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Environmental parameters, and not phylogeny, determine the composition of extracellular polymeric substances in microbial mats from extreme environments

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ABSTRACT

The ability to establish biofilms is a key trait for microorganisms growing in extreme environments. The extracellular polymeric substances (EPS) present in biofilms provide not only surface attachment, but also protection against all kinds of environmental stressors, including desiccation, salinity, temperature or heavy metal pollution. The acquisition of suitable biofilm characteristics might thus be an important process mediating the adaptation of microorganisms to novel environmental conditions. In this work we have characterized the EPS of 20 phylogenetically diverse biofilms collected *in situ* from five contrasting extreme environments, including two geothermal areas (Copahue, Argentina; Seltun, Iceland), two cold areas (Pastoruri glacier, Peru; Byers Peninsula, Antarctica) and one extremely acidic river (Río Tinto, Spain). Biofilms were subjected to biochemical characterization, glycan profiling and immunoprofiling with an antibody microarray. Our results showed that environmental conditions strongly influence biofilm characteristics, with microorganisms from the same environment achieving similar EPS compositions regardless of the phylogeny of their main species. The concentration of some monosaccharides in the EPS could be related to environmental conditions such as temperature or heavy metal toxicity, suggesting that in some cases stress resistance can be mediated by specific sugars. Overall, our results highlight the existence of conserved EPS compositional patterns for each extreme environment, which could in turn be exploited to engineer ecological adaptations in genetically modified microorganisms.

1. Introduction

Exploration of extreme terrestrial environments has turned up a diverse assortment of microorganisms that not only live, but also thrive, at environmental extremes previously thought to be inhospitable to terrestrial life. These conditions include extremes of pH, temperature, pressure, radiation, water availability, salinity or nutrient concentration (Rothschild and Mancinelli, 2001). However, in spite of the apparent hostility of such habitats, they contain a higher level of biodiversity and biomass than expected. Additionally, most of the microbial communities found in extreme environments are distributed and assembled in extensive biofilms and microbial mats. Although the development of these structures in aquatic and terrestrial environments has been well documented (reviewed by Bolhuis et al., 2014), the distribution pattern and factors controlling their development in extreme environments are not yet well understood.

The growth of microbial mats and biofilms is promoted by the excretion of extracellular polymeric substances (EPS) by the cells, which serves as an adhesive agent enabling cellular attachment and form the biofilm matrix embedding the cells (Wolf et al., 2007). EPS are a complex mixture of biomolecules, *e.g.*, proteins, humic-like substances, polysaccharides, uronic acids, nucleic acids, lipids, and glycoproteins, surrounding the cells (Sheng et al., 2010). EPS make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix. The key functions of EPS comprise the mediation of the initial attachment of cells to different substrates and protection against environmental stress and dehydration (Sutherland, 2001). Besides, EPS can afford a stable environment, promoting the growth of organisms. Thus, it prevents the losses of organisms and retains diversity over a long period of time, allowing the development of synergistic relationships among species (Ras et al., 2011).

These EPS secretions may constitute up to 60% of the dry biomass (Hill et al., 1997), and their presence is considered tightly related to the capability of the organisms to successfully cope with environmental constraints and with the formation of complex microbial mats on a great variety of substrates. Thus, since the extracellular matrix is a vital and complex component of all biofilms, it has to play an even more important role in the development of microbial communi-

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ties in extreme environments by providing a suitable architectural structure, mechanical stability and protection against external conditions, which are incompatible with most life forms.

Although literature on biofilm characterization is abundant, a large part of it is focused on either clinical isolates (Hall-Stoodley et al., 2008; Gunn et al., 2016; Zhang et al., 2018) or man-made systems such as water treatment plants or microbial fuel cells (Miqueleto et al., 2010; Sheng et al., 2010, Read et al., 2010, Cao et al., 2011, Salama et al., 2016). On the other hand, studies focusing on natural environments are often performed on environmental isolates rather than in biofilms collected *in situ* from their native habitats (Nichols et al., 2005; Ortega-Morales et al., 2007; Ren et al., 2015). Finally, studies often focus on a single species and/or on a single environment (Nichols et al., 2005; Goltsman et al., 2015; Caruso et al., 2018), which precludes the detection of phylogenetic or environmentally determined patterns in biofilm composition.

In this work we have characterized the microbial EPS composition in 20 phylogenetically distinct benthic biofilms from different extreme environments. Since temperature and pH are among the most restrictive environmental parameters for the presence of microorganisms, five different extreme environments were selected taking into consideration these parameters, (i) two extreme geothermal areas, the Copahue Volcano region (Neuquen, Argentina) and the Seltun area (Reykjanes, Iceland), where water temperature can reach up to 77 °C and pH range from 3 to 7 (Aguilera et al., 2010; Urbieta et al., 2014, 2015), (ii) Pastoruri Glacier area (Huascarán National Park, Perú), an extreme cold acidic ecosystem (pH 2–5, T^a 4–10 °C) (González-Toril et al., 2015), (iii) Río Tinto (Huelva, Spain), a temperate extremely acidic river (pH 1.5–3, 20–25 °C) showing high levels of heavy metals (mg/L in most cases) (Aguilera et al., 2007; Aguilera, 2013), (iv) Byers Peninsula (Livingston Island, Antarctica), an extreme cold natural environment (pH 6–7, T^a 4–5 °C) (Quesada et al., 2009).

The environmental parameters (pH, temperature, conductivity, and heavy metal content) were measured at each sampling point, and the biofilms were collected and further analyzed in the laboratory. The biofilms were classified based on their phylogeny, using a combination of microscopic and molecular techniques, and their EPS were extracted and characterized by a mixture of biochemical techniques, glycan profiling and immunoprofiling with an antibody microarray. Our results highlight the existence of conserved EPS compositional patterns for each extreme environment, which are independent of the phylogenetic composition of the biofilm. This suggests that the acquisition of optimal biofilm characteristics is a hallmark of microbial adaptation to novel environments.

2. Material and methods

2.1. Field sites and sample collection

Twenty microbial mats and biofilms from five different extreme environments were analyzed (Fig. S1). *In situ* measurements of water pH, temperature, redox potential and conductivity, were carried out as described previously (Santofimia et al., 2013) (Table 1). Water samples were filtered through 0.45 μm Millipore membranes. Total concentrations of nine recoverable metals were measured for each water sample (Zn, Cu, Fe, Co, Ni, As, Cd, Cr and Pb) using X-ray Fluorescence Reflection (TXRF) and Inductively Coupled Plasma-mass Spectrometry (ICP-MS) (Santofimia et al., 2013). Microbial mats and biofilms were taken using a sterile plastic spatula and then placed in 50 mL Falcon tubes. The samples were immediately frozen in dry ice, freeze-dried and kept in the laboratory at −20 °C until the analyses were carried out.

2.2. Morphotype and molecular identification

Since eighteen samples were mainly formed by photosynthetic species (algae and cyanobacteria), microscopic identification and quantification of species were carried out. Thus, subsamples of *ca.* 2 cm^2 from each mat or biofilm were taken, resuspended in 5 mL of BG11 media (Rippka et al., 1979) at the different environmental pH and preserved at 4% formaldehyde. Species identification was carried out by direct microscopic observation down to the lowest possible taxonomic level using different phenotypic features based on previous studies of these communities (Aguilera et al., 2007; Souza-Egipsy et al., 2008; Aguilera et al., 2010; González-Toril et al., 2015; Urbieta et al., 2014; Urbieta et al., 2015). Cell counts were performed in triplicate in a Sedwerick-Rafter chamber. A Zeiss Axioscope 2 microscope equipped with phase-contrast was used in this work.

Biofilms IC14 and RT21 were predominantly composed by bacteria. In these cases, DNA extraction, PCR amplification, cloning and sequencing of the 16S RNA gene were carried out in order to identify the prokaryotic microbial diversity, following previously reported

Table 1
Means and standard errors of water physicochemical parameters at each sampling site. Temperature (°C), redox (mV), conductivity (mS cm^{-1}).

