Science of the Total Environment 829 (2022) 154472

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Short Communication

Sulfated glycosaminoglycan-like polymers are present in an acidophilic biofilm from a sulfidic cave

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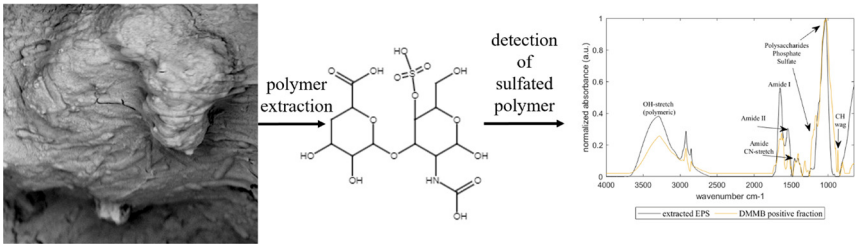
HIGHLIGHTS

- Biofilm from Sulfur Cave is a unique environment to study sulfated polymers.
- Sulfated glycosaminoglycan-like polymers were present in Sulfur Cave biofilm.
- Sulfated polymers might be produced internally by biofilm inhabitants.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 December 2021
Received in revised form 21 February 2022
Accepted 7 March 2022
Available online 8 March 2022

Editor: Huu Hao Ngo

Keywords:
EPS
Extremophiles
Sulfur Cave
Romania

ABSTRACT

Sulfated glycosaminoglycans (sGAG) are negatively charged extracellular polymeric substances that occur in biofilms from various environments. Yet, it remains unclear whether these polymers are acquired from the external environment or produced by microbes in the biofilm. To resolve this, we analyzed the presence of sGAGs in samples of an acidophilic biofilm collected from Sulfur Cave in Puturosu Mountain (Romania), an environment that is largely inaccessible to contamination. A maximum of 55.16 ± 2.06 μg sGAG-like polymers were recovered per mg of EPS. Enzymatic treatment with chondroitinase ABC resulted in a decrease of the mass of these polymers, suggesting the structure of the recovered sGAG is similar to chondroitin. Subsequent FT-IR analysis of these polymers revealed absorbance bands at 1230 cm^{-1}, 1167 cm^{-1} and 900 cm^{-1}, indicating a possible presence of polysaccharides and sulfate. Analysis of genomic sequences closely related to those predominant in the acidophilic biofilm, contained genes coding for sulfotransferase (an enzyme needed for the production of sGAG), which supports the hypothesis of microbial synthesis of sGAGs within the biofilm.

1. Introduction

Biofilms are aggregations of microorganisms that are immobilized in a self-produced matrix composed of extracellular polymeric substances (EPS). EPS provide mechanical stability and scaffolds for microbial growth and protect microbes in the biofilm from e.g. desiccation, biocides, heavy metals, ultraviolet radiation, protozoan grazers (Flemming and Wingender, 2010). Negatively charged polymers, such as sulfated glycosaminoglycans (sGAG), are particularly important for increased water retention, due to their ability to form hydrogels by cross-linking with cations (Zykwinska et al., 2019). These glycans are large polysaccharide chains built from disaccharide blocks of an amino sugar and either uronic acid or hexose sugar. Sulfation of hydroxyl groups in the disaccharide building block makes the sGAGs amongst the most negatively charged polymers in nature (Köwitsch et al., 2018). The high negative charge make sGAGs have diverse bioactivities in multicellular organisms, and sGAGs like heparin are used pharmaceutically as an anticoagulant. The chemical production of sGAGs is

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http://dx.doi.org/10.1016/j.scitotenv.2022.154472

difficult and time consuming and therefore sGAGs are mainly sourced from animal tissue (Soares da Costa et al., 2017).

