activity from a marine costal environment from New Caledonia

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Abstract :

Marine bacteria are a rich source of bioactive metabolites. However, the microbial diversity of marine ecosystem still needs to be explored. The aim of this study was to isolate and characterize bacteria with antimicrobial activities from various marine coastal environment of New Caledonia. We obtained 493 marine isolates from various environments and samples of which 63 (12·8%) presented an antibacterial activity against a panel of reference pathogenic strains (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis)*. Ten out of the most promising strains were cultured, fractionated and screened for antibacterial activity. Four of them (NC282, NC412, NC272 and NC120) showed at least an activity against reference and multidrug-resistant pathogenic strains and were found to belong to the genus *Pseudoalteromonas,* according to the 16S phylogenetic analysis. The NC282 strain does not belong to any described *Pseudoalteromonas* species and might be of interest for further chemical and biological characterization. These findings suggest that the identified strains may contribute to the discovery for new sources of antimicrobial substances to develop new therapies to treat infections caused by multidrug-resistant bacteria.

Significance and Impact of the Study

With the constant increasing of bacterial resistance against known antibiotics in worldwide public health, it is now necessary to find new sources of antimicrobials. Marine bacteria from New Caledonia were isolated, tested for antibacterial activity and characterized to find new active molecules against multidrug-resistant bacteria. This study illustrates the diversity of the marine ecosystem with potent new bacteria species. Also the potential of marine bacteria as a rich source of bioactive molecule, for example antibiotics, is highlighted.

Keywords : antibacterial activity ; Characterization ; marine bacteria ; New Caledonia ; screening

Introduction

The sea, covering more than 70% of the surface of the planet, contains an exceptional biological diversity, accounting for more than 95% of the whole biosphere (Spížek *et al.* 2010). The ocean has recently been demonstrated as an ecosystem with many unique forms of microorganisms. Thus microbial diversity constitutes an infinite pool of novel chemistry, making up a valuable source for innovative biotechnology (Fenical and Jensen 2006). A number of valuable molecules, antibiotics and metabolites have been derived from terrestrial microorganisms (99% of the known microbial compounds) although efforts in this area have diminished since the late 1980s because this resource has been considered to be exhaustively studied (Spížek *et al.* 2010). In this respect, researchers switched over to new environments for novel pharmaceutical compounds (Moellering 2010). With the constant increasing of bacterial resistance against known antibiotics in worldwide public health, it is now necessary to find new sources of antimicrobials (Overbye and Barrett 2005; Moellering 2010) . In 2004, the Infectious Disease Society of America (IDSA) reported that the majority (over 70%) of bacterial pathogens responsible for fatal infections are likely to be resistant to at least one of the drugs commonly used in the treatment for bacterial infections (Boyle-Vavra and Daum 2007). Several preventive measures have been taken to avoid the microbial resistance development, but there is still an urgent need for new antimicrobial agents and new strategies to overcome the problematic of resistant pathogens. Historically, pharmaceutical companies have focused their research on terrestrial microorganisms and the study of antibacterial from the sea were left aside (Hughes and Fenical 2010), principally due to a lack of technology. During the last decades marine microorganisms have demonstrated their potential for antimicrobials production with not less than 660 marine bacterial compounds identified and characterized between 1997 and 2008 (Williams 2009). It is now widely admitted that atypical environments like marine biotopes are a promising reservoir of original

microorganisms and potentially new antimicrobials compounds. Among these microorganisms, bacteria are the third most producers (37% from sponges, 21% for coelenterates and 18% microorganisms) (Berdy 2005). Prospections of marine bacteria have shown that these organisms are widely present in the marine areas from the intertidal zones to the deep seas and even found in the most extreme places of the world: hydrothermal sources or Polar seas. This presence illustrates their incredible capacity of adaptation to the environment by developing strategies to survive. Those strategies includes metabolic pathways, especially in a world where competition is very selective (Harvey 2008). This may explain why the majority of marine bacteria antimicrobial producers described in the literature are originated from surface attached bacteria (on marine macro-organisms like algae, corals, etc…) (Anand *et al.* 2006; Gandhimathi *et al.* 2008; Wietz *et al.* 2010; Wilson *et al*. 2010). The Pacific Ocean is the biggest oceanic division on Earth that regroups a great diversity of intertidal environments largely unstudied and may therefore be a great reservoir for new bacterial molecules but also a promising source for marine biotechnology developments. For that purpose a bioprospection started a few years ago on microbial mats of some French Polynesian atolls where microorganisms are exposed to high variation of abiotic factors (Guezennec *et al.* 2011) and in New Caledonia which is known for its high endemic biodiversity and a large variety of marine costal environments (Chalkiadakis *et al.* 2013). The present study describes the analysis of bioactive marine bacteria strains collected among the New Caledonia coast. The purpose of this study was to screen the strains for their antibacterial activity, to provide phylogenetic analyses and partially characterize their bioactivity.