Location	Sampling site	GPS coordinates	Sample	pH	Temperature	Redox	Conductivity	Toxicity index
Argentina	Copahue Area	37°52′11″S, 71°05′12″W	AR2	5.8±0.3	30.1±0.4	110.4±12.1	0.32±0.04	1.4
		37°14′27″S, 70°21′12″W	AR3	6.1±0.1	32.2±0.3	98.3±13.1	0.31±0.03	1.2
		37°18′11″S, 70°21′16″W	AR4	5.7±0.5	33.1±0.8	88.6±11.4	0.22±0.05	1.1
Antarctica	Byers Peninsula	62°38′03″S, 61°5′01″ W	ANT5	6.8±0.2	4.0±0.3	112.3±9.1	0.05±0.01	0.3
		62°38′22″S, 61°5′19″ W	ANT6	7.0±0.3	5.1±0.5	131.6±10.1	0.03±0.01	0.3
		62°38′34″S, 61°5′08″ W	ANT7	6.9±0.2	3.2±0.3	144.2±8.4	0.04±0.01	0.3
		62°37′12″S, 61°4′02″ W	ANT8	7.0±0.3	6.5±0.2	98.9±3.1	0.03±0.01	0.3
		62°37′04″S, 61°5′06″ W	ANT9	7.0±0.2	5.3±0.1	154.5±11.6	0.04±0.01	0.3
Iceland	Seltun Area	63°53′42″N, 22°3′21″W	IC11	2.4±0.2	32.3±0.2	354.1±16.1	1.85±0.01	1.5
		63°53′05″N, 22°3′17″W	IC12	5.2±0.1	33.2±0.2	98.1±2.1	0.33±0.12	0.2
		63°52′12″N, 22°3′01″W	IC13	6.7±0.2	35.6±0.3	136.5±10.2	0.15±0.05	0.2
		63°52′08″N, 22°3′11″W	IC14	6.2±0.3	35.3±0.3	292.3±12.2	0.09±0.02	0.2
		63°53′09″N, 22°3′07″W	IC15	6.8±0.2	38.6±0.4	86.2±5.3	0.44±0.03	0.1
Río Tinto	Nerva Area	37°42′33″N, 6°33′17″W	RT21	3.1±0.1	21.3±0.6	413.5±14.3	5.3±0 0.5	3.3
		37°43′22″N, 6°33′12″W	RT22	3.2±0.2	22.1±0.2	446.1±10.1	11.3±2.5	6.2
		37°43′28″N, 6°33′36″W	RT23	3.2±0.3	20.2±0.5	430.2±15.8	10.4±1.3	6.2
		37°43′14″N, 6°33′09″W	RT24	3.2±0.2	23.4±0.3	450.3±19.2	11.8±4.1	6.2
		37°43′19″N, 6°33′03″W	RT25	3.2±0.3	22.0±0.2	439.1±17.5	12.4±3.1	6.2
Peru	Pastoruri Glacier	9°20′18″S, 77°24′04″W	PE2	5.1±0.2	6.1±0.2	403.2±19.5	0.27±0.05	2.9
		9°21′03″S, 77°23′11″W	PE4	5.4±0.3	10.1±0.3	99.6±9.2	0.07±0.02	1.1

methodology (Souza-Egipsy et al., 2008; González-Toril et al., 2015; Urbieta et al., 2014; Urbieta et al., 2015). Briefly, Fast DNA Spin kit for soil (Q-Bio Gene Inc., CA, USA) was used for DNA extraction according to the manufacturer's instructions. DNA obtained was purified by passage through a GeneClean Turbo column (Q-Bio Gene Inc., CA, USA). The 16S rRNA genes were amplified according to previously described methodologies (Souza-Egipsy et al., 2008; García-Moyano et al., 2012) using the universal Bacteria-specific primers 27f and 1492r (Achenbach and Woese, 1995; Lane, 1991). Archaea-specific primers 21f and 1492r (DeLong, 1992) were also assayed. PCR amplified genes were purified by GeneClean Turbo Column (Q-Bio Gene Inc., CA, USA) and cloned using the Topo TA Cloning Kit (Invitrogen, CA, USA). Primers used for sequencing were primers M13f and M13r recommended by TOPO TA kit. PCR products were directly sequenced using a Big-Dye sequencing kit (Applied Biosystem) according to manufacturer's instructions.

Partial sequences were assembled with DNAbaser (DNA Sequence Assembler v4, Heracle BioSoft) and the taxonomic assignation was carried out by BLAST at the NCBI database (http://ncbi.nlim.nih.gov/BLAST) (Altschul et al., 1990).

2.3. Isolation and purification of EPS

The protocol used for EPS extraction was modified from previously described methods (Aguilera et al., 2008a, 2008b). Two fractions of exopolymers, colloidal and capsular, were extracted from each biofilm (Staats et al., 1999). The colloidal fraction includes compounds that are loosely bound to microorganisms, while capsular fraction contains tightly carbohydrates and proteins that require a lengthier extraction protocol (Hirst and Jordan, 2003). Briefly, 30 mL of deionized water were added to 10 g of each biofilm and continuously agitated for 30 min at room temperature at 10 rpm. Samples were then centrifuged (15 min, 5000 ×*g*) and the supernatant was lyophilized, weighted and used for analysis of the colloidal fraction. In parallel, 30 mL of 0.2 M EDTA were added to each pellet to carry out the capsular fraction extraction. Mixtures were maintained continuously agitated for 2 h as the same conditions above and then centrifuged at 8000 ×*g* for 10 min. The resulting lyophilized and weighted supernatants were used further as capsular fractions.

2.4. Biochemical composition of EPS

The protein and carbohydrate content of colloidal and capsular fractions were quantified by Bradford (1976) and Dubois et al. (1956) using BSA and glucose as standards, respectively. The total DNA content in both EPS fractions was measured by using Qubit® ds-DNA HS Assay kit and Qubit® 2.0 fluorometer from Invitrogen (Oregon, USA) according to the manufacturer's instructions. The total concentrations of nine recoverable metals were also measured (Zn, Cu, Fe, Co, Ni, As, Cd, Cr and Pb) using X-ray Fluorescence Reflection (TXRF) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Santofimia et al., 2013).

For the identification of monosaccharides and related compounds, the following protocol was used: (i) freeze-dried colloidal and capsular fractions were dialyzed thoroughly against ddH_2O, newly lyophilized and the weights corresponding to 100 μg of total carbohydrate were hydrolyzed with 6 M HCl at 75 °C overnight and freeze dried to remove water and HCl as described in Blanco et al. (2010); (ii) the hydrolyzed samples in 75 μL of Power Syl-Prep [TMSIM (*N*-Trimethylsilylimidazole): BSA: TMCS (Trimethylchlorosilane) (3:3:2), from Alltech (Grace, Deerfield, IL, USA)] were heated at 70 °C for 15 min to obtain the corresponding TMS derivatives; (iii) The derivatized samples were extracted with 400 μL of water and 100 μL of hexane; (iv) The organic layers were collected and analyzed by GC–MS with the following GC oven program: 100 °C (initial temperature) with a hold of 1 min, heated to 200 °C at 30 °C min^{-1} with a hold time of 15 min heated to 270 °C at 15 °C min^{-1} with a hold time of 20. 2 μL of each sample were injected. The temperature of the injector was 300 °C, and the injections were made in splitless mode. The detector temperature was 300 °C, and the flow rate was 1.1 mL min^{-1}. The GC–MS analyses were done in the full-scan mode and were carried out on a 6850 network GC system coupled to a 5975 VL MSD with a triple-axis detector operating in electronic impact mode at 70 eV (Agilent Technologies), with an HP-5MS column (30 m × 0.25 mm i.d. × 0.25 mm film thickness) and He as carrier gas. As a rule, the identification of the GC–MS peaks attributed to monosaccharides and related compounds was verified by comparison with the retention times and mass spectra of external standards, purchased from Sigma-Aldrich.

2.5. Profiling of protein and glycan variations in EPS by using antibody microarrays

2.5.1. Production, purification and fluorescent labelling of antibodies. EMChip80 construction

The 80 antibodies used in this work were produced against extracts from cell cultures, some purified proteins and natural samples (Table S1). They can detect specific bioanalytes from bacteria (Table S1, antibodies 9–22, 24–64 and 73), archaea (antibodies 66–73) and eukaryota (antibody 23), and specific proteins related with iron storage (antibodies 75 and 78–80), transporters (antibody 74) or metal reductases (antibodies 76–77). Their purification with protein-A, their fluorescent labeling with Alexa-647 (Molecular Probes) and EMChip80 (Environmental Monitoring Chip) design and construction have been reported previously in Parro et al. (2011) and Blanco et al. (2012).

Briefly, for EMChip80 construction 0.5 mg/mL of protein A-purified antibodies and some of their corresponding pre-immune sera diluted in Protein Printing Buffer 1× (Whatman, Schleicher & Schuell, Sanford, ME) supplemented with 0.02% Tween 20 (v/v) were printed in duplicate on epoxy-activated glass slides (Arrayit Corp., Sunnyvale, CA) using a MicroGrid II TAS arrayer (Biorobotics, Genomic Solutions, UK). Every slide contained 9 microarrays each of them fitting with one of the nine flow cells in a multi array analysis module (MAAM) device (Parro, 2010; Blanco et al., 2016).

