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Conteúdo

UNRAVELLING TREASURES FROM THE NATURAL HERITAGE OF AZORES: GENETIC RICHNESS OF MICROORGANISMS FROM EXTREME ENVIRONMENTS

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SUMMARY

Bacterial secondary metabolites are an important source of natural products with considerable effects on health, nutrition and economy of human societies. The search for compounds such as antibiotics has increasingly gained relevance with the rise in bacterial resistance. Microorganisms from extreme environments are especially valuable as reservoirs for novel bioactive compounds for various biotechnological applications. Biogeographic isolation, accessibility to sites with extreme conditions, and their immense bacterial diversity, make the Azores an excellent scenario to screen for the presence of interesting compounds.

We aimed at detecting the presence of antibiotic-producing bacteria in volcanic cavities and fumarole fields of the Azores.

The obtained isolates were grown and purified in conditions resembling those of the sampling location. They were characterized on the basis of their morphological and physiological characters. Antimicrobial activity screening was performed against human pathogens by the cross-streak assay. For the isolates with larger antimicrobial spectra, antimicrobial activity was confirmed by the well-assay test and their taxonomic identity was assessed by sequencing of the 16S rRNA gene.

Isolates displaying antimicrobial activity against at least one of the pathogens under study were found in all sampling locations. Actinobacteria, Firmicutes and Proteobacteria were the most represented phyla among the isolates of interest. Azorean extreme environments are home to a vast diversity of bacteria, from which molecules that are useful from the biotechnological point of view can be obtained. The economic value of Azorean natural resources needs to be better understood and duly promoted.

Palavras-chave: antibiotics; bacteria; biotechnology; extreme environments

1. CONTEXT AND INTRODUCTION

Extreme habitats represent a widely unexplored ecological niche with a vast potential for the discovery of novel antibiotics (Spížek *et al*., 2010), resulting from several billion years of evolution of their biosynthetic reactions. The demanding conditions of life in these habitats might suggest the presence of mutualistic interactions to support community growth under such starved conditions (Barton and Jurado, 2007; Barton *et al*., 2007). Secondary metabolites are used as informational cues to instigate a collective behavioral change to environmental challenges (Atkinson and Williams, 2009; Lopez *et al*., 2010). Some of these metabolites have bioactive, antibacterial, antifungal, antiviral, anticancer, insecticidal, algicidal and immunosuppressive properties. To maximize the chemical diversity available from microorganisms, new sources of antimicrobials are needed, providing new scaffolds of bioactive compounds (Fischbach and Walsh, 2009).

Although considerable progress can be achieved by chemical synthesis and engineered biosynthesis, mining natural habitats for antimicrobial compounds is still regarded as the richest and most versatile source for the discovery of novel antibiotic molecules (Bredholt *et al*., 2008). More than two-thirds of clinically-used antibiotics are natural products or their semi-synthetic derivatives (Fischbach and Walsh, 2009). The demand for new antibiotics has increased, fuelled by the spread of antibiotic resistance among pathogenic bacteria (Davies and Davies, 2010).

In spite of their barren, lifeless, appearance to the casual observer, extreme environments contain indeed a wealth of life at a closer look. In particular, they harbour considerable bacterial biodiversity: six bacterial phyla (Acidobacteria, Bacteroidetes, Firmicutes, Actinobacteria, α- and β-Proteobacteria) were found to be dominant in soils (Fierer *et al*., 2007). Sulphur vents are home to extremophilic microorganisms adapted to survive in harsh ecological niches with stressful conditions (temperature, pH and sulphur concentrations) showing a high occurrence of antibiotic producing microorganisms (Riquelme *et al*., 2010). Azorean volcanic lava tubes are extremophilic habitats, due to low nutrient availability. Lava tubes have been studied and are especially rich in life, both macroscopic and microscopic (Hathaway *et al*., 2013; Northup *et al*., 2012; Reboleira *et al*., 2012; Northup *et al*., 2011). Our previous studies on lava cave microbial mats have shown the presence of 14 bacterial phyla (Actinobacteria, Proteobacteria, Acidobacteria, Chloroflexi, Nitrospirae, Verrucomicrobia, Gemmatimonadetes, Planctomycetes, Bacteroidetes, Chlamydiae, OD1/OP11, Firmicutes and TM7) (Northup *et al*., 2011). This biodiversity represents a still untapped resource for antibiotic mining.

The objectives of the present work were: 1) Search for antibiotic compounds in extreme volcanic environments of the Azores, in response to the present needs because of increasing antibiotic resistance in human pathogens. 2) Contribute for the sustainable exploitation of unique volcanic environments by bringing new knowledge on the microbial communities they harbour. 3) Lead the initial steps for future developments in the area of biotechnology by Azorean industry by strengthening the liaison between industry and University.