In the past, microorganisms were thought to be incapable of producing sGAG-like polymers (Schiraldi et al., 2010). Yet, recent studies suggest that sGAG-like polymers are present in a wide variety of microbial biofilms (Boleij et al., 2020; Bourven et al., 2015; Felz et al., 2020; van Vliet et al., 2020; Xue et al., 2019). This suggests that the production of sulfated polymers could occur within the biofilm, and is potentially linked with important functions in the biofilm. However, previous studies targeted biofilms in natural environments or microcosms where organic substrates containing sulfated polysaccharides were present and/or supplied. Thus, the question arises as to the metabolic potential of prokaryotic cells to synthesize sulfated polymers.

Our research aims to investigate if sGAG-like polymers can be microbially produced. For this we studied a microbial biofilm growing in the Puturosu Sulfur Cave (Romania), an extreme environment where the possibility of obtaining sGAG-like polymers from the environment is very unlikely. In the Puturosu Sulfur Cave, biofilms grow on the cave walls at the level of the interface between volcanic gas (CO_2, CH_4, and H_2S) and overlying atmospheric air. Stable isotope measurements showed that the organic carbon is autotrophic in nature (Sarbu et al., 2018). Deposits of elemental sulfur are present on the walls below this gas-gas interface. The measured pH on the cave wall range between 0.5 and 1, indicating possible sulfide oxidation to sulfate. No direct water source was found in the cave, implying that water is only available through condensation inside the cave (Sarbu et al., 2018). This avoids external input of organic sulfated polymers from the environment to the biofilm. If sGAG-like polymers are detected in this biofilm, it would support the hypothesis that sulfated polymers are produced in situ by the microorganisms. This hypothesis was tested by measuring the presence of sGAG in the EPS of biofilm growing at the gas-gas interface. EPS was extracted from the biofilm and highly negatively charged polymers were isolated and detected using a specific cationic dye for sGAG. Further validation of sGAG presence was performed with enzymatic treatment with chondroitinase ABC and FT-IR measurements of the biofilm, EPS, pellet and dye precipitated fraction. Lastly, the possibility of the microorganisms in the biofilm to produce sulfated polymers was explored by performing genome database searches for sulfotransferase, which is an important enzyme needed for sGAG-like polymer production. The detection of sGAG-like polymers in this extreme environment provides support that sulfated polymers can be produced exclusively by microorganisms and could be an important step in understanding the role of specific EPS compounds in the biofilm.

2. Materials and methods

2.1. Biofilm sampling

Biofilm was sampled from Sulfur Cave located on Puturosu Mountain ("Stinky Mountain" in English translation), located within the Ciomadul volcanic edifice area in the East Carpathian Mountains of Romania. Sulfur Cave provides a unique extreme environment, as it displays microbial life within a gas chemocline (Fig. 1a). The cave penetrates approximately 14 m into the volcanic bedrock and shows a gradual downward slope. The bottom section of the cave is filled by a continuous inflow of 2000 m^3/day with an anoxic gaseous mixture (CO_2, CH_4, and H_2S), while an ambient air layer (containing O_2) floats on top of the heavier gases (Sarbu et al., 2018). At the back end of the cave, the depth of the anoxic gas layer is the highest and reaches about 2 m. Elemental sulfur (S°) and sulfuric acid (H_2SO_4) cover the cave walls at and below this gas chemocline. The pH drops to very low values (<1) near the chemocline, suggesting oxidation of H_2S. At and above the chemocline, microbial biofilms are present on the cave wall, which can be described as a white/brown colored slimy layer ranging in thickness of a few mm (Sarbu et al., 2018).

Eight biofilm samples were collected from the cave wall at the back end of the cave (Fig. 1a). Samples were collected by scraping the biofilm from the cave wall into sterile eppendorf tubes (2 mL), which were closed inside the cave. All samples were frozen (−20 °C) until further processing. One intact biofilm sample was imaged with JEOL 5600 scanning electron microscope (SEM) operated at 10 keV and 14,500× magnification under low vacuum (Fig. 1b).