2.5.2. Preparation of antigens from capsular fraction for Fluorescent Sandwich Microarray Immunoassay (FSMI)

25 mg of each lyophilized capsular fraction was resuspended in 1 mL of 10 mM Tris-HCl pH 8 and centrifuged at 2000 ×*g* at 4 °C for 15 min to remove debris. Supernatants were diluted with TBSTRR (0.4 M Tris-HCl pH 8, 0.3 M NaCl, 0.1% Tween 20) to obtain a total protein concentration of 4 μg mL^{-1} to 12 μg mL^{-1} to be immunoassayed in 50 μL assay volume.

2.5.3. FSMI procedure and clustering

FSMI were carried out as reported previously in Blanco et al. (2016). Each chip was blocked in 5% (w/v) BSA in 0.5 M Tris-HCl pH 9 and 2% (w/v) BSA in 0.5 M Tris-HCl pH 8 for 5 min and 30 min, respectively. Then, the chip was set up into a MAAM device and 50 μL of the environmental extracts prepared at different concentrations (between 200 and 600 ng of total protein) were inoculated into one of the nine flow cells to flood the EMChip80. After 1 h of incubation and washing with TBSTRR, the immunoassay was re-

vealed by 1 h incubation with a cocktail of the 80 different fluorescent antibodies and then scanned for fluorescence at 635 nm using a GenePix 4100A scanner. The images were analyzed and quantified by Genepix Pro Software (Genomic solutions) as described in Rivas et al. (2011) with some modifications. Two parallel FSMI using only buffer as antigen and the tracer antibody cocktail at 20–30 ng mL^{-1} for each antibody were made as a blank control in all cases. At least three experimental replicates were carried out for each environmental extract concentration. The replicate averages were filtered (see Experimental Procedures in Rivas et al., 2011) and transformed into a binary matrix (1 for values higher than background and 0 for those lower than background) and, then, visualized as a heat map based on unsupervised hierarchical clustering using Cluster 2.11 and Tree View 1.60 programs (Eisen et al., 1998).

2.6. Statistical analysis

Differences in bulk composition (colloidal/capsular fraction weight and DNA, sugar, protein and metal content) between biofilms from different environments was assessed by ANOVA followed by post hoc pairwise *t*-tests corrected for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

In turn, the relationships between environmental parameters (pH, temperature, conductivity, and toxicity index) and biofilm composition were analyzed as follows. Firstly, Principal Component Analysis (PCA) was used to cluster the biofilms according to the environmental parameters at their sampling locations. Toxicity index (TI) was used as a measure of relative heavy metal presence in each sampling station following the equation:

$$T_i = \frac{1}{K}\Sigma\frac{\log\left[Me_i\right]}{\log\left[Me_{min}\right]} \quad (1)$$

where Me_i is the concentration of each metal in sample i, Me_{min} its minimal concentration in all samples, and K stands for the total number of samples. Eight total recoverable metals were used for the calculation (Zn, Cu, Fe, Co, Ni, As, Cd and Cr) (González-Toril et al., 2015). Redundancy Analysis (RDA) was used to summarize the variation in biofilm properties (content in proteins, carbohydrates, DNA and metals, as well as total weight of the colloidal and capsular fractions of the exopolymers) in response to the environmental parameters at their sampling locations. For both the PCA and the RDA the data were log-normalized and standardized following Kenkel (2006). Further, Canonical Correspondence Analysis (CCA) was used to assess the relationships between the monosaccharide composition of the capsular fractions of the biofilms and the environmental parameters. Individual linear models were fitted in order to detect significant correlations between the proportion of individual sugars in the capsular fraction of the biofilms, and the different environmental parameters. The colloidal fraction was not used for these analyses as it was nearly non-existent in several of the biofilms. Finally, Correspondence Analysis was used to cluster the biofilms according to their antigen profile, and CCA to assess the relationship between the antigen profile of the biofilms and the environment. In both RDA and CCA the significance of the models was assessed by ANOVA-like permutation tests. Convex hulls enclosing the samples from each environment were calculated using the *ordihull* function. All statistical analyses were performed with *R* and the *vegan* package (Oksanen et al., 2007).

3. Results

3.1. Sampling locations and water physicochemical analyses

Data concerning pH, temperature and toxicity index of the water column at each location are shown in Table 1. The three sampling sites from Argentina showed a pH between 5.7 and 6.1, with a temperature between 30 and 33 °C and a toxicity index between 1.1 and 1.4. Sample sites from Antarctica were the coldest with a temperature between 3 and 6 °C and they were neutral (pH *ca.* 7). Toxicity index was 0.3 in every case. Samples from Iceland presented pH and toxicity index similar to those from Argentina. Temperature was between 32 and 38 °C, pH between 5.2 and 6.8 and toxicity index between 0.1 and 0.2. Sample IC11 from Iceland was the exception with a pH of 2.4 and a toxicity index above the rest (1.5). All sampling sites from Río Tinto showed an acidic pH (3.2). Temperature of water was between 20 and 23 °C. Toxicity index was the highest (6.2 in every sample site and 3.3 in sample RT21). Finally, the two samples from Peru, presented a pH of 5.1 and 5.4, a temperature of 6 and 10 °C and a toxicity index of 2.9 and 1.1.

3.2. Microbial diversity analysis

Most of the biofilms analyzed were dominated by one or two phototrophic species, easily differentiated by their color and microscopic characteristics (Fig. S1). In these cases, species identification and quantification was carried out by direct microscopic observations and cell counts. Only biofilms IC14 and RT21 were composed by non-photosynthetic prokaryotes, and their species characterization was carried out by cloning and sequencing the 16S RNA gene. In order to evaluate possible changes in the extracellular matrix composition among different biofilms, the samples were selected taking into account their dominant species as well as the physicochemical water characteristics at their sampling sites (Fig. S2). Samples from hot geothermal areas in Argentina were mainly formed by cyanobacteria, being *Mastigocladus*, *Leptolyngbya* and *Chloroflexus* the main genera present in these microbial mats (AR2, AR3 and AR4 respectively), representing more than the 80% of the biomass. Cyanobacteria belonging to the *Phormidium* and *Chloroflexus* genera were also the leading constituents in samples from a cold neutrophilic environment in Antarctica (mats ANT8 and ANT9, respectively), while filamentous chlorophytes from the *Klebsormidium* and *Zygnemopsis* genera were found in the ANT6 and ANT7 mats, reaching amounts higher than 80% of the total biomass. Diatoms from the genera *Nitzschia* were the most abundant species in sample ANT5. *Chloroflexus* and *Phormidium* were also found in mats from Iceland (IC13), although in this case microalgae were also present in three of the biofilms analyzed: *Cyanidium* (IC11), *Chlorella* (IC12) and *Klebsormidium* (IC15). Besides, proteobacteria related to *Geobacillus* was the main component in IC14 sample.

Biofilms from the acidic Río Tinto and Pastoruri areas were mainly formed by eukaryotic protists such as *Euglena mutabilis* (RT23), acidic microalgae *Cyanidium* and *Pinnularia* diatoms (RT24) and filamentous algae from the *Klebsormidium* and *Zygnemopsis* genus (RT22, RT25 and PE4) as well as members of the *Spirogyra* and *Zygogonium* genus (PE2). Finally, biofilm RT21 was also prokaryotic. Bacterial 16S rRNA gene clone libraries showed the presence of species related to *Leptospirillum* and *Acidiphilium*, usually found in extreme acidic environments.

3.3. EPS quantification and chemical characterization

The EPS composition of the different biofilms is shown in Fig. 1 and Table S2. The biofilms studied in this work had variable amounts of total EPS, ranging from a minimum of 49.9 (ANT6) to a maximum of 553.9 (RT25) mg g^{-1} of biofilm dry weight. Samples from Río Tinto showed the highest values of extracted capsular EPS, followed by the biofilms collected in Peru and most of the microbial mats analyzed from Iceland (IC12, IC14 and IC15). Most of the remaining samples from Argentina, Antarctica and Iceland showed total EPS concentrations lower than 200 mg g^{-1} of biofilm dry weight. The colloidal fractions represented less than the 10% of the total weight for most of the biofilms, with the exception of the Río Tinto samples (*ca.* 48%).