Results and Discussion

From 205 environmental samples a collection of 493 marine bacterial isolates was obtained as previously described (Chalkiadakis *et al.* 2013). The antibacterial activity of each isolate was tested against the four reference strains (Table 1): *Staphylococcus aureus*, *Enterococcus faecalis, Pseudomonas aeruginosa*, *Escherichia coli*. Agar-diffusion assays showed that 63 out of the 493 isolates (12.8%) possessed an antibacterial activity against at least one of the reference strains. This percentage is closed to what was observed in other marine environment, such as surface-attached bacteria, corals, invertebrates… (Nissimov *et al.* 2009; Penesyan *et al.* 2009; Wilson *et al.* 2010). Among the 63 isolates, 26 were active against *S. aureus*, 4 were active against three reference strains (*E. coli*, *E. faecalis* and *S. aureus* or *E. faecalis*, *S. aureus* and *P. aeruginosa*), and 14 against two reference strains (*E. coli* and *S. aureus* or *E. faecalis* and *S. aureus*) and one against *P. aeruginosa* (Table 2). Of these 63 isolates, 10 displayed high anti-bacterial activity mainly against *S. aureus*, *E. faecalis* and *E. coli* reference strains, gram negative and positive bacteria, suggesting that several compounds may be produced by these marine isolates. These 10 isolates were selected for further characterization (Table 2). The five culture supernatant (S) and pellet (P) residues obtained for each of the 10 marine isolates were used for testing against reference strains and MDR strains (Table 3). No activity was detected in the fractions of decreasing polarity S1 to S3 for all the isolates tested (data not shown). The antibacterial activity was recovered mainly in the less polar fraction P1 for all the isolates tested. Among the 10 strains selected, three strains are of interest: NC 272, NC282 and NC412, as antibacterial activity of the P1 fraction is found against both references strains and MDR strains. Unexpectedly, NC120, which was the most active isolate, presented a weak activity after the fractionation procedure. Fractionation often result in improved activity but in some cases a loss of activity has been reported depending on the nature of the interaction (antagonism or synergism) between the constituent compounds extracted (Nwodo *et al.* 2011; Joray *et al.* 2013). Moreover the fractionation

procedure may have affected the stability of the antibacterial compounds produced, thus resulting in a loss of activity. It is to note that the strains NC272, NC282 and NC412 are presenting an antibacterial activity against at least one of these MDR strains: BLSE *E. coli*, *E. faecium* vancomycin resistant and *P. aeruginosa* carbapenem resistant, suggesting the presence of molecules with potential pharmaceutical interest.

The 16S sequences obtained for the 10 selected strains were compared using a BLAST algorithm (http://blast.ncbi.nlm.nih.gov) and allowed us to determine the strains genus as followed: *Salinivibrio* (NC 15, 143), *Photobacterium* (NC 17) and *Pseudoalteromonas* (NC 49, 120, 257, 271, 272, 282, 412). All three genera are belonging to the phylum *Proteobacteria* and the class *Gammaproteobacteria*. Only the family differs: *Vibrionaceae* for *Salinivibrio* and *Photobacterium* or *Pseudoalteromonadaceae* for *Pseudoalteromonas*. This distribution suggests that the standard cultivation procedure used in this study is favoring the isolation of close species from the marine environment. Based on these results, a phylogenetic tree (Figure 1) was constructed with the 1100 bp of the 16S sequence obtained. The tree showed that the closest neighbour of strain NC15, NC49 and NC143 were *Salinivibrio costicola-subsp*. According to the phylogenetic data obtained, the closest specie of strain NC17 is *Photobacterium jeanii.* Figure 1 and Figure S1 showed that the strains NC 120, 257, 271, 272, and 412 are clustering together and are closed to *Pseudoalteromonas piscicida.* However, strain NC282 is clearly out grouping from the other NC *Pseudoalteromonas* isolates with the nearest identified strain *Pseudoalteromonas viridis*. Despite these interesting results, further investigations are needed on NC282 in order to conclude in a probable new member of the *Pseudoalteromonas* genus.