2.2. Extraction of extracellular polymeric substances

In the lab, seven biofilm samples were lyophilized. Extraction of extracellular polymeric substances (EPS) was performed on the lyophilized samples following an alkaline extraction protocol (Pinel et al., 2020). In brief, 10 mg of lyophilized sample were added to 1 mL of 0.1 M NaOH solution and stirred vigorously for 30 min at 80 °C. Then, mixture was cooled to 4 °C and centrifuged at 4000 rpm for 30 min. The supernatant was dialyzed with a 3.5 kDa cut-off dialysis bag against demi-water overnight at room

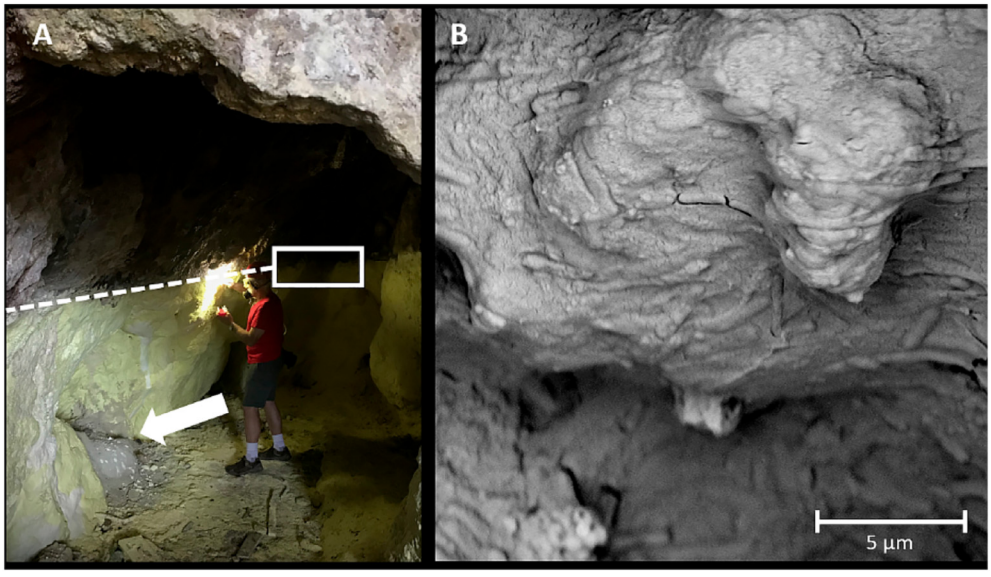

Fig. 1. A) Sulfur Cave with white rectangle showing sampling location. The grey zone/white arrow at the bottom of the wall is the point where the volcanic gas emanates. Yellow deposits are sulfur deposits and show the height of the gas/gas interface (indicated by dashed white line). B) Scanning electron microscope (SEM) image of collected biofilm where numerous rod shaped cells embedded in the EPS are visible.

temperature and, subsequently, lyophilized. The EPS extraction yield was determined by dividing the weight of lyophilized supernatant by the weight of the lyophilized biofilm sample.

2.3. Extracellular polymeric substances characterization

2.3.1. Protein and carbohydrate analysis

The protein content of two EPS samples was measured in triplicate with a commercially available protein quantification kit (BC assay protein quantification kit, Interchim, Montluçon FRANCE) with bovine serum albumin (BSA) as the standard. Samples were dissolved in 0.01 M NaOH to a 2 mg/mL concentration and protein quantified according to the manufacturer's instructions. Absorbance was measured at 562 nm using a multimode plate reader (TECAN Infinite M200 PRO). The sugar content of the same samples was measured in triplicate with a phenol-sulfuric acid assay (Dubois et al., 1956), by measuring the absorbance at 482 nm with a VIS-spectrophotometer (HACH DR3900). A mixture of glucose, xylose, fucose, rhamnose, galactose, glucose, xylose, mannose and ribose was used as standard to ensure accurate quantification of sugar concentrations (see detailed explanation in Felz et al., 2019).