Biofilms from different locations had significant ($p < 0.05$) differences in their bulk composition for all parameters except for the capsular EPS weight (Fig. 1). These differences were not related to the phylogeny of the main species in the biofilm (Fig. S2). For example, biofilms formed by filamentous algae (*Zygnemopsis* and *Klebsormidium*) showed higher amounts of total EPS when collected from Río Tinto (*ca.* 450 and 550 mg g^{-1} in RT22 and RT25 biofilms, respectively) than when collected from Peru (*ca.* 270 mg g^{-1} in PE4), Iceland (*ca.* 300 mg g^{-1} in IC15) or Antarctica (*ca.* 50 and 350 mg g^{-1} in ANT6 and ANT7) (Welch's *t*-test, $p = 0.051$). Similar results were obtained in the biofilms mainly formed by *Chloroflexus* (*ca.* 100 mg g^{-1} in AR4, *ca.* 140 mg g^{-1} in ANT9 and *ca.* 85 mg g^{-1} in IC13) and *Cyanidium* (*ca.* 120 mg g^{-1} in IC11 and *ca.* 450 mg g^{-1} in RT24).

The EPS composition of the biofilms studied in this work (carbohydrates, proteins, DNA and metals) is reported in Fig. 1 and Table S2. Carbohydrates accounted for, at least, 60% of all samples analyzed. Carbohydrate concentrations (colloidal + capsular fraction) varies from *ca.* 7 μg mg^{-1} of EPS dry weight in Iceland hot spring IC14 sample, to *ca.* 840 μg mg^{-1} of EPS dry weight in Antarctica ANT8 biofilm. Besides, Antarctica samples showed the highest amounts of carbohydrates followed by Río Tinto and Peru samples, representing >80% of the total EPS composition in these biofilms. Proteins accounted for *ca.* 20% of the total EPS composition in all samples except for those from Río Tinto, in which they reached <5%. Values varied from *ca.* 5 μg mg^{-1} of EPS dry weight in Río Tinto to *ca.* 70 μg mg^{-1} of EPS dry weight in the neutral cold samples from Antarctica.

Heavy metals, together with proteins, were the second main constituents of the EPS in the acidic biofilms from Río Tinto, being significantly higher in that environment (Fig. 1). To a lesser extent, metal content was also high in samples IC15, ANT8, ANT5 and PE2 from Iceland, Antarctica and the Pastoruri glacier (Peru), respectively. Finally, DNA showed the lowest values in the EPS composition. DNA concentrations were significantly higher in the Antarctica than in other environments, albeit their overall concentration was always low (lesser than 1 μg mg^{-1} of EPS dry weight) (Fig. 1).

To further characterize the carbohydrate composition of the EPS samples, abundances of monosaccharide components in the capsular fraction were analyzed and presented as percentages of total monosaccharides (Table S3). Glucose and mannose represented the most abundant neutral sugar in the EPS representing each one *ca.* 21% of the identified monosaccharides. Glucose was especially abundant in IC13 accounting for the 53% while mannose showed the highest percentage in PE2, reaching up to 49% of the total monosaccharide composition.

3.4. Profiling protein and glycan variations in EPS by using antibody microarrays

In a Fluorescent Sandwich Microarray Immunoassay (FSMI), selected antibodies recognize and bind to biomolecules with high specificity, in a similar manner to how the immune system recognizes foreign antigens. Taking into account that proteins and polysaccharides (the primary compounds of the EPS) are good immunogens and DNA and lipids non- and poorly immunogenic (Cruse and Lewis, 2004), protein and glycosidic immunoprofiles in the capsular fractions extracted from the diverse biofilms were estimated by FSMI. While this assay can only detect predefined antigens and thus does not provide a precise taxonomic characterization, it still serves as a quick fingerprinting technique for comparing the microbial composition of different samples. The fluorescent intensity values from those experiments that rendered the maximum number of positive antibody spot signals in a non-saturating fluorescence signal conditions (values < 60,000 units) were transformed into a binary matrix and visualized as a heatmap (Fig. 2).

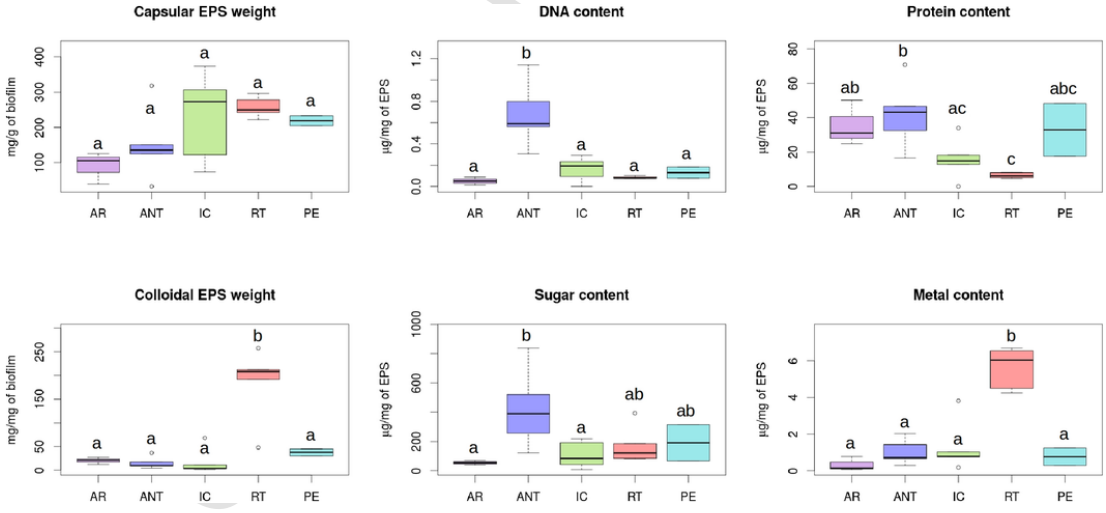

Fig. 1. Biofilm compositions at five different extreme environments. Boxplots representing the distribution of total colloidal and capsular EPS weights, and DNA, protein, sugar and metal contents of biofilms from the five different extreme environments (AR: Argentina, ANT: Antarctica, IC: Iceland, RT: Río Tinto and PE: Peru). Letters represent statistical differences among biofilms from the five different environments, that is, environments sharing the same letter are not significantly different based on pairwise *t*-tests (fdr > 0.05).

The antigens or targets for the 80 antibodies used in this work are described in Table S1. EMChip80 revealed the presence of biomolecules recognized by antibodies produced against Alpha-, Beta-, Gamma- and Deltaproteobacteria, Acidobacteria, Actinobacteria, Firmicutes, Nitrospirae, bacteria from the phylum Deinococcus-Thermus, Bacteroidetes, Aquificales, Planctomycetes, Thermotogales and Verrucomicrobiales, as well as against halophilic, methanogenic and thermophilic archaea and the only eukaryotic microorganism included in the chip (*Rhodotorula* sp.). Also, positive signals were detected in antibodies to proteins related to iron storage, metal reductases and transporters and against crude environmental extracts from the Río Tinto area, which might contain biomarkers from iron and sulfur oxidizers (*e.g. Leptospirillum ferrooxidans*, *Acidithiobacillus* sp. and others (Amils et al., 2002)) (Fig. 2). Signals against cyanobacteria were not detected in EPS from biofilms from locations with an ultra-acidic pH (pH < 3.5) (such as IC11 and those from Río Tinto). Astonishingly, no positive signals were revealed in spots from psychrophiles when EPS from biofilms collected from the coldest zones of Antarctica (ANT5, ANT6 and ANT7) were immunoassayed.

Biofilms were separated into three major clusters according to their immunoprofiles (Fig. 2), which concur with EPS from two biofilms from Argentina (AR2 and AR3) (cluster I); from Iceland, together with AR4 and the samples with higher temperatures within Antarctica (ANT8 and ANT9) (cluster II); and from Río Tinto, Peru and the samples with lower temperatures within Antarctica (ANT5, ANT6 and ANT7) (cluster III). The unsupervised hierarchical clustering grouped the EPS based on the place where they were collected, although two pairs of biofilms with the same phylogenetic composition (AR4-ANT9 and IC13-ANT8) (within the cluster II) (Fig. S2) were clustered out of the rest of biofilms collected in the same environment. However, other biofilms sharing the phylogenetic composition, such as AR4 and ANT9 (mainly composed of *Chloroflexus* spp.), or IC11 and RT24 (*Phormidium* spp. biofilms), or IC15, ANT6, RT 22 and PE4 (*Klebsormidium* spp. biofilms), were grouped together with other biofilms from the same environments.