2. METHODOLOGY

a. Soil sampling in the fumarole field of Furnas do Enxofre (Terceira, Portugal)

Sampling was performed on 29/10/2009 in six sites. Soil temperatures varied from 45ºC at sampling sites 1 and 2, 69ºC at sampling sites 3 and 4, and 73ºC at sampling sites 5 and 6. Samples of soil around degassing systems were retrieved into sterile containers and brought back to the laboratory, where isolation was carried out at once. Soil samples were diluted in physiological saline (1:10) and, using a sterile swab, spread over ½R2A medium (AES). Plates were incubated at 46ºC until sufficient growth was achieved.

b. Lava tubes sampling

Four volcanic caves from Pico Island (Gruta das Torres, Furna do Lemos, Gruta dos Montanheiros and Gruta da Ribeira do Fundo) representing a variety of geological and physical conditions were sampled, including three to six sample points for bacterial community analysis. Samples were collected from white, yellow, and tan microbial mats (Figure 3) and from apparently uncolonized surfaces. Air temperature inside the caves ranged from 11–16ºC and relative humidity was generally close to saturation. Cave walls were constantly covered by condensed water. Sampling for isolation of microorganisms was carried out by swabbing cave walls covered with microbial mats, oozes and moonmilk as well as bare rock. Samples of drip water were also aseptically

collected for analysis. Swabs were plated out on ½R2A medium, which is more suitable to the oligotrophic conditions of the lava tubes. The plates were incubated in situ, in the lava tubes for 1day, after which they were collected and brought, under refrigeration, by air transport, to the laboratory of the research unit for further analysis. Isolated colonies were subcultured onto the same medium on which they were originally inoculated and incubated at 11°C.

c. Morpho-physiological characterization

Isolates were characterized on the basis of seven morphology traits and physiological tests to classify them into morpho-physiological groups using standard microbiological methods (Winn Jr. *et al*., 1997). Prior to the characterization, pure cultures were grown in ½R2A medium for three days and were tested for i) Gram status, ii) presence of catalase, iii) presence of oxidase, iv) production of colony pigment (absence or presence of pigment), v) cell shape/arrangement (rods, filaments, cocci, coccobacilli, curved rods, filament-like rods), vi) nitrate reductase activity and, vii) colony color (white, tan, yellow, beige, pink, purple). Gram status was determined using KOH by the method of Ryu (1940). Catalase presence was assessed using 3% H_2O_2 and oxidase presence was determined with oxidase test strips (Bactident). Nitrate reduction to nitrite was assayed with Nitrate Reduction Test Broth (Fluka). The method used to search the nitrate reduction has been described by Benson (2001).

d. Screening isolates for antimicrobial activity

Cross-streak assay: Initial screening of the antimicrobial spectrum of all isolates was evaluated by the cross-streak assay, using eight human pathogenic bacteria as target strains: *Listeria monocytogenes* ATCC 7466, *Listeria innocua* ATCC 33090, *Salmonella* Typhimurium ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29523, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 9144 and *Proteus* sp. (from the collection of Serviços de Desenvolvimento Agrário da Ilha Terceira). Each isolate was streaked with a single line, top-to-bottom, on ½ R2A medium and incubated at the adequate temperature until sufficient growth was achieved. Afterwards, plates were set to dry in a biosafety chamber and overlaid with PCA medium (BioKar). Target strains, previously grown overnight in Nutrient Broth, were streaked perpendicularly to the isolate. Plates were then incubated at 37ºC for 16h and visually assessed for growth inhibition of the target strains.

Well diffusion assay: Isolates with clear antimicrobial activity towards at least 3 pathogens were subsequently tested for production of antibacterial metabolites in 25-day old liquid cultures by the well diffusion assay, modified from Mathabe *et al*. (2006). *L. monocytogenes* ATCC 7466 was used as the target strain. Antimicrobial activity was tested for cell-free supernatants (CFS). CFS were obtained by centrifuging the whole cell cultures at 4500×g during 10 minutes at 4ºC, in an Eppendorf 5804 R centrifuge. Supernatants were retrieved and aseptically filtered through sterilized filters with 0.2 µm pores. Target strains, previously grown overnight in Nutrient Broth at 37ºC, were adjusted to a cell density of 0.5 McFarland with sterile Nutrient Broth, incorporated in PCA medium at a concentration of 0.05% (v/v) and poured on Petri plates. Plates were solidified at 4ºC to slow down pathogen growth. Wells were perforated in the inoculated agar using an inverted Pasteur pipette and the bottom of the wells was sealed with a drop of agar at 14% (w/v) (Pastagar, AES). Sixty µL of each sample were introduced into each well. Plates were stored at 4ºC during 4h to allow sample to diffuse in the agar and incubated at 37ºC during 12 to 16 hours. Each sample was run in duplicate. After the incubation period, plates were inspected visually for pathogen inhibition and the diameter of the inhibition zones was measured with digital calipers.

