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Molecular Characterization of Protease Producing *Idiomarina* Species Isolated from Peruvian Saline Environments

Carol N. Flores-Fernández[1]*, Elizabeth Chávez-Hidalgo[1], Marco Santos[1], Amparo I. Zavaleta[1], and David R. Arahal[2]

[1]*Laboratorio de Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad Nacional Mayor de San Marcos, Jr. Puno 1002, Lima, Perú*
[2]*Departamento de Microbiología y Ecología; y Colección Española de Cultivos Tipo, Universidad de Valencia, 46010 València, Spain*

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All *Idiomarina* species are isolated from saline environments; microorganisms in such extreme habitats develop metabolic adaptations and can produce compounds such as proteases with an industrial potential. ARDRA and 16S rRNA gene sequencing are established methods for performing phylogenetic analysis and taxonomic identification. However, 16S-23S ITS is more variable than the 16S rRNA gene within a genus, and is therefore, used as a marker to achieve a more precise identification. In this study, ten protease producing *Idiomarina* strains isolated from the Peruvian salterns were characterized using biochemical and molecular methods to determine their bacterial diversity and industrial potential. In addition, comparison between the length and nucleotide sequences of a 16S-23S ITS region allowed us to assess the inter and intraspecies variability. Based on the 16S rRNA gene, two species of *Idiomarina* were identified (*I. zobellii* and *I. fontislapidosi*). However, biochemical tests revealed that there were differences between the strains of the same species. Moreover, it was found that the ITS contains two tRNA genes, $tRNA^{Ile\ (GAT)}$ and $tRNA^{Ala\ (TGC)}$, which are separated by an ISR of a variable size between strains of *I. zobellii*. In one strain of *I. zobellii* (PM21), we found nonconserved nucleotides that were previously not reported in the $tRNA^{Ala}$ gene sequences of *Idiomarina* spp. Thus, based on the biochemical and molecular characteristics, we can conclude that protease producing *Idiomarina* strains have industrial potential; only two *I. zobellii* strains (PM48 and PM72) exhibited the same properties. The differences between the other strains could be explained by the presence of subspecies.

Keywords: *Idiomarina*, protease, Peruvian saline environments, 16S-23S ITS, tRNA

Introduction

The genus *Idiomarina* belongs to the family *Idiomarinaceae*, order *Alteromonadales*, class *Gammaproteobacteria* and phylum *Proteobacteria*. This genus was proposed by Ivanova *et al.* to accommodate two strains isolated from deep seawater samples collected at the Pacific Ocean, which were described as two different species, *Idiomarina abyssalis* and *I. zobellii* [1, 2]. At the time of writing, the genus *Idiomarina* comprises twenty eight recognized species including all *Pseudidiomarina* species [3–7]; *Idiomarina*-like organism without motility or flagella, unable to grow at pH below 6, without DNAse activity and with a nucleotide substitution in the 16S rRNA gene; that were reclassified by Taborda *et al.* and Wang *et al.* (Table 1) [8, 9]. All *Idiomarina* species have been isolated from saline environments, mainly from marine habitats (seawater), and they share several phenotypic characteristics with other heterotrophic, oxidative, marine and halophilic members of the

***Corresponding author**
Tel: +00-51-980553650, Fax: +00-51-1-6197000
E-mail: nathalifloresf@gmail.com
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Table 1. Species of *Idiomarina* isolated from different saline environments.