3.5. Relating biofilm properties to environmental factors through multivariate analysis

Constrained and unconstrained ordinations were used to group biofilms according to their EPS properties, and relate those properties to environmental factors. Biofilms obtained from the same environment clustered together according to the environmental parameters present at their sampling point (Fig. 3a). The toxicity index was negatively correlated with the pH, depicting the increased solubility of heavy metals under acidic conditions. The environmental parameters also determined the bulk composition of the biofilms (Fig. 3b, RDA, $p = 0.004$). In particular, biofilms from low temperature sampling points had significantly larger amounts of carbohydrates ($R^2 = 0.26$, $p = 0.012$) and DNA ($R^2 = 0.36$, $p = 0.003$). On the other hand, biofilms from sampling points with a high toxicity index had a significantly larger amount of colloidal exopolysaccharides ($R^2 = 0.84$, $p < 0.001$) and heavy metals ($R^2 = 0.71$, $p < 0.001$).

The environmental parameters also drove the proportions of monosaccharides in the biofilms (Fig. 4a, CCA, $p = 0.009$). The relative abundance of individual monosaccharides was significantly correlated with different environmental parameters (Fig. 4b). In particular, a higher toxicity index resulted in higher inositol and 3-*O*-methylglucose abundances and lower abundances of an unidentified aldohexose. On the other hand, the relative abundance of galactose in the EPS was negatively correlated with temperature.

Finally, the antigen profile of the different biofilms showed a less marked clustering by the source environment than the bulk biofilm and exopolysaccharide compositions (Fig. 5a), albeit it was still found to be significantly dependent on the environmental conditions (Fig. 5b, CCA, $p = 0.004$).

4. Discussion

4.1. EPS composition in microbial mats from extreme environments

Extracellular polymeric substances (EPS) are critical in establishing the physical and functional properties of a biofilm, and are in-

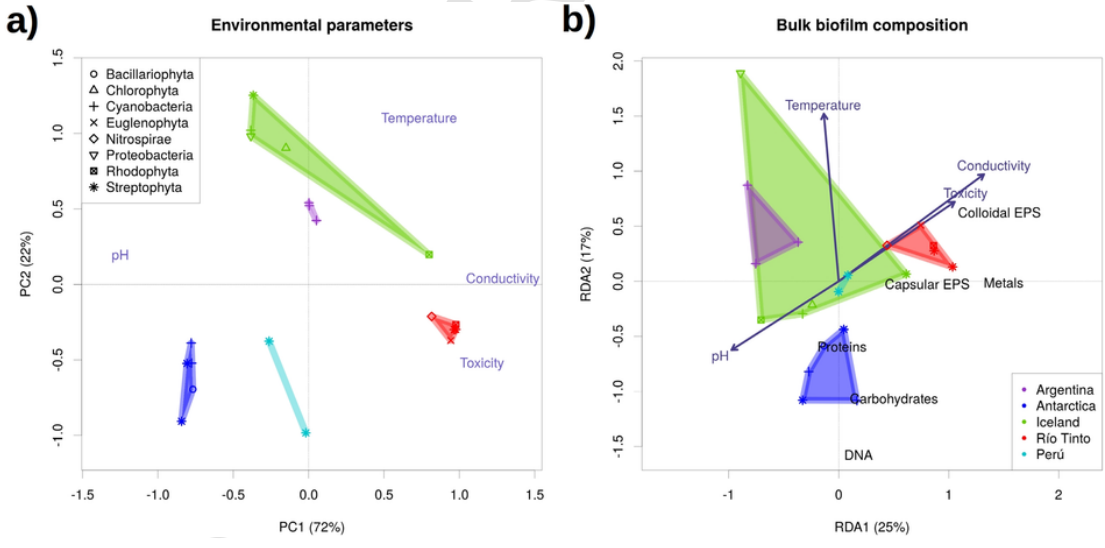

Fig. 3. Environmental effect on bulk EPS composition. (a) Principal component analysis of biofilms according to the environmental parameters at their respective sampling points, (b) redundancy analysis showing the relationship between the bulk environmental parameters and biofilm composition. For each environment, the convex hull enclosing all its samples is shown as a colored area. Species scores are scaled by eigenvalues. Symbol: most abundant phylum in the biofilm. Symbol color: Isolation source. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

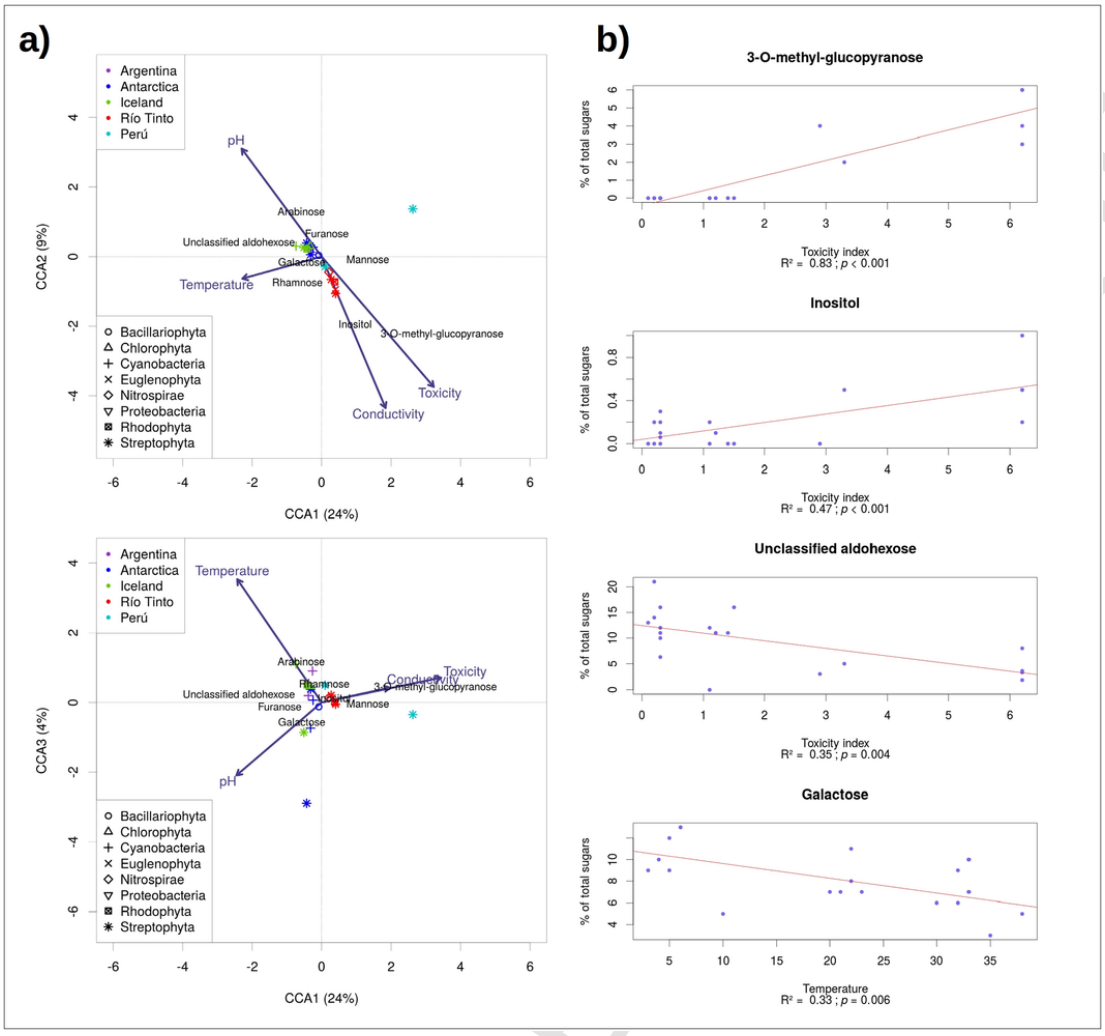

Fig. 4. Environmental effect on EPS sugar composition (a) Constrained correspondence analysis showing the relationship between environmental parameters and the sugar composition of the capsular fraction of different environmental biofilms. Only sugars with a significant ($p<0.05$) correlation with at least one environmental parameter are shown in the figure. Site and species scores are scaled symmetrically by the square root of eigenvalues. Symbol: most abundant phylum in the biofilm. Symbol color: Isolation source, (b) linear correlation between the percentage of different sugars in biofilms and the toxicity index (first three plots) or the temperature (bottom-right plot). pH and conductivity were omitted as they were highly correlated with the toxicity index. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

volved in the ecological adaptation of microbial communities to their host environments. This is particularly relevant for extremophilic organisms, which face several challenges such as desiccation, osmotic stress, temperature and pH extremes, and low nutrient availability. While several studies on extremophilic EPS composition have been performed to date (Ewert and Deming, 2013; Zhao et al., 2014; Chrismas et al., 2016), to the best of our knowledge we are the first to compare the EPS of several biofilms taken *in situ* from different extreme environments and using the same methodology. In addition, since most of these previous studies have been focused exclusively on prokaryotes (Nicolaus et al., 2010; Casillo et al., 2018), references related to EPS production and composition of eukaryotic extremophiles are even more scarce.