e. Identification of the active isolates

Isolates that showed broad antimicrobial activity (active against at least three pathogens) by the cross-streak assay were identified by sequencing of the 16S rRNA gene.

Extraction of nucleic acids: Genomic DNA was extracted from cultures growth in Nitrate Broth with the UltraClean Microbial DNA isolation kit (MoBio Laboratories, Inc.) using manufacturer's protocol.

16S rDNA PCR amplification and sequencing: The 16S rRNA gene was amplified by PCR with universal primers, 8F forward (5'-AGAGTTTGATCCTTGGCTCAG-3') and p1492 reverse (5'-GCYTACCTTGTTACGACTT-3') using Amplitaq. Reactions were carried out in a 50 µl volume with 1X PCR buffer with 1.5mM Mg^{2+}, 0.4µM of each primer, 0.2mM each dNTP, 5µg BSA and 1U AmpliTaq LD (Applied Biosystems, Foster City, CA, USA). Amplification was carried out under the following thermocyling conditions on an Eppendorf Mastercycler 5333 (Eppendorf, Hauppauge, NY): 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1.5 min, followed by a final extension of 72°C for 7 min. Amplicons were cleaned and purified using the Qiagen PCR cleanup kit (Qiagen, Germantown, Maryland). Isolates were sequenced using the Big Dye_ Terminator Kit (Applied Biosystems) with primer p46 forward (5'-GCYTAAYACATGCAAGTCG-3') and p1409 reverse (5'-GTGACGGGCRGTGTGTRCAA-3'). DNA fragments from each Big Dye reaction for each primer were precipitated with Sodium Acetate and Ethanol and sequences were read by the ABI 377 Sequencer (Applied Biosystems). Closest relatives of the twelve isolates with broad antimicrobial spectrum were obtained using the Basic Local Alignment Search Tool (nucleotide BLAST) (www.ncbi.nlm.nih.gov/BLAST/).

3. RESULTS AND DISCUSSION

Four hundred thirty seven isolates were retrieved from the extreme environments under study, most of which from the lava tubes (Fig. 1). In the cave environments, several types of colonisations as well as bare rock were sampled. Most of the isolates were obtained from different colours of bacterial mats on the walls and ceilings of the caves (Fig. 2). Fig. 3 shows the percentage of isolates from the fumarole fields that were obtained for each sampling site temperature. More than half of the isolates were obtained from 45°C sites. Isolates from true thermophilic conditions (69 and 73°C) represented 44% of the total.

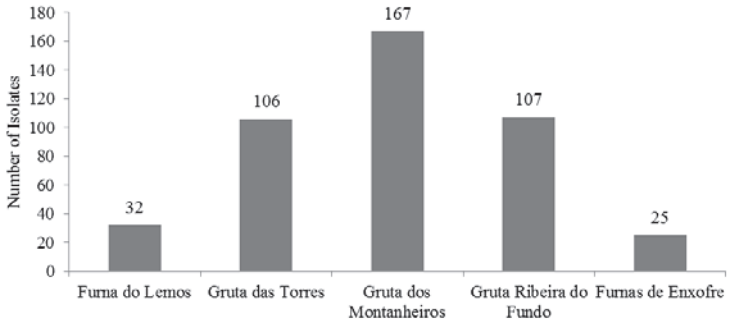

Fig. 1 – Numbers of isolates obtained from each sampling site

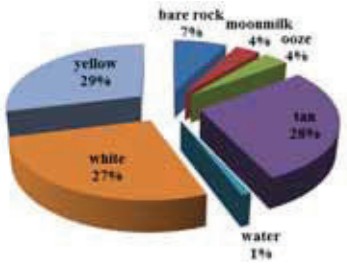

Fig. 2 – Percentage of isolates from each type of sampling site within the Pico Island lava tubes under study

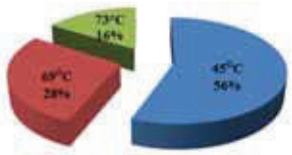

Fig. 3 – Percentages of isolates obtained from the fumarole field of Furnas do Enxofre by sampling site temperature