It is interesting to note that in our work, seven out of 10 isolates are belonging to the *Pseudoalteromonas* genus (Figure S1) and are presenting the more interesting antibacterial activity against reference and MDR strains. This observation was expected as antibacterial

activity has already been reported in this family (Gram *et al.* 2010; Vynne *et al.* 2011). For example, previous work evaluated the *in vitro* activity of MC21-B, an antibiotic produced by the marine bacterium *Pseudoalteromonas phenolica* O-BC30(T), against methicillin-resistant *S. aureus* (Isnansetyo and Kamei 2003; 2009). Also, the protein p-153 secreted by *Pseudoalteromonas* sp. X153 displays good inhibitory activity against human pathogenic strains involved in dermatologic diseases and marine bacteria, suggesting that *Pseudoalteromonas* sp. X153 may be useful in aquaculture as a probiotic bacterium (Longeon *et al.* 2004). Finally the seven NC *Pseudoalteromonas* sp isolates were colored strains (yellow, orange or red). This observation is of interest as among the *Pseudoalteromonas* species, the best bioactivity was found in pigmented strains (Vynne *et al.* 2011). Considering the 16S phylogenetic analyses and the antibacterial activity observed, NC282 (the only red strain collected) presents an interesting antibacterial profile and might be further investigated.

In summary, the current study has identified from NC coastal environment a range of culturally marine bacteria with antibacterial activity. Phylogenetic analysis showed that those of most interest are closely related. Complementary studies will be undertaken to better characterize the bioactive compounds.

Materials and methods

Bioprospection, sampling and strain isolation

New Caledonia is a Melanesian archipelago situated in the South West Pacific and possesses one of the largest lagoons in the world with exceptionally diverse marine areas, i.e. reef flats, mangrove swamps, sandy beaches etc. During bioprospection, samples of waters, sediments, intertidal rocks, invertebrates, plants, fishes... were collected, mainly in the western part of the main island of New Caledonia. The samples collected were inoculated into liquid Zobell

(ZoBell 1941) medium at pH 7.6 and incubated at 28°C for 24 h. Successful cultures were subcultured on a solid medium under the same conditions; this procedure was repeated several times until pure cultures were obtained (Chalkiadakis *et al.* 2013). API 20NE (BioMérieux, France) test kit was used to determine metabolic properties.

DNA extraction and 16S PCR

One colony from an overnight culture of each strain was suspended in 100 µL of sterile water. The bacterial suspensions were lysed by heating to 99°C for 10 min. The lysed suspensions were centrifuged at 12,000 rpm for 5 min, the supernatant removed and stored at -20°C for further analysis. To identify isolates, 16S ribosomal RNA gene sequences were amplified by PCR. The 16S ribosomal RNA gene was amplified using two primers: SADIR (AGA GTT TGA TCA TGG CTC AGA) and S17REV (GTT ACC TTG TTA CGA CTT) (Cambon-Bonavita *et al.* 2002). The reaction was heated to 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 s with a final extension of 72°C for 7 min.

Nucleotide sequencing and phylogenetic analyses

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The subsequent sequencing reactions were performed with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) using the same primers as previously and loaded on the 3130xl Genetic analyzer (Applied Biosystems, USA). Overlapping fragments were assembled with Staden Package (MRC Cambridge, England). The nucleotide sequences obtained have been deposited in the GenBank database (accession numbers KC843419 to KC843428).

The phylogenetic analyses were then done by comparing 16S sequences of type strains obtained on Genbank sequence database and the partial 16S sequences from the isolates of interest. DNA sequences of strains were aligned using the BioEdit software package (Hall 1999). The phylogenetic analyses were performed using the Phylo_win program (Galtier *et al.* 1996) for the Neighbor-joining (Saitou and Nei 1987) and maximum-likehood (Felsenstein 1992) methods corrected with the Kimura two-parameter model (Kimura 1979). Bootstrap values were determined after 500 replications. Finally, the tree was plotted using the njplot program (Perrière and Gouy 1996) to obtain a clear tree representation.

Bacterial strains and growth conditions

The strains used for this study are listed in Table 1. The reference strains and the multidrug resistant (MDR) strains provided by the Collection of Institut Pasteur de Nouvelle-Calédonie were maintained on Muller Hinton (MH) medium at 37°C. The marine isolates were maintained on Zobell agar medium at 28°C. For fractionation purpose, isolates of interest were grown on Zobell liquid medium for 48h at 28°C under vigorous agitation.

Extraction of antimicrobial metabolites

250 ml of marine bacterial cultures were centrifuged for 30 min at 9000 rpm. Pellets and culture supernatants were collected and lyophilised before further analyses. Both lyophilised pellets and culture supernatants were submitted to extraction by Accelerated Solvent Extraction (ASE), and the culture supernatants extracts were fractionated by Flash-chromatography.