Permutation tests ran on constrained ordinations confirmed that the properties of the biofilm (bulk composition, sugar composition, antigen profile) depended on the environmental conditions (see Section 3.5), mainly the combination of low pH and presence of heavy metals, but to a lesser extent also temperature (Figs. 3–5). EPS composition of different biofilms taken from the same environment had similar properties, regardless of the dominant species, suggesting that EPS composition plays a critical role in the adaptation to extreme conditions, and is thus subjected to strong selection in natural communities. This idea is also supported by the fact that biofilms isolated from different environments sharing similar species composition showed very different EPS characteristics. Thus, *Klebsormidium and Zygnemopsis* biofilms from Río Tinto (RT22 and RT25) showed between c.a. 1.5 and 10 times higher amounts of total EPS than the same mats from cold environments (ANT6, ANT7, PE4) and geothermal areas (IC15) (Welch's *t*-test, $p=0.051$). In the same manner, *Chloroflexus* mats from cold environments (ANT9) showed c.a. seven times higher amounts of carbohydrates than the same mats from geothermal areas (AR4) (Tables S2, S3).

Biofilms from the extremely acidic Río Tinto environment had a significantly higher overall EPS weight and metal content, while also having a significantly lower protein content than biofilms from other environments (Fig. 1). This is consistent with previous observations of high metal and low protein contents in both eukaryotic and prokaryotic biofilms from acid mine drainages (AMD), in which EPS serve not only to protect against heavy metal toxicity, but also to cap-

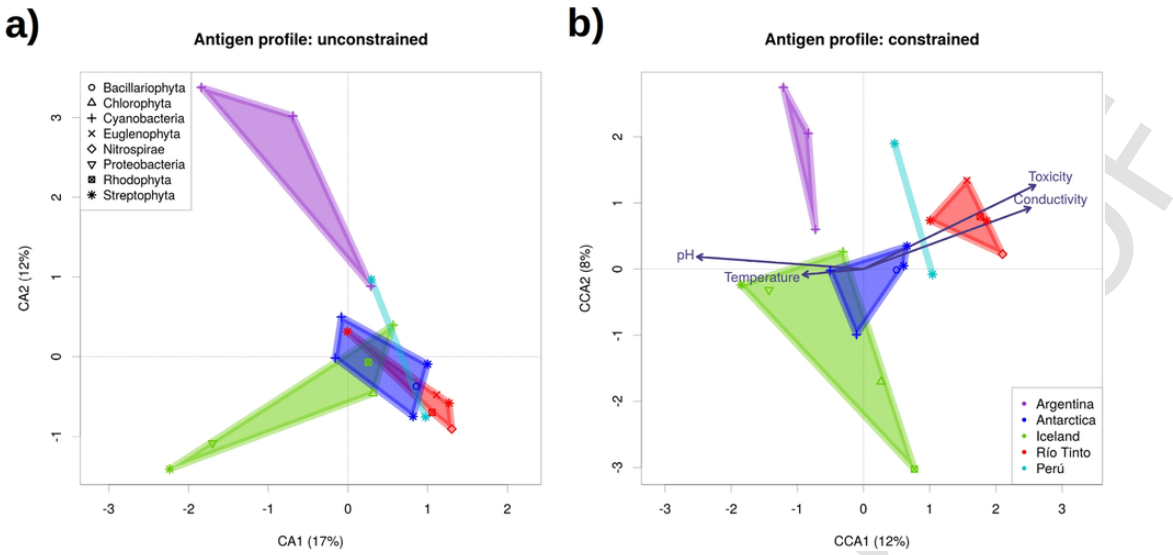

Fig. 5. Environmental effect on EPS immunogenic profile using an antibody microarray. (a) Correspondence analysis showing the clustering of biofilms according to their antigenic profile, (b) Canonical correspondence analysis showing the relationship between the antigenic profile of the biofilms and the environmental parameters present at their respective sampling points. For each environment, the convex hull enclosing all its samples is shown as a colored area. Species scores are scaled by eigenvalues. Symbol: most abundant phylum in the biofilm. Symbol color: Isolation source. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ture and enrich trace elements (Aguilera et al., 2008a, 2008b; Jiao et al., 2010).

The contributions of inositol and 3-*O*-methylglucose to the total carbohydrate fraction of the EPS were significantly correlated with the toxicity index ($p<0.001$; Fig. 4b). This suggests that the protective effect of biofilms in extremely acidic environments is, at least in part, mediated by specific sugars. In particular, inositol polyphosphates have been shown to inhibit the formation of hydroxyl radicals by ferric iron (Hawkins et al., 1993), which would decrease its toxicity. On the other hand, methylglucose-containing polysaccharides from marine bacteria have been exploited toward the removal of heavy metals from solutions (Shah et al., 2000; Bramhachari and Nagaraju, 2017). Our results suggest that they might also be involved in the adaptation to extremely acidic environments, although further work is needed in order to elucidate their specific mechanism of action.

Biofilms from the Antarctica, on the other hand, had significantly higher DNA and sugar contents (Figs. 1; 3b). A recent report by Liao et al. (2016) has highlighted the quantitative and qualitative importance of extracellular DNA in biofilm formation in antarctic archaea. Sugars, on the other hand, have been shown to act as cryoprotectants in psychrophilic biofilms (Nichols et al., 2005; Caruso et al., 2018). This agrees well with our results, and reinforces our hypothesis that biofilm composition is dictated by the environment rather than by phylogeny. We found a significant ($p<0.006$) negative correlation between temperature and galactose content in the biofilms analyzed in this work (Fig. 4b). Several cold-active β-galactosidases have been retrieved from psychrophilic bacteria, pointing to a role of galactose-containing carbohydrates in biofilms from cold environments (Schmidt and Stougaard, 2010). However, a study characterizing ten Antarctic marine bacterial strains revealed variable galactose concentrations in their EPS (Nichols et al., 2005). Further work, preferably characterizing biofilms collected *in situ* as opposed to cultured strains, would require to determine whether the cryoprotectant effect of EPS from psychrophilic organisms can be traced back to the presence of particular sugars.

The antigen profile of the biofilms was also significantly dependent on the environmental parameters (Figs. 2, 5), although the clustering by environments was less marked than for the bulk and sugar compositions (Figs. 3, 4). This is not surprising, as the antibody microarray used in this work included not only antibodies against EPS components, but also against other cellular components such as membrane transporters or energy transduction proteins. Those additional components might be dependent on the phylogeny or on the main metabolism of the species in the biofilm thus resulting in lower correlations of the antigen profile with the environmental parameters.

4.2. Optimization of biofilm properties as a hallmark for environmental adaptation

In this study we have focused on acidic, cold, and thermal habitats, but similar patterns might be inferred for other environments from the available literature. For example, uronic acids are abundant in the EPS from different halophilic organisms, such as *Halomonas* (Mata et al., 2006), *Alteromonas* (Zhang et al., 2017) or the photosynthetic *Cyanospira* (Cesàro et al., 1990). On the other hand, EPS from hyperthermophilic organisms appear to be structurally complex and of high molecular weight (Nicolaus et al., 2002; Kambourova et al., 2009; Zhao et al., 2014; Panosyan et al., 2018), which could increase biofilm stability at very high temperatures (Kambourova et al., 2009). Overall, our and others' results indicate that biofilm composition in extreme environments is strongly influenced by environmental parameters. Thus, the acquisition of optimal biofilm properties might be necessary for the colonization of novel environments.

4.3. Horizontal gene transfer of EPS biosynthesis genes might enable rapid adaptation to novel environments

The fact that similar EPS properties are achieved by phylogenetically-distinct organisms living in the same environment suggests the action of either convergent evolution over long time scales or horizontal gene transfer (HGT). Several studies have indeed reported the occurrence of HGT in EPS biosynthesis gene clusters, not only between closely related bacteria (Forde and Fitzgerald, 2003; Cefalo et al., 2011), but also between different bacterial groups (Patil and Sonti, 2004; Hidalgo-Cantabrana et al., 2014), and even from bacteria

to eukaryotes (Whitaker et al., 2009). The lateral acquisition of environment-specific sets of EPS production genes might thus be a critical step in microbial adaptation to novel environments. We propose that the heterologous expression of EPS biosynthesis clusters might be a promising strategy for engineering environmental adaptations in microorganisms.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.08.440.