A total of 98 different morphotypes (MPT) were recovered from lava tubes and fumaroles according to seven traits (Gram status, Oxidase, Catalase, Nitrates, Colony color, Pigment diffusion and Cell morphology). Gram-positive, oxidase-negative, catalase-positive, non-nitrate reducing rods were prevalent among the studied isolates. Most colonies were beige and did not produce diffusible pigments (Table 1). Morphotyping large numbers of isolates provides an early, relatively fast, low-cost means of de-replication at an early stage of the study of bioactivities such as antimicrobial activity (Goodfellow and Fiedler, 2010). Sixty-three of the morphotypes were observed just in one of the sampling sites, whereas only two appeared in all of the sampled locations (Fig. 4).

Table 1 – Results of the morpho-physiological characterization of the 437 isolates obtained from extreme environments of the Azores. MPT – morphotypes.

MPT	Outcome	% isolates
Gram staining	Negative	43,9
	Positive	56,1
Oxidase	Negative	65,4
	Positive	34,6
Catalase	Negative	15,8
	Positive	84,2
Nitrates	Negative	53,8
	Positive	46,2
Colony Color	beige	33,2
	pink	1,1
	purple	0,2
	tan	24,7
	white	34,3
	yellow	6,4
Pigment diffusion	No pigment diffusion	83,7
	Pigment diffusion	16,3
Morphology	cocci	1,1
	coccobacilli	4,4
	curved rods	7,8
	filament-like rods	3,2
	filaments	24
	rods	59,5

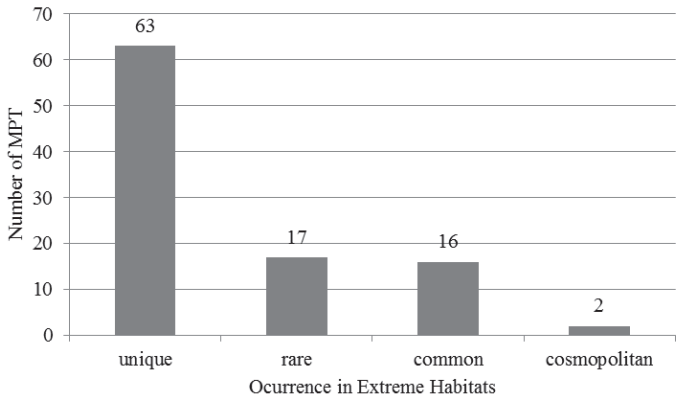

Fig. 4 – Occurrence of the different morphotypes within the sampled extreme environments.

When tested by the cross-streak assay method, a methodology which is useful for the fast screening of large numbers of isolates for their antimicrobial activity, 10,5% of the isolates displayed antimicrobial activity against at least one of the target strains under study (Figs. 5 and 6). Most of the lava tube isolates with antimicrobial activity came from yellow mats, whereas in the fumarole field most were obtained from the 45°C sampling sites. A high percentage of isolates with anticrobial activity were recovered from bare rock samples.

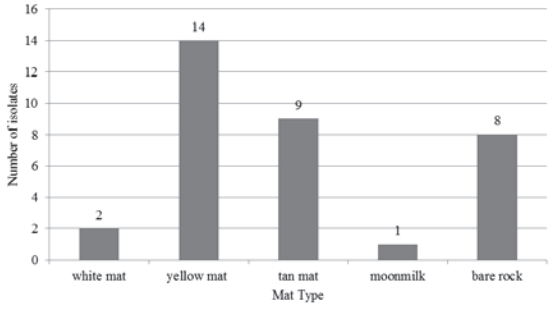

Fig. 5 – Numbers of isolates obtained from lava tubes of Pico Island (Azores, Portugal) with antimicrobial activity against at least one of the tested target strains

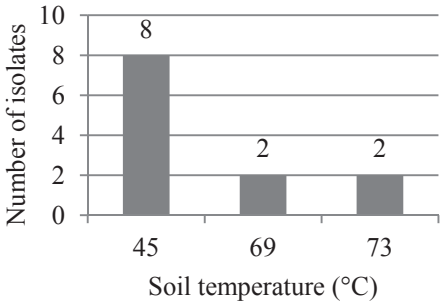

Fig. 6 – Numbers of isolates obtained from the fumarole field of Furnas do Enxofre (Terceira Island, Azores, Portugal) with antimicrobial activity against at least one of the tested target strains

All target strains were inhibited by some of the tested isolates. However, the sensitivity to the tested isolates varied among target strains (Fig. 7). The most sensitive was *S. aureus* ATCC 29523 (inhibited by 20,5% of the isolates), while only 3,5% of the isolates were active against *P. aeruginosa*.