Bacteria	Source	Year	Ref.
Idiomarina abyssalis	Deep seawater (The Pacific Ocean)	2000	1
Idiomarina zobellii	Deep seawater (The Pacific Ocean)	2000	1
Idiomarina baltica	Surface water (The Baltic Sea)	2003	39
Idiomarina loihiensis	Submarine volcano (Hawaii)	2003	40
Idiomarina fontislapidosi	Soil sample from the temporally emerged banks of a hypersaline pool (Spain)	2004	10
Idiomarina ramblicola	Hypersaline water (Spain)	2004	10
Idiomarina seosinensis	Hypersaline water of a solar saltern (Korea)	2005	41
Idiomarina homiensis	Seashore sand (Korea)	2006	8, 14
Idiomarina salinarum	Marine solar saltern (Korea)	2007-2009	8, 42
Idiomarina insulisalsae	Soil of a sea salt evaporation pond (The Cape Verde Archipelago)	2009	8
Idiomarina marina[a]	Shallow coastal seawater (Taiwan)	2009	6, 8
Idiomarina maritima[a]	Coastal seawater (China)	2009	5, 8
Idiomarina donghaiensis[a]	Coastal seawater (China)	2009	5, 8
Idiomarina sediminum[a]	Coastal sediment (China)	2007-2009	4, 8
Idiomarina tainanensis[a]	Shallow coastal seawater (Taiwan)	2009	6, 8
Idiomarina taiwanensis[a]	Shallow coastal water (Taiwan)	2006-2009	3, 8
Idiomarina aestuarii[a]	Shallow coastal seawater (Korea)	2010-2011	7, 9
Idiomarina xiamensis	Crude oil degrading consortium enriched from surface sea water (China)	2011	9
Idiomarina maris	Sediment of sea (China)	2012	43
Idiomarina aquimaris	Reef-building coral *Isopora palifera* (Taiwan)	2012	11
Idiomarina piscisalsi	Thai fermented fish (pla-ra)	2013	44
Idiomarina indica	Seawater (The Indian Ocean)	2013	45
Idiomarina planktonica	Saline lake (China)	2014	46
Idiomarina atlantica	Deep sea sediment (The Atlantic Ocean)	2014	12
Idiomarina woesei	Seawater (The Indian Ocean)	2014	15
Idiomarina halophile	Solar saltern sediment (Korea)	2015	13
Idiomarina aquatica	Water of saltern pond (Spain)	2015	47
Idiomarina tyrosinivorans	Estuarine surface water (Taiwan)	2016	48

[a]*Pseudidiomarina* reclassified species.

class *Gammaproteobacteria* [10]. They are Gram-staining-negative, rod-shaped cells, colonies are non-pigmented or are slightly yellowish, strictly aerobic, catalase-and oxidase-positive, require NaCl or seawater base to grow, cannot utilize sugars as a unique source of carbon and energy and most are motile by means one polar flagella [8]. Species of *Idiomarina* can be distinguished from other marine bacteria by their physiological properties such as being able to grow within a broad range of temperatures, pH values and NaCl concentrations [11–13]. In addition, another eminent feature of the genus *Idiomarina* is its uniquely high content of iso-branched fatty acids, which is atypical of *Proteobacteria* with the exception of the *Xanthomonas* branch [10, 14, 15].

Saline environments are widely distributed in the world, and a growing interest in the study of their microbial diversity has been developed. These are habitats of halophilic microorganisms which have adapted physiological and genetic strategies to grow under extreme conditions such as high salinity, broad range of temperature and pH, and low oxygen conditions. As a result of their adaptation to these environments, halophiles have developed the capacity to produce extremophilic enzymes such as proteases with biotechnological potential [16–18]. It is important to highlight that bacteria isolated from saline environments produce extracellular prote-

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Table 2. Proteases produced by bacteria isolated from saline environments and their potential industrial application.

Strain	Source	Protease	Application	Ref.
Idiomarina sp. C9-1	Soda lake	Alkaline, thermostable	Eco-friendly dehairing of animal skins in the leather industry	36
Bacillus subtilis	Sea water	Alkaline	Laundry industry	49
Bacillus subtilis BLK-1.5	Salt mines	Alkaline, halotolerant	Industrial and biotechnological research	50
Bacillus sp. NPST-AK15	Hyper saline soda lakes	Alkaline, halotolerant	Laundry industry and others	51
Bacillus alcalophilus LW8	Hyperalkaline-saline soda lake	Alkaline	Biotechnological industries	52
Bacillus licheniformis KBDL4	Soda lake	Alkaline, thermostable	Laundry industry	53
Bacillus licheniformis MP1	Sea water	Alkaline, thermostable	Laundry detergents	54
Bacillus *pumilus* M3-16	Shallow salt lake	Alkaline, thermostable and high salt-tolerant	Biotechnology alimentary and agronomy industries	55
Streptomyces rutgersensis SCSIO 11720	*Porites lutea* from a reef	Alkaline, thermostable	Antibacterial peptides production	56
Streptomyces sp. MAB18	Marine sediments	Alkaline, thermostable, halotolerant	Production of antioxidant compounds for animal feed formulations	57
Pseudomonas aeruginosa BC1	Saline wastewater	Alkaline, salt-tolerant	Tannery saline wastewater treatment	25
Pseudomonas aeruginosa	Sea water	Alkaline	Laundry industry	49

ases that are not limited to their stability at high salt concentrations, since they are also tolerant to high temperatures and they are stable in presence of organic solvents, metal ions and surfactants [19–21].