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References

Achenbach, L., Woese, C.R., 1995. 16S and 23S rRNA-like primers. In: Sower, K.R., Schreier, H.J. (Eds.), Archaea. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 521–523.

Aguilera, A., 2013. Eukaryotic organisms in extreme acidic environments, the Río Tinto Case. Life 3, https://doi.org/10.3390/life30x000x, (1-x manuscripts).

Aguilera, A., Zettler, E., Gómez, F., Amaral-Zettler, L., Rodríguez, N., Amils, R., 2007. Distribution and seasonal variability in the benthic eukaryotic community of Río Tinto (SW Spain), and acidic, high metal extreme environment. Syst. Appl. Microbiol. 30, 531–546.

Aguilera, A., Souza-Egipsy, V., San Martín-Úriz, P., Amils, R., 2008. Extracellular matrix assembly in extreme acidic eukaryotic biofilms and their possible implications in heavy metal adsorption. Aquat. Toxicol. 88, 257–266.

Aguilera, A., Souza-Egipsy, V., San Martín-Úriz, P., Amils, R., 2008. Extraction of extracellular polymeric substances from extreme acidic microbial biofilms. Appl. Microbiol. Biotechnol. 78, 1079–1088.

Aguilera, A., Souza-Egipsy, V., González-Toril, E., Rendueles, O., Amils, R., 2010. Eukaryotic microbial diversity of phototrophic microbial mats in two Icelandic geothermal hot springs. Int. Microbiol. 13, 29–40.

Altschul, S.F., et al., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Amils, R., González-Toril, E., Fernández-Remolar, D., Gómez, F., Rodríguez, N., Durán, C., 2002. Interaction of the sulfur and iron cycles in the Tinto River ecosystem. Rev. Environ. Sci. Biotechnol. 1 (4), 299–309.

I.B., J.. Curr. Opin. Biotechnol.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57, 289–300.

Blanco, Y., Legaz, M.E., Vicente, C., 2010. Gluconacetobacter diazotrophicus, a sugarcane endophyte, inhibits xanthan production by sugarcane-invading Xanthomonas albilineans. J. Plant Interact. 5, 241–248.

Blanco, Y., Prieto-Ballesteros, O., Gómez, M.J., Moreno-Paz, M., García-Villadangos, M., Rodríguez-Manfredi, J.A., Cruz-Gil, P., Sánchez-Román, M., Rivas, L.A., Parro, V., 2012. Prokaryotic communities and operating metabolisms in the surface and the permafrost of Deception Island (Antarctica). Environ. Microbiol. 14, 2495–2510.

Blanco, Y., Moreno-Paz, M., Aguirre, J., Parro, V., 2016. Multiplex fluorescent antibody microarrays and antibody graphs for microbial and biomarker detection in the environment. In: McGenity, T.J., Timmis, K.N., Nogales, B. (Eds.), Hydrocarbon and Lipid Microbiology Protocols. Springer-Verlag, Berlin, Heidelberg.

Bolhuis, H., Cretoiu, M.S., Stal, L.J., 2014. Molecular ecology of microbial mats. FEMS Microbiol. Ecol. 90, 335–350.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bramhachari, P.V., Nagaraju, G.P., 2017. Extracellular polysaccharide production by bacteria as a mechanism of toxic heavy metal biosorption and biosequestration in the marine environment. In: Marine Pollution and Microbial Remediation. Springer, Singapore, pp. 67–85.

Caruso, C., Rizzo, C., Mangano, S., Poli, A., Di Donato, P., Finore, I., Giudice, A.L., 2018. Production and biotechnological potential of extracellular polymeric substances from sponge-associated Antarctic bacteria. Appl. Environ. Microbiol. 84, e01624-17.

Casillo, A., Lanzetta, R., Parrilli, M., Corsaro, M.M., 2018. Exopolysaccharides from marine and marine extremophilic bacteria: structures, properties, ecological roles and applications. Mar. Drugs 16, 238–245.

Cefalo, A.D., Broadbent, J.R., Welker, D.L., 2011. Intraspecific and interspecific interactions among proteins regulating exopolysaccharide synthesis in Streptococcus thermophilus, Streptococcus iniae, and Lactococcus lactis subsp. cremoris and the assessment of potential lateral gene transfer. Can. J. Microbiol. 57, 1002–1015.

Cesàro, A., Liut, G., Bertocchi, C., Navarini, L., Urbani, R., 1990. Physicochemical properties of the exocellular polysaccharide from Cyanospira capsulata. Int. J. Biol. Macromol. 12 (2), 79–84.

Chrismas, N.A., Barker, G., Anesio, A.M., Sánchez-Baracaldo, P., 2016. Genomic mechanisms for cold tolerance and production of exopolysaccharides in the Arctic cyanobacterium Phormidesmis priestleyi BC1401. BMC Genomics 17, 533.

Cruse, J.M., Lewis, R.E., 2004. Antigens and Immunogens in Atlas of Immunology, 2ª ed. CRC Press, Boca Raton, Florida, USA, 105–126.

DeLong, E.F., 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. U. S. A. 89, 5685–5689.

M., D.B.. Environ. Microbiol.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U. S. A. 95, 14863–14868.

Ewert, M., Deming, J.W., 2013. Sea ice microorganisms: environmental constraints and extracellular responses. Biology (Basel) 2, 603–628.

Forde, A., Fitzgerald, G.F., 2003. Molecular organization of exopolysaccharide (EPS) encoding genes on the lactococcal bacteriophage adsorption blocking plasmid, pCI658. Plasmid 49, 130–142.

J.V.C., W..

García-Moyano, A., González-Toril, E., Aguilera, , Amils, R., 2012. Comparative microbial ecology study of the sediments and the water column of the Río Tinto, an extreme acidic environment. FEMS Microbiol. Ecol. 81, 303–314.

Goltsman, D.S.A., Comolli, L.R., Thomas, B.C., Banfield, J.F., 2015. Community transcriptomics reveals unexpected high microbial diversity in acidophilic biofilm communities. ISME J. 9 (4), 1014.

, .

González-Toril, E., Santofimia, E., Blanco, Y., López-Pamo, E., Gómez, M.J., Bobadilla, M., Cruz, R., Palomino, E.J., Aguilera, A., 2015. Pyrosequencing-based assessment of the microbial community structure of Pastoruri Glacier area (Huascarán National Park, Perú), a natural extreme acidic environment. Microb. Ecol. 70, 936–947.

Gunn, J.S., Bakaletz, L.O., Wozniak, D.J., 2016. What's on the outside matters: the role of the extracellular polymeric substance of Gram-negative biofilms in evading host immunity and as a target for therapeutic intervention. J. Biol. Chem. 291 (24), 12538–12546.

, .

Hall-Stoodley, L., Nistico, L., Sambanthamoorthy, K., Dice, B., Nguyen, D., Mershon, W.J., … Post, J.C., 2008. Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in Streptococcus pneumoniae clinical isolates. BMC Microbiol. 8 (1), 173.

Hawkins, P.T., Poyner, D.R., Jackson, T.R., Letcher, A.J., Lander, D.A., Irvine, R.F., 1993. Inhibition of iron-catalysed hydroxyl radical formation by inositol polyphosphates: a possible physiological function for myo-inositol hexakisphosphate. Biochem. J. 294, 929.

S., D.B..

Hidalgo-Cantabrana, C., Sánchez, B., Milani, C., Ventura, M., Margolles, A., Ruas-Madiedo, P., 2014. Genomic overview and biological functions of exopolysaccharide biosynthesis in Bifidobacterium spp. Appl. Environ. Microbiol. 80, 9–18.

Hill, D.R., Keenan, T.W., Helm, R.F., Potts, M., Crowe, L.M., Crowe, J.H., 1997. Extracellular polysaccharide of Nostoc commune (Cyanobacteria) inhibits fusion of membrane vesicles during desiccation. J. Appl. Phycol. 9, 237–248.

Hirst, C.N., Jordan, I.A., 2003. Distribution of exopolymeric substances in the littoral sediments of an oligotrophic lake. Microb. Ecol. 46, 22–32.

Jiao, Y., Cody, G.D., Harding, A.K., Wilmes, P., Schrenk, M., Wheeler, K.E., Banfield, J.F., Thelen, M.P., 2010. Characterization of extracellular polymeric substances from acidophilic microbial mats. Appl. Environ. Microbiol. 76, 2916–2922.

.

. In: , pp.