Twelve isolates displayed a broad antimicrobial activity spectrum (active against at least three of the target strains under study). For these isolates, antimicrobial activity of cell-free supernatants was studied by the well-assay method. The CFS obtained from five of the studied isolates upon prolonged incubation (25 days) inhibited *L. monocytogenes* (Fig. 8). These are promising as sources of antibiotics for the treatment of a severe infection (listeriosis). Further studies leading to the purification and chemical

characterization of the compound(s) responsible for these antimicrobial activities are presently under way.

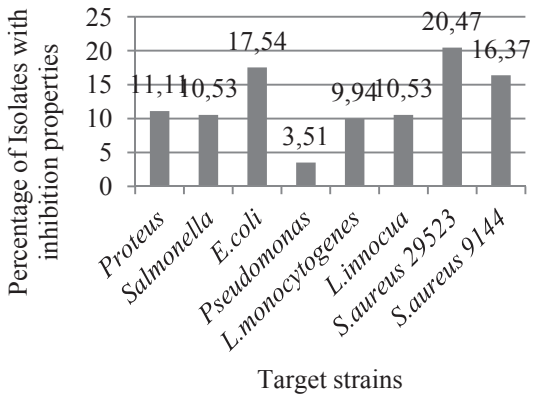

Fig. 7 – Percentage of isolates displaying antimicrobial activity against each of the target strains

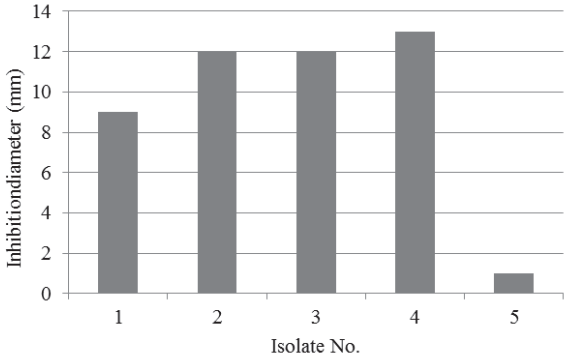

Fig. 8 – Inhibition diameters of CFS from five isolates from extreme volcanic environments of the Azores against *L. monocytogenes* ATCC 7466

Table 2 – Closest relatives of the twelve isolates with broad antimicrobial spectrum

Isolate no.	Closest Relative	% identity	CR_GenBank Acc. Number
1	*Ensifer adhaerens*	98%	AB681162.1
2	*Streptomyces mauvecolor*	99%	GU166430.1
3	*Streptomyces mauvecolor*	99%	GU166430.1
4	*Streptomyces xanthophaeus*	99%	HQ202828.1
5	*Streptomyces mauvecolor*	99%	GU166430.1
6	*Streptomyces spororaveus*	98%	EU593746.1
7	*Collimonas pratensis*	99%	AY281143.1
8	*Streptomyces mauvecolor*	99%	GU166430.1
9	*Streptomyces xanthophaeus*	98%	HQ202828.1
10	*Streptomyces avidinii*	98%	EU593657.1
11	*Streptomyces avidinii*	96%	HQ202833.1
12	*Paenibacillus elgii*	99%	AY090110.1

The 16S rRNA gene was sequenced for all twelve isolates with broad antimicrobial spectra. Closest relatives were found and are given in Table 2. *Streptomyces*, a well-known genus of antibiotic producers that belongs to the Actinobacteria Phylum (Baltz, 2008), was prevalent, representing three quarters of the active isolates. The remaining three isolates were a *Paenibacillus* (Phylum Firmicutes), *Ensifer* and *Collimonas* (Phylum Proteobacteria). Whereas antibiotic production has been reported in the genera *Paenibacillus* (Wu *et al*., 2010) and *Collimonas* (Hakvåg *et al*., 2009), no reports were published to date on antibiotic production by *Ensifer*. This genus appears, thus, as a promising candidate for novel antibiotic discovery.

4. CONCLUSIONS

Screening the untapped microbial community of the Azorean volcanic habitats may lead to the discovery of new generation antimicrobials, contributing to boost the

development of the Portuguese Biotechnology Industry. Improving our communication to industries to get to know which their real needs are is a fundamental step to guarantee the applicability of scientific research to improve citizens' life. Furthermore, the potential economic value associated with extremophile microbial biodiversity provides a strong argument for habitat conservation. In this respect, the present proposal also constitutes a contribution for the recognition of biodiversity and natural heritage values associated with volcanic environments.

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