The industrial demand of proteases with special properties continues to stimulating the search for new enzymes. The genus *Bacillus* is the main producer of industrial alkaline proteases, however other genus such as *Pseudomonas* and *Streptomyces* isolated from various environments have been reported as producers of alkaline and thermostable proteases with industrial importance (Table 2) [22–25]. Likewise, *Idiomarina* could represent an important source of novel proteases with commercial applications. In this sense, the exploration of saline environments microbial diversity and the finding of novel enzymes, showing activity and stability in wide ranges of temperature, pH and at high salt concentrations, are of great relevance.

The 16S rRNA gene sequence has been established as a powerful marker for phylogenetic analysis; however, in some studies, it has been encountered apparent resolution limitations due to the high level of similarity in the sequences [26]. It promoted the search for a new phylogenetic marker such as the 16S-23S internal transcribed spacer (ITS). ITS regions show higher variability than 16S rRNA genes within a genus, and they allow finding inter and intraspecies variability based on length and nucleotide sequence. The length variations are due to the type and number of tRNA genes interspersed in the ITS sequence, and most Gram-negative bacteria contain $tRNA^{Ala}$ and $tRNA^{Ile}$ genes [27–29].

In this study, protease producing *Idiomarina* strains were isolated from two Peruvian saline environments and characterized by using biochemical and molecular methods in order to assess bacterial diversity and to determine their industrial potential. In addition, comparison between length and nucleotide sequences of a 16S-23S ITS region allowed to determine inter and intraspecies variability within these *Idiomarina* strains.

Materials and Methods

Isolation and phenotypic characterization of protease producing *Idiomarina* sp.

Soil samples were collected from two terrestrial saline environments, Pilluana and San Blas salterns, located in the north and in the centre of Peru, respectively. For the isolation, the samples were cultured in a broth containing salt water (SW) 5% and yeast extract 0.5% at 37℃ for 7 days. SW contains in g/l: NaCl 40; $MgSO_4 \cdot 7H_2O$ 5.83, $MgCl_2 \cdot 6H_2O$ 5; KCl 1.17; NaBr 0.13; $CaCl_2$ 0.083 and $NaHCO_3$ 0.03. After incubation, serial dilu-

tions were made in sterile phosphate buffer saline (PBS) 1 X containing NaCl 5% up to 10^{-12}. One hundred microliters of 10^{-8}, 10^{-10} and 10^{-12} dilutions were spread on agar plates containing SW 5% and yeast extract 0.5%, and kept for incubation at 37℃ for 24 h. Colonies were isolated based on their morphological characteristics and stored at -80℃ in the isolation broth containing glycerol 30%. Screening of protease producing bacteria was carried out on agar plates with SW 5% and yeast extract 0.5% supplemented either with gelatin or skim milk (1% w/v). Proteolytic activity was visualized as clear zones around the colonies due to substrate hydrolysis after 24 h of incubation at 37℃. The strains were characterized by Gram staining and cultured at different NaCl concentrations (0.5, 5, 7.5, 10, 15 and 20%), pH (5–9) and temperature (20, 37 and 45℃). In addition, they were subjected to an antimicrobial susceptibility assay. The inhibition of the strains by several antimicrobials was tested by standard disc diffusion technique (Bauer *et al.* 1996) [30]. Briefly, the cultures were grown in agar plates containing SW 5% and yeast extract 0.5%, and the following antimicrobial discs with their concentrations indicated in parenthesis were used: amoxicillin (25 μg), penicillin G (10 U), trimethoprim-sulfamethoxazole (25 μg), chloramphenicol (30 μg), bacitracin (10 U), novobiocin (30 mcg), erythromycin (30 μg) and rifampin (30 μg).