Kambourova, M., Mandeva, R., Dimova, D., Poli, A., Nicolaus, B., Tommonaro, G., 2009. Production and characterization of a microbial glucan, synthesized by Geobacillus tepidamans V264 isolated from Bulgarian hot spring. Carbohydr. Polym. 77 (2), 338–343.

Kenkel, N.C., 2006. On selecting an appropriate multivariate analysis. Can. J. Plant Sci. 86, 663–676.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley, Chichester, pp. 115–176.
W.W., H.Q.. Bioresour. Technol.
Liao, Y., Williams, T.J., Ye, J., Charlesworth, J., Burns, B.P., Poljak, A., Cavicchioli, R., 2016. Morphological and proteomic analysis of biofilms from the Antarctic archaeon, Halorubrum lacusprofundi. Sci. Rep. 6, 37454.
, .
, .
W.O.R.. Nucleic Acids Res.
Mata, J.A., Béjar, V., Llamas, I., Arias, S., Bressollier, P., Tallon, R., … Quesada, E., 2006. Exopolysaccharides produced by the recently described halophilic bacteria Halomonas ventosae and Halomonas anticariensis. Res. Microbiol. 157 (9), 827–835.
Miqueleto, A.P., Dolosic, C.C., Pozzi, E., Foresti, E., Zaiat, M., 2010. Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment. Bioresour. Technol. 101 (4), 1324–1330.
Nichols, C.M., Lardière, S.G., Bowman, J.P., Nichols, P.D., Gibson, J.A., Guézennec, J., 2005. Chemical characterization of exopolysaccharides from Antarctic marine bacteria. Microb. Ecol. 49, 578–589.
Nicolaus, B., Lama, L., Panico, A., Moriello, V.S., Romano, I., Gambacorta, A., 2002. Production and characterization of exopolysaccharides excreted by thermophilic bacteria from shallow, marine hydrothermal vents of Flegrean Ares (Italy). Syst. Appl. Microbiol. 25 (3), 319–325.
Nicolaus, B., Kambourova, M., Oner, E.T., 2010. Exopolysaccharides from extremophiles: from fundamentals to biotechnology. Environ. Technol. 31, 1145–1158.
D.K., C.N.. Proc. Natl. Acad. Sci. U. S. A.
, . moIn: , , pp.
J., N.. Biotechnol. Adv.
Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J., Suggests, M.A.S.S., 2007. The Vegan Package, (Community ecology package).
Ortega-Morales, B.O., Santiago-García, J.L., Chan-Bacab, M.J., Moppert, X., Miranda-Tello, E., Fardeau, M.L., … Guezennec, J., 2007. Characterization of extracellular polymers synthesized by tropical intertidal biofilm bacteria. J. Appl. Microbiol. 102 (1), 254–264.
Panosyan, H., Di Donato, P., Poli, A., Nicolaus, B., 2018. Production and characterization of exopolysaccharides by Geobacillus thermodenitrificans ArzA-6 and Geobacillus toebii ArzA-8 strains isolated from an Armenian geothermal spring. Extremophiles 1–13.
Parro, V., 2010. Antibody microarrays for environmental monitoring. In: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer-Verlag, Berlin, Germany, pp. 2699–2710.
Parro, V., de Diego-Castilla, G., Rodríguez-Manfredi, J.A., Rivas, L.A., Blanco-López, Y., Sebastián, E., Romeral, J., Compostizo, C., Herrero, P.L., García-Marín, A., Moreno-Paz, M., García-Villadangos, M., Cruz-Gil, P., Peinado, V., Martín-Soler, J., Pérez-Mercader, J., Gómez-Elvira, J., 2011. SOLID3, a multiplex antibody microarray-based optical sensor instrument for in situ life detection in planetary exploration. Astrobiology 11, 15–28.
Patil, P.B., Sonti, R.V., 2004. Variation suggestive of horizontal gene transfer at a lipopolysaccharide (lps) biosynthetic locus in Xanthomonas oryzae pv. oryzae, the bacterial leaf blight pathogen of rice. BMC Microbiol. 4, 40.
Quesada, A., Camacho, A., Rochera, C., Velázquez, D., 2009. Byers Peninsula: a reference site for coastal, terrestrial and limnetic ecosystem studies in maritime Antarctica. Polar Sci. 23, 181–187.
Ras, M., Lefebvre, D., Derlon, N., Paul, E., Girbal-Neuhauser, E., 2011. Extracellular polymeric substances diversity on biofilms grown under contrasted environmental conditions. Water Res. 45, 1529–1538.
Read, S.T., Dutta, P., Bond, P.L., Keller, J., Rabaey, K., 2010. Initial development and structure of biofilms on microbial fuel cell anodes. BMC Microbiol. 10 (1), 98.
Ren, D., Madsen, J.S., Sørensen, S.J., Burmølle, M., 2015. High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. ISME J. 9 (1), 81.
Rippka, R., Deruelles, J., Waterbury, J., Herdman, M., Stanier, R., 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111, 1–61.
Rivas, L.A., Aguirre, J., Blanco, Y., Gonzalez-Toril, E., Parro, V., 2011. Graph-based deconvolution analysis of multiplex sandwich microarray immunoassays: applications for environmental monitoring. Environ. Microbiol. 13, 1421–1432.
Rothschild, L.J., Mancinelli, R.L., 2001. Life at extreme environments. Nature 409, 1092–1101.
Salama, Y., Chennaoui, M., Sylla, A., Mountadar, M., Rihani, M., Assobhei, O., 2016. Characterization, structure, and function of extracellular polymeric substances (EPS) of microbial biofilm in biological wastewater treatment systems: a review. Desalin. Water Treat. 57 (35), 16220–16237.
Santofimia, E., González-Toril, E., López-Pamo, E., Gomariz, M., Amils, R., Aguilera, A., 2013. Microbial diversity and its relationship to water physicochemical characteristics in two extreme acidic pit lakes from the Iberian Pyrite Belt (SW Spain). PLoS One 8, e66746.
Schmidt, M., Stougaard, P., 2010. Identification, cloning and expression of a cold-active β-galactosidase from a novel Arctic bacterium, Alkalilactibacillus ikkense. Environ. Technol. 31, 1107–1114.
Shah, V., Ray, A., Garg, N., Madamwar, D., 2000. Characterization of the extracellular polysaccharide produced by a marine cyanobacterium, Cyanothece sp. ATCC 51142, and its exploitation toward metal removal from solutions. Curr. Microbiol. 40, 274–278.
Sheng, G.P., Yu, H.Q., Li, X.Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological waste water treatment systems: a review. Biotechnol. Adv. 28, 882–894.
Souza-Egipsy, V., González-Toril, E., Zettler, E., Amaral-Zettler, L., Aguilera, A., Amils, R., 2008. Prokaryotic community structure in algal photosynthetic biofilms from extreme acidic streams in Río Tinto (southwestern Spain). Int. Microbiol. 11, 251–260.
Staats, N., Winder, B.D., Stal, L.J., Mur, L.R., 1999. Isolation and characterization of extracellular polysaccharides from the epipelic diatoms Cylindrotheca closterium and Navicula salinarum. Eur. J. Phycol. 34, 161–169.
Sutherland, I.W., 2001. The biofilm matrix - an immobilized but dynamic microbial environment. Trends Microbiol. 9, 222–227.
. Bioresour. Technol.
Urbieta, M.S., González-Toril, E., Aguilera, A., Giaveno, M.A., Donati, E.R., 2014. Archaeal and bacterial diversity in five different hydrothermal ponds in the Copahue region in Argentina. Syst. Appl. Microbiol. 37, 429–436.
Urbieta, M.S., Gonzalez-Toril, E., Aguilera, M.A., Giaveno, M.A., Donati, E.R., 2015. Comparison of the microbial communities of hot springs waters and the microbial biofilms in the acidic geothermal area of Copahue (Neuquen, Argentina). Extremophiles 19, 437–450.
Whitaker, J.W., McConkey, G.A., Westhead, D.R., 2009. The transferome of metabolic genes explored: analysis of the horizontal transfer of enzyme encoding genes in unicellular eukaryotes. Genome Biol. 10, R36.
Wolf, G., Picioreanu, C., van Loosdrecht, M.C.M., 2007. Kinetic modeling of phototrophic biofilms: the PHOBIA model. Biotechnol. Bioeng. 97, 1064–1079.
Zhao, S., Cao, F., Zhang, H., Zhang, L., Zhang, F., Liang, X., 2014. Structural characterization and biosorption of exopolysaccharides from Anoxybacillus sp. R4-33 isolated from radioactive radon hot spring. Appl. Biochem. Biotechnol. 172, 2732–2746.