Extraction by Accelerated Solvent Extraction:

Extractions were performed under the following conditions: temperature 40°C, no preheat, heat 5 min, static time 7 min, cycles 3, flush 60%, purge 300 s, under a 100 bars pressure.

Regarding the lyophilized culture supernatants, each sample was mixed to Fontainebleau sand in a-66ml-stainless steel cell. Extractions were performed on an ASE (Dionex, ASE 300), on-line filtered on a 10µm stainless steel frit and on a glass filter. For each sample, two fractions were obtained successively with two solvents of increasing polarity: Ethyl acetate (EtOAc) 100% and Methanol (MeOH)/H_2O 80:20. Both extracts were then pooled, evaporated under vacuum at 40°C and then purified by Flash-Chromatography. The same procedure was applied to the pellets, with the exception of the solvent used. In this case, solvents were Dichloromethane (DCM)/EtOAc 1:1, MeOH/H_2O: 1.1. The two extracts obtained were named respectively P1 and P2. Organic fractions were evaporated under vacuum at 40°C in a rotative evaporator (EZ2, GENEVAC).

Fractionation by Flash-Chromatography (Spot Flash Chromatography System, ARMEN):

The fractionation of the extracts obtained from the culture supernatant was performed on a RP18/11g column from Merck (SVF D22 RP18 25-40µm). The column was activated by MeOH flow prior to water conditioning. The extracts were injected and eluted successively by 120ml of H2O (fraction S1), 120ml of MeOH (fraction S2), and 120ml of DCM (fraction S3). The different fractions obtained were evaporated by EZ2 evaporator.

Screening for antibacterial activity

The bacterial marine isolates

Antimicrobial activity of isolates was assessed using the agar diffusion method. The isolates were plated on Zobell agar medium and incubated for. 24-48h. Plugs of 6 mm in diameter were cut and deposited upside down onto MH plates previously inoculated with a reference strain according to the McFarland protocol (production of a suspension of about 10^5 CFU.mL$^-$

[1]). After 24-48h incubation at 37°C, the diameter of inhibition zones around the agar plug was measured and recorded.

The residues after fractionation

An amount of 10 mg of each extract was dissolved in ethanol/water (50/50) and directly loaded on MH plates previously inoculated with a reference strain or a MDR strain. The concentrations were ranging from 10 mg/mL (S2 and S3) to 66 mg/mL (S1 and P1-2). A volume of 10 µL of each solution was used for testing against reference strains and MDR strains. Positive and negative controls were respectively a disk impregnated with chloramphenicol (BioRad, Marne-la-Coquette) and 10µl of the ethanol/water solvent. After 24-48h incubation at 37°C, the diameter inhibition zones around the extract was measured and recorded.

All the experiments were run in duplicate, and the results are presented as mean values of the two measurements.

Acknowledgments

We would like to thank O. O'Connor and S. Mermond for scientific support, E. Calvez for sequencing experiments, C. Goarant for helpful discussions, M. Greene for technical support and the bacteriology laboratory staff from Institut Pasteur of New Caledonia. All the sequencing experiments were done at "La Plateforme du Vivant", Noumea, New Caledonia. This work was partially supported by a grant from the Overseas French Ministry, SEOM, n°09/1217976/BF and 12/024079/D.

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Table 1 List of the reference and MDR pathogenic strains used for antibacterial testing

Strain	Identification	Characteristics
Staphylococcus aureus	ATCC 25923	reference strain
Enterococcus faecalis	ATCC 29212	reference strain
Pseudomonas aeruginosa	ATCC 27853	reference strain
Escherichia coli	ATCC 25922	reference strain
Methycillin-resistant *Staphylococcus aureus*	SA 139	MDR strain, * Pen, Oxa, Gm, K, Tm, Ofx, E, Lin, Te, Sxt
Vancomycin-resistant *Enterococcus faecium*	DIV 369	MDR strain, * Amp, Gm, K, S, Lvx, Mox, E, Cli, Va, Sxt
Carbapenem-resistant *Pseudomonas aeruginosa*	DIV 302	MDR strain, * Tic, Cla, Pip, Caz, Fep, Imp, Gm, Sxt
BLSE *Escherichia coli*	BS 183	MDR strain, * Amp, Amc, Tic, Cf, , Nal, Nor, Ofx

* Antibiotics susceptibility profile obtained on the Vitek II (BioMérieux), only the resistant antibiotics are listed. MDR = multi-drug resistant