DNA extraction

Genomic DNA extraction was performed according to the method described as follows. Isolates cultures were grown overnight at 37℃ in 2 ml of broth containing SW 5% and yeast extract 0.5%. After that, they were centrifuged at 10,000 g for 10 min, and the resultant pellet was resuspended in PBS 1 X containing NaCl 3% and centrifuged at 10,000 g for 5 min. This procedure was carried out twice for washing the cells. Then, 600 μl of TSE 1 X buffer, 60 μl of SDS 10% and 10 μl of Proteinase K were added and incubated at 50℃ for 1 h. The mixture was extracted once each with 600 μl of phenol-chloroform-isoamyl alcohol (25:24:1) and 600 μl of chloroform. Afterwards, DNA was precipitated from the aqueous phase using 100 μl of sodium acetate 3 M and 600 μl of ethanol. The mixture was maintained at -20℃ for 2 h and then was centrifuged at 10,000 g for 15 min. The pellet was washed twice with 500 μl of ethanol 70%. The DNA pellet obtained after final centrifugation was dried and dissolved in 50 μl of TE buffer. Finally, DNA was analyzed by electrophoresis in 1% (w/v) agarose gel in 1 X TBE buffer (45 mM Tris-HCl, 45 mM boric acid and 1 mM EDTA pH 8.0), stained with ethidium bromide (0.5 μg/ml) and visualized under UV transilluminator. The 1 kb DNA Ladder was used as molecular weight standard.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The 16S rRNA gene of each strain was amplified by Polymerase Chain Reaction (PCR) using the universal primers 16SBF 5'-AGAGTTTGATCATGGCTCAG-3' and 16SBR 5'-GGTTACCTTGTACGACTT-3'. PCR reactions were performed with 50 ηg of genomic DNA, 10 X PCR buffer (10 mM Tris-HCl pH 8.4, 50 mM KCl), 20 ρmoles of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, and 1 U *Taq* DNA polymerase in a total volume of 25 μl. The reaction mixtures were incubated in a thermal cycler at 94℃ for 4 min and then cycled 35 times: 94℃ for 45 s, 55℃ for 1 min and 72℃ for 45 s. A final extension at 72℃ for 7 min was used. PCR products were resolved by electrophoresis in 1% (w/v) agarose gels. For the restriction of the amplified fragment, a 10 μl aliquot of each PCR product (1 μg) was incubated overnight with 4 U of one of the following restriction enzymes: *Hae* III, *Cfo* I, or *Rsa* I at 37℃ in a total volume of 20 μl. Restriction fragments were analyzed by electrophoresis in 3% (w/v) agarose gels in 1 X TBE using 100 bp DNA Ladder as molecular size marker, and staining as above conditions. For the analysis of ARDRA patterns bands, PyElp program version 1.4 was used.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA PCR products obtained as described above were subjected to commercial sequencing (Macrogen, Korea). The sequence of each strain was searched in the GenBank using the algorithm BLASTn. Gene sequences of 16S rRNA of microorganisms with the highest homology were obtained from GenBank and aligned with gene sequence of our isolates using ClustalX2 software. Phylogeny was inferred using the Maximum Likelihood method based on the Jukes-Cantor evolutionary model with 100 bootstrap replicates. Tree building along with visualization were done using the MEGA6 program. After the analysis, the sequences were deposited in Gen-

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Bank nucleotide sequence data libraries.

PCR of 16S-23S rRNA internal transcribed spacer (ITS) and sequencing

The primers 16S14F 5' CTTGTACACACCGCCCGTC3' and 23S1R 5' GGGTTTCCCCATTCGGAAATCA 3' were used for PCR amplification of 16S-23S rDNA ITS and the conditions were the same as used for 16S rRNA gene amplification. Then, the PCR products were subjected to commercial sequencing (Macrogen). The 16S-23S ITS nucleotide sequences of the bacterial strains were aligned using ClustalX2, and analyzed by tRNAscan -SE v.2.0 and tRNADB-CE (http://trna.ie.niigata-u.ac.jp/cgi-bin/trnadb/index.cgi).

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences derived in this study have been deposited in GenBank under the accession numbers MH588073, MH588074, MH588075, MH588076, MH588077, MH588078, MH588079, MH588080, MH588081 and MH588082.

Results

Isolation and characterization of protease producing *Idiomarina* sp.

A total of ten protease producing *Idiomarina* strains were isolated, eight from Pilluana and two from San Blas. All were able to hydrolyze both used substrates (gelatin and skim milk) showing hydrolysis clear zones between 20 and 25 mm of diameter. Growth conditions are summarized in Table 3. The strains isolated from Pilluana generally grew in a broad salt concentration range from 5 to 20% (except PM70), whereas of the two strains from San Blas, one grew from 5 to 10% (ESB68) and the other from 0.5 to 15% (ESB71). In addition, some strains grew at pH 5 and all of them grew in alkaline pH range up to pH 9. Finally, most of them grew up to 45℃.

Table 3. Growth conditions of protease producing *Idiomarina* sp. isolated from Pilluana and San Blas Salterns in Peru.