2.3.2. FT-IR analysis

FT-IR spectra were collected for 5 samples: one intact biofilm, two recovered EPS samples, one pellet (after EPS extraction) and one DMMB positive fraction (see Section 2.3.3 below). Spectra were recorded with a Perkin-Elmer spectrum 100 FTIR spectrometer (Perkin-Elmer, Shelton, USA), over the wavenumber range from 4000 cm^{-1} to 650 cm^{-1} with 32 accumulations and 4 cm^{-1} resolution. Spectral data processing was performed in MATLAB and consisted of baseline correction (linear baseline correction) and feature scaling of the individual spectra.

2.3.3. sGAG-like polymers quantification

The total carbohydrate assay (e.g. phenol-sulfuric acid assay in Section 2.3.1) does not extract a number of carbohydrates, including amino sugars, sialic acids and sulfated glycosaminoglycans (sGAG), as those carbohydrates do not form a furfural structure when they react with concentrated sulfuric acids (Felz et al., 2019; de Graaff et al., 2019). Dye-spectrometry with DMMB a cationic dye is a conventional method for measuring polyanionic carbohydrates like sGAG (Kubaski et al., 2017). It is a simple and fast method for detecting the presence of sGAG, but no additional structural information is available. This is why additional analysis was added to validate the presence of sGAG-like polymers in the acidophilic biofilm. The presence of polyanionic carbohydrates such as sulfated glycosaminoglycans was evaluated in the intact biofilm (one sample) and in the extracted EPS (two samples) by conventional DMMB dye-spectrometry with the Blyscan™ glycosaminoglycan assay according to the manufacturer's instructions (Biocolor, UK). In brief, 20–50 mg of samples was treated with papain protein digestion solution (Sigma Aldrich) overnight. The supernatant was collected after centrifuging at 13,000 rpm for 10 min. Supernatant (50 μL) was mixed with 1 mL of DMMB-dye reagent, in triplicate per sample. The DMMB dye is a cationic dye with a pH of 1.7 to selectively bind sulfated groups (Boleij et al., 2020). It forms precipitates with sulfated polysaccharides (e.g. sGAG). After resolubilization, the DMMB bound sGAG was quantified by measuring its absorbance at 656 nm with a multimode plate reader (TECAN Infinite M200 PRO). Chondroitin-6-sulfate is used as the standard. For the FT-IR measurement, the sample which had the highest concentration of sGAG-like polymers was selected. 200 μL of the remaining supernatant was mixed with 4 mL of DMMB-dye reagent. The sGAG-DMMB precipitates were lyophilized for FT-IR analysis.

2.3.4. DNase and chondroitinase ABC digestion of enriched sulfated glycosaminoglycan-like polymer

The Blyscan™ glycosaminoglycan assay is designed to be selective for sGAGs like chondroitin-6-sulfate. Other polyanions can interfere and cause a false positive signal. Therefore the effect of enzymatic digestion was used to further investigate any structural similarity between DMMB positive fraction of the EPS and chondroitin-6-sulfate, EPS samples were digested with chondroitinase ABC (ChABCase from *Proteus vulgaris* (Sigma-Aldrich, Zwijndrecht, Netherlands)). ChABCase catalyzes the cleavage of chondroitin-4 sulfate, chondroitin-6 sulfate, dermatan sulfate and to a lesser extent hyaluronic acid. The sGAG concentration was measured after enzymatic treatment to determine the effectiveness of the enzymatic digestion, and thus the similarity of the sulfated polymer to the chondroitin-6-sulfate standard.

The presence of DNA may cause interference to sGAG quantification as it is also a polyanionic molecule. Thus, a DNase I treatment was performed to evaluate the interference of DNA. One papain digested sample was divided into three fractions, with one fraction as control and the other two fractions treated by chondroitinase ABC and DNase I, respectively, according to Zheng and Levenston (2015). Chondroitinase ABC was prepared at 20 U/mL in 0.01% BSA, 50 mM Tris and 60 mM sodium acetate at pH 8 (Felz et al., 2020). DNase I was prepared at 400 U/mL working solution in 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 50% (*v*/v) glycerol (Zheng and Levenston, 2015). For the enzymatic treatment, 90 μL of papain digested samples were digested with 5 μL of enzyme working solution. After enzymatic treatment, sGAG concentration was measured with Blyscan™.