Amc: Amoxicillin/Clavulanate, Amp: Ampicillin, Caz: Ceftazidime, Cla: Ticarcillin/Clavulanate, Cf: Cefalotin, Cli: Clindamycin, E: Erythromycin, Fep: Cefepime, Gm: Gentamycin, Imp: Imipenem, K: Kanamycin, Lvx: Levofloxacin, Lin: Lincomycin, Mox: Moxifloxacin, Nal: Nalidixic acid, Nor: Norfloxacin, Ofx: Ofloxacin, Oxa: Oxacillin, Pen: Benzylpenicillin, Pip: Piperacillin, Te: Tetracycline, Tic: Ticarcillin, Tm: Tobramycin, S: Streptomycin, Sxt: Trimethropim/Sulfamethoxazole, Va: Vancomycin

Table 2 Antibacterial activity on reference strains of the ten selected NC marine isolates

NC marine isolate	Inhibition zone (mm) *E. coli* ATCC 25922	*E. faecalis* ATCC 29212	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 27853
NC 15	8	8	10	-
NC 17	9	-	13	-
NC 49	-	13	11	-
NC 120	16	26	26	-
NC 143	-	-	24	-
NC 257	7	-	27	-
NC 271	10	-	16	-
NC 272	7	-	15	-
NC 282	-	10	11	-
NC 412	-	14	15	8

Results are the mean of two independent experiences. - : no activity detected.

Table 3 Antibacterial activity of the pellet fractions P1 on reference and MDR strains

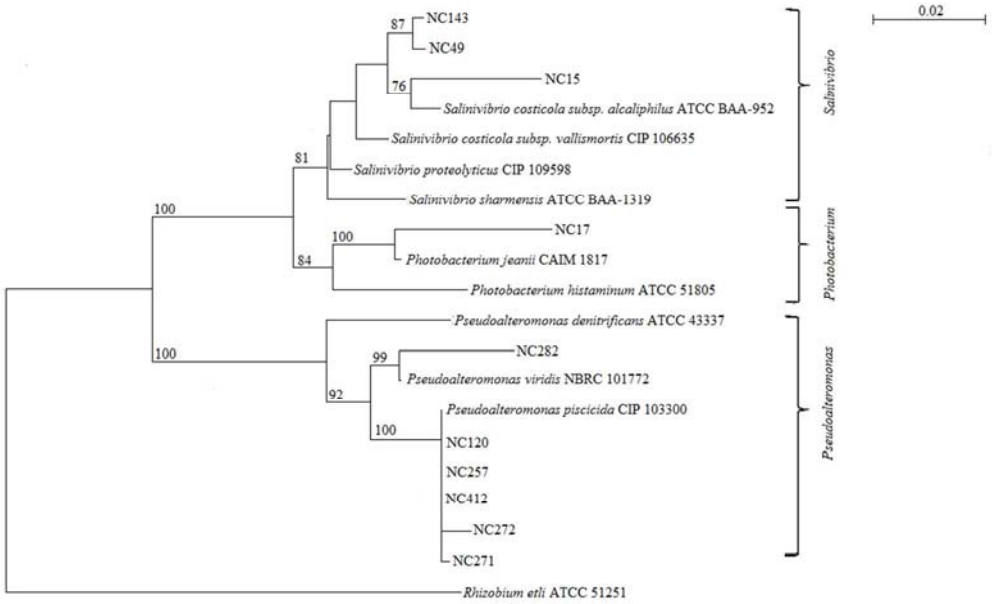
	E. coli ATCC 25922	*E. faecalis* ATCC 29212	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 27853	BLSE *E. coli*	Vancomycin-resistant *E. faecium*	Methycillin-resistant *S. aureus*	Carbapenem-resistant *P. aeruginosa*
NC 15	++	-	-	-	-	-	-	-
NC 17	+	-	-	-	-	-	-	-
NC 49	+	+	-	-	-	-	-	-
NC 120	+	+	-	-	+	-	-	-
NC 143	+	++	-	+	-	-	-	-
NC 257	+	++	+	-	+	-	-	-
NC 271	++	++	+	-	+	-	-	-
NC 272	+	++	+	++	+	+	-	+
NC 282	+	++	+	-	+	-	-	+
NC 412	+	++	-	-	+	+	-	-
EtOH/H2O	-	-	-	-	-	-	-	-

Results are the mean of two independent experiences.

- : no activity detected, + : inhibition zone between 6mm and 12mm, ++ : inhibition zone over 12 mm

Fig 1. Phylogenetic tree based on partial 16S rRNA gene (1100 bp) sequence analysis of *Pseudoalteromonas*, *Salinivibrio* and *Photobacterium* strains.

This tree has been build using the Neighbor-joining method with the Kimura 2 algorithm and based on 500 replicats. Only supportive bootstraps (over 75) are represented.



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