Saltern	Strain	Salt concentration[a] (%, w/v)	pH[a]	Temperature[a] (℃)
Pilluana	PM21	5-20	6-9	20-45
	PM23	5-20	6-9	20-45
	PM39	5-20	5-9	20-45
	PM48	5-20	5-9	20-45
	PM70	5-15	5-9	20-45
	PM72	5-20	5-9	20-45
	PM75	5-20	6-9	20-37
	PM76	5-20	6-9	20-37
San Blas	ESB68	5-10	5-9	20-45
	ESB71	0.5-15	5-9	20-37

[a]Range of growth.

Antimicrobial susceptibility assay

In this test, all the strains exhibited resistance to bacitracin. In addition, strains PM39, PM76 and ESB68 were resistant to erythromycin, trimethoprim-sulfamethoxazole and amoxicillin, respectively. The strain PM75 also exhibited resistance to penicillin, trimethoprim-sulfamethoxazole and erythromycin (Table 4). It could be observed five different profiles based on this assay.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

As expected, fragments of approximately 1400 bp corresponding to the 16S rRNA genes were obtained. For each restriction enzyme (*Hae* III, *Cfo* I, or *Rsa* I), the banding patterns for the ten strains were similar. Fragments smaller than 50 bp were not visualized on agarose gels and they were not included in the analysis. *Hae* III produced the same eight bands in the 50 to 350 bp range for all strains, except for PM70 that produced seven bands. *Cfo* I produced four bands in the 50 to 1000 bp range for all the strains, except for PM21, PM39 and ESB71 that produced three bands with the same profile. *Rsa* I gave six bands in the 50 to 550 bp range and in this case the profile for all the strains looked identical (data not shown).

16S rRNA gene sequencing and phylogenetic analysis

As result of BLASTn analysis, the nucleotide sequences of the 16S rRNA genes revealed 99% homology with *Idiomarina* genus. For the phylogenetic analysis, the alignment included 16S rRNA gene sequences from *Idiomarina* species retrieved from the GenBank with a close relationship with the strains of this study. In the phylogenetic tree (Fig. 1), strains PM70 and ESB68 grouped with *Idiomarina fontislapidosi* and the others with *Idiomarina zobellii.*

Table 4. Antimicrobial susceptibility to protease producing *Idiomarina* isolated from Pilluana and San Blas Salterns in Peru.

Strain	Antimicrobials							
	AMX	PEN	STX	CHL	BAC	NOVO	ERY	RIF
PM21[c]	S	S	S	S	R	S	S	S
PM23[c]	S	S	S	S	R	S	S	S
PM39[d]	S	S	S	S	R	S	R	S
PM48[c]	S	S	S	S	R	S	S	S
PM70[c]	S	S	S	S	R	S	S	S
PM72[c]	S	S	S	S	R	S	S	S
PM75[e]	S	R	R	S	R	S	R	S
PM76[f]	S	S	R	S	R	S	S	S
ESB68[g]	R	S	S	S	R	S	S	S
ESB71[c]	S	S	S	S	R	S	S	S

AMX, amoxicillin; PEN, penicillin; STX, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; BAC, bacitracin; NOVO, novobiocin; ERY, erythromycin; RIF, rifampicin. S, susceptible; R, resistant.
[c-g]: Profiles based on antimicrobial susceptibility assay.

PCR amplification of 16S-23S rRNA ITS and sequencing

After amplification and analysis of 16S-23S rRNA ITS sequences, it was found that all *Idiomarina* strains presented a 16S-23S rRNA ITS sharing the first and the last region. The interspersed sequences between these conserved ends were composed of sequences that contain tRNA genes (tRNA$^{Ile\ (GAT)}$ and tRNA$^{Ala\ (TGC)}$) separated by an intergenic spacer region (ISR) of variable size: 170,