2.4. BLAST analysis of enzymes related to sulfated glycosaminoglycans synthesis

The Basic Local Alignment Search Tool (BLAST) was used, with blastp from the NCBI website to find homologs of sulfotransferases, an enzyme known to be used by eukaryotes to synthesize sGAGs. To this end, two reference protein sequences from *Pseudomonas ogarae* (NCBI:txid1114970) and from *Sinorhizobium fredii* (NCBI:txid380) were matched against a protein database that includes the NCBI sequences from four major phylotypes (*Mycobacterium* (NCBI:txid1763), *Acidomyces* (NBCI:txid245561), *Acidithiobacillus* (NCBI:txid119977), *Ferroplasmaceae* (NCBI:txid90142)) that were previously shown to be present in the acidophilic biofilm of Sulfur Cave (Sarbu et al., 2018). The reference protein sequences were chondroitin 4-O sulfotransferase from *Pseudomonas ogarae* (accession number: WP_014336261.1) and heparan sulfate glucosamine 3-O sulfotransferase from *Sinorhizobium fredii* (accession number: WP_015887312.1) (Boleij et al., 2020). The matched sequences were considered significantly similar when the *E*-value was below 1E-8.

3. Results

3.1. EPS, carbohydrate and protein content of Sulfur Cave biofilms

During EPS extraction of the biofilm samples, the biological fraction was solubilized and separated from mineral components (pellet). Through dialysis, polymers larger than 3.5 kDa were separated from smaller molecules. The average EPS extraction yield was 5.0 ± 2.7% of the lyophilized biofilm weight. The insoluble fraction amounted to an average of 62.7 ± 1.0% of the lyophilized biofilm weight (supplemental Table 1). The remaining 32.3% were dissolved and smaller than 3.5 kDa, and thus passed through the dialysis bag. The total protein and carbohydrate content in the extracted EPS were on average 32.3 ± 0.5% (BSA equivalents) and 26.3 ± 6.5% (carbohydrate standard mix equivalents), respectively. Underestimation of the polysaccharides in the extracted EPS can occur due to inability to measure amino sugars with the phenol-sulfuric acid method, and this underestimation could explain part of the unquantified fraction of EPS (Felz et al., 2019). Other EPS components can be e.g. lipids and humic substances (Flemming and Wingender, 2010).

3.2. FT-IR analysis of pellet, biofilm and EPS fractions

FT-IR spectroscopy was used to identify and compare main components in the insoluble pellet, intact biofilm and EPS fractions (Fig. 2). Organic and

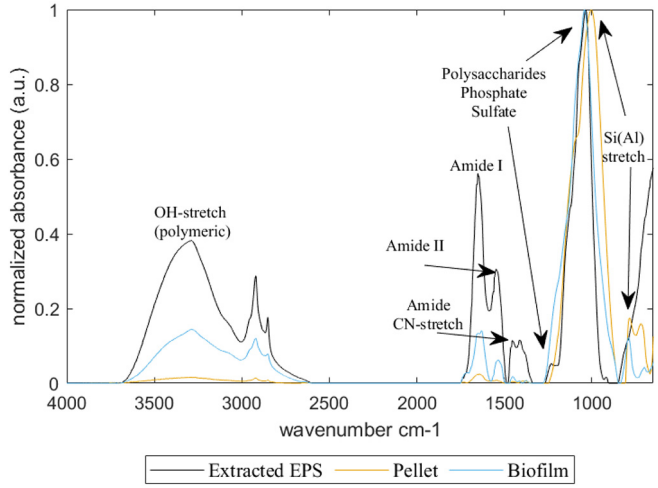

Fig. 2. Normalized FT-IR spectra of insoluble pellet, intact biofilm and extracted EPS. Spectra were recorded from 4000 to 650 cm^{-1} and measured absorbances normalized by dividing by the maximum absorbance (shown in the figure as arbitrary unit a.u).

inorganic functional groups could be assigned from the measured spectra (Table 1). In the insoluble pellet, a low absorbance (0.02 a.u.) was measured in amide I bands, which can be used as an indicator for organic components. Inorganic fractions were more prevalent as the normalized absorbance of inorganic functional groups reached 1.0 (a.u.), thus implying that the insoluble pellet contains mainly minerals.