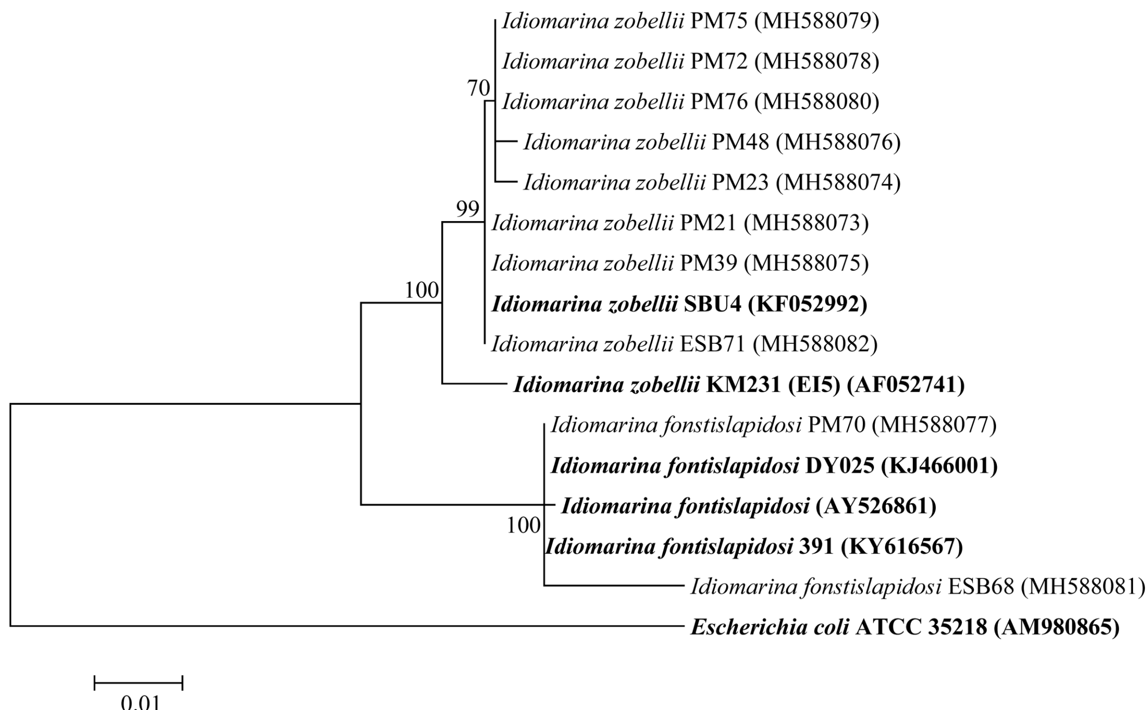

Fig. 1. Phylogenetic relationship between the 16S rRNA gene sequences of *Idiomarina* strains isolated from Pilluana and San Blas Salterns in Peru, and *Idiomarina zobellii* and *Idiomarina fontislapidosi* strains from the GenBank. *Escherichia coli* was used as outgroup taxon strain. The numbers in brackets are the GenBank accession numbers. The tree was constructed using the Maximum Likelihood method based on the Jukes-Cantor evolutionary model using MEGA 6 software. The scale bar shows 0.01 substitutions per base position. Numbers refer to bootstrap values for each node out of a total of 100 replicate resampling.

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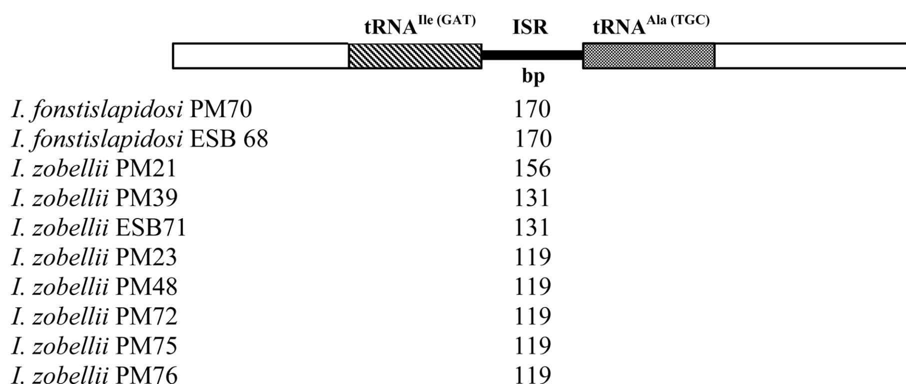

Fig. 2. Schematic representation of 16S-23S rRNA Internal Transcribed Spacer (ITS) of *Idiomarina* species isolated from Pilluana and San Blas salterns in Peru. The non-filed boxes represent regions of homologous nucleotide sequences, filed boxes represent tRNA genes and the solid line represents the non-conservation region between tRNA genes.

156, 131 and 119 bp (Fig. 2). The presence of tRNA genes was also confirmed by secondary structure predictions.

Table 5 shows nucleotide sequences of $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in ITS of the *Idiomarina* strains. $tRNA^{Ile}$ has a length of 74 bp and it is highly conserved in all *I. zobellii* strains as well as with $tRNA^{Ile}$ of *I. loihiensis*

Table 5. Nucleotide sequences of $tRNA^{Ile}$ and $tRNA^{Ala}$ genes of *Idiomarina* strains isolated from Pilluana and San Blas Salterns in Peru.

Idiomarina species	$tRNA^{Ile\ (GAT)}$ (74 bp)
I. fonstislapidosi PM70	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**T**AGTTCAAGTCT**A**C**T**CAGACCCA
I. fonstislapidosi ESB 68	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**T**AGTTCAAGTCT**A**C**T**CAGACCCA
I. zobellii PM21	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
I. zobellii PM39	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
I. zobellii ESB71	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
I. zobellii PM23	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
I. zobellii PM48	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
I. zobellii PM72	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
I. zobellii PM75	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
I. zobellii PM76	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
I. loihiensis GSL 199	GGGTCTGTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGG**C**AGTTCAAGTCT**G**C**C**CAGACCCA
Idiomarina species	$tRNA^{Ala\ (TGC)}$ (73 bp)
I. fonstislapidosi PM70	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. fonstislapidosi ESB 68	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. zobellii PM21	GGGGCCATA**T**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. zobellii PM39	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. zobellii ESB71	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. zobellii PM23	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. zobellii PM48	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. zobellii PM72	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. zobellii PM75	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. zobellii PM76	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA
I. loihiensis GSL 199	GGGGCCATA**G**CTCAGCTGGGAGAGCGCCTGCCTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTTGGCTCCA

Highlighted and underlined letters: no conserved nucleotides.

which is a very studied *Idiomarina* species [31]. However, in both *I. fontislapidosi* strains it could be observed no conserved nucleotides. Likewise, $tRNA^{Ala}$ has a length of 73 bp and it could be saw that it is highly conserved in all *Idiomarina* strains except in *I. zobellii* PM21.

Discussion

All *Idiomarina* species have been isolated from saline environments mainly from marine habitats. Interestingly, the *Idiomarina* strains or this work were isolated from terrestrial saline environments. This genus has been found in the Pacific, Atlantic and Indian Oceans; and countries such as Hawaii, Spain, Korea, China and Taiwan (Table 1). The study of halophiles biodiversity is of great relevance since these microorganisms have developed metabolic strategies to survive under extreme conditions, and as consequence they are considered as an important source of industrially useful enzymes [32]. Several protease producing bacteria with biotechnological and industrial potential application have been isolated from different saline environments including genus *Bacillus, Pseudomonas, Streptomyces* and *Idiomarina*, whose enzymes are mainly alkaline, thermostable and halotolerant (Table 2). *Idiomarina* strains of this study produced hydrolysis clear zones up to 25 mm of diameter, which were higher than those reported for some *Bacillus* strains [33]. In addition, they grew at alkaline conditions (pH up to 9), high temperatures (up to 45℃) and high salt concentrations (up to 20%) similar to other protease producing *Bacillus* species [34, 35]. Likewise, Zhou et al. reported a novel serine alkaline protease from alkaliphilic *Idiomarina* sp. C9-1 isolated from a soda lake. This enzyme showed optimum activity at pH 10.5 and 60℃, and it was active and stable in a wide range of pH and temperature. Moreover, the protease presented high activity in presence of Ca^{2+}, surfactants and oxidizing and reducing agents. Finally, they suggested that this protease may have great potential for eco-friendly dehairing of animal skins in the leather industry [36]. Hence, similar to other bacterial genus, *Idiomarina* strains could represent an important source of proteases with commercial applications. In this study, ten members of protease producing bacteria belonging to the *Idiomarina* genus were isolated from soil samples from Peruvian saline environments. *Idiomarina* strains were characterized by using biochemical and molecular methods in order to study bacterial diversity, inter and intraspecies variability and to determine their industrial potential.

The 16S rRNA gene sequencing analysis of the strains revealed 99% similarity with the species *I. zobellii* and *I. fontislapidosi* (Fig. 1). Members of *Idiomarina* genus are able to grow in a wide range of salt concentrations, pH and temperature [11–13]. Thus, Martínez-Cánovas *et al.*, described that *I. fontislapidosi* is capable of growing at NaCl concentrations up to 25% (w/v), temperature up to 45℃ and pH values between 5 and 10 [10]. These physiological properties are similar to those found in this study for both *I. fontislapidosi* strains. On the other hand, most of *I. zobellii* strains of this work grew up to 20% salt concentration (%, w/v) and up to 45℃ (Table 3). These findings are relevant since Ivanova *et al.* reported that this species grows only up to 10% salt concentration and up to 30℃ [1].

In antimicrobial susceptibility assay, it has been reported that *I. zobellii* is susceptible to erythromycin and resistant to penicillin [1]. However in this research *I. zobellii* strains (PM39 and PM75) exhibited resistance to erythromycin and only one of them was resistant to penicillin (PM75). Likewise, it has been found that *I. fontislapidosi* is susceptible to amoxicillin, chloramphenicol, erythromycin, rifampicin and trimetroprim-sulfamethoxazole [10]. This information is in agreement with the results obtained for both *I. fontislapidosi* strains, except for the strain ESB68 which exhibited resistance to amoxicillin. It could be observed that *I. zobellii* strains have three different profiles in this test (c, d, e and f), whereas *I. fontislapidosi* strains evidenced two profiles (c and g) (Table 4).

Bacterial phylogeny and taxonomic identification of a group of strains could be achieved by ARDRA and by phylogenetic analysis of 16S rRNA gene sequences [26]. ARDRA results (data not shown) revealed that all the strains tested were similar or identical. The analysis of 16S rRNA gene sequences classified the strains within *Idiomarina* genus (*I. zobellii* and *I. fonstislapidosi*). However, due to differences found in physiological and biochemical characteristics between strains from the same species and the lack of resolution of the 16S rRNA genes for the differentiation of them, a more precise

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molecular identification was achieve by an ITS region amplification and sequencing. ITS region shows a high degree of variability between species, both in length and nucleotide sequence, and has been successfully used to differentiate closely related bacteria [27, 28, 37]. In the present study it was found that 16S-23S rRNA ITS of *Idiomarina* strains consists of constant and variable regions. All the strains share the first and the last region and, similar to most Gram-negative bacteria, all of them contain $tRNA^{Ala}$ and $tRNA^{Ile}$ genes. Earlier studies reported that the number of tRNA genes coexisting in a bacterial 16S-23S rRNA ITS varies from zero to four [27]. Moreover, it was observed that both *I. fontislapidosi* strains presented an ISR region of 170 bp, however, variation in length was observed between *I. zobellii* strains (156, 131 and 119 bp), which evidence that intra-species variability exists (Fig. 2).

The tRNA genes exhibit variability within a genus, but they are highly conserved among species [38]. In tRNADB-CE database, 37 sequences of $tRNA^{Ile}$ genes (all with GAT anticodon) for different *Idiomarina* species have been reported. Most of them (27) are identical, including $tRNA^{Ile}$ gene of *I. zobellii* KMM 23 which is the same as $tRNA^{Ile}$ gene of *I. zobellii* strains of this study. However, it is observed that both strains of *I. fontislapidosi* have three no conserved nucleotides (Table 5). These nucleotides have been reported in strains such as *I. baltica* OS145, *Idiomarina xiamenensis* 10-D-4, *I. salinarum*, *I. bacterium* HL-53 and *Idiomarina woesei* DSM 27808. In case of $tRNA^{Ala}$ genes, 60 sequences with two types of anticodons (TGC or GGC) containing no conserved nucleotides in several positions have been reported. It can be noted in Table 5 that most of sequences for this gene are identical to $tRNA^{Ala\ (TGC)}$ reported in *I. zobellii* KMM 231. However, in $tRNA^{Ala}$ gen of *Idiomarina zobellii* PM21, one not conserved nucleotide in a position that has not been reported before for $tRNA^{Ala}$ genes was found. Finally, it is important to mention that $tRNA^{Ile}$ and $tRNA^{Ala}$ genes sequences have not been reported for *I. fontislapidosi*.

In conclusion, *Idiomarina* strains isolated from Pilluana and San Blas salterns in Peru exhibited different biochemical properties that show their potential to produce proteases with biotechnological and industrial applications. In addition, molecular characteristics revealed that bacterial diversity exists not only between species but also between strains of the same species. Overall, based on biochemical and molecular profiles we can conclude that it was found two species of genus *Idiomarina*, *I. zobellii* and *I. fontislapidosi*; nevertheless, only *I. zobellii* PM48 and *I. zobellii* PM72 exhibited the same characteristics. The others strains can be differentiated by growth conditions, antimicrobial susceptibility and 16S-23S ITS sequences analysis. This might be explained by the presence of different subspecies.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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