The spectrum of the biofilm displayed high normalized absorbance both at the regions indicating mineral (0.75 a.u.), and polysaccharide components (1.0 a.u.), however overlap of these bands interfere with functional group assignment. Alternatively, higher absorbance in the amide I protein band and OH polymeric stretch of the biofilm spectrum (0.14 and 0.14 a.u., respectively), suggested more organic components compared to the insoluble pellet (0.02 and 0.02 a.u., respectively). The EPS spectrum showed that the extraction is successful in extracting organic polymeric components from the biofilm. The absorbance of protein bands and OH-polymeric stretch increased from 0.14 to 0.56 and 0.14 to 0.38 a.u., respectively. Additionally, the polysaccharide absorbance peak was sharper and a shoulder band indicating possible sulfate presence became apparent.

3.3. Detection of sulfated-glycosaminoglycans-like polymers

The detection and quantification of the sulfated polymers was performed using the Blyscan™ glycosaminoglycan assay. Considering the extreme low pH in the cave, the appearance of strong polyanionic carbohydrates, with strong acidic groups, can be expected in the biofilm. Therefore, the occurrence of polyanionic carbohydrates such as sulfated glycosaminoglycans (sGAG) in the biofilm was evaluated. The sGAG concentration was measured in triplicate on one biofilm sample that did not undergo EPS extraction. On average, 5.45 ± 0.02 μg/mg of the lyophilized biofilm material was composed of sGAG-like polymers (chondroitin sulfate equivalent). In the extracted EPS, the content of sGAG-like polymers 21.5 and 56.2 μg/mg EPS for the two samples analyzed.

Since the quantification assay is based on precipitation of the DMMB–sulfated glycosaminoglycan complex, it also served as a method for sGAG-like polymer isolation. In theory, the isolated DMMB positive fraction is enriched with sGAG-like polymer. To validate this, the FT-IR spectra of the recovered EPS and the DMMB positive fraction were compared (Figure 3). Both spectra contained bands around 1023 and 1047 cm^{-1}, implying CO stretch in carbohydrates. The intensity of absorbance bands of OH-polymeric stretch and amide I and II bands, is decreased in the spectrum of DMMB positive fraction in comparison to that of extracted EPS, which is due to the removal of proteins during the pretreatment of DMMB assay. Interestingly, bands at around 1230 and 1167 cm^{-1} in the spectrum of DMMB positive fraction became apparent, which are indicators for sulfate half-esters and polysaccharide, respectively (Bombalska et al., 2011; Cabassi et al., 1978).

Table 1
Overview of absorbance bands with their respective spectral assignments.

Band (cm^{-1})	Assignment	Reference
720	Si-Si(Al) and SiSi stretch	(Chen et al., 2015; Theodosoglou et al., 2010).
770	Si-Si(Al) and SiSi stretch	(Chen et al., 2015; Theodosoglou et al., 2010).
1005	Si(Al) stretch	(Chen et al., 2015; Theodosoglou et al., 2010).
1040–1160	C-O stretch (proteins and carbohydrates)	(Talari et al., 2017)
1222–1265	Phosphate asymmetric stretch	(Talari et al., 2017)
1230–1260	S=O asymmetric stretch	(Cabassi et al., 1978; Devlin et al., 2019)
1540–1570	Amide II (protein)	(Talari et al., 2017)
1620–1680	Amide I (protein)	(Talari et al., 2017)
2921–2923	CH_2 symmetric stretch	(Talari et al., 2017)
3200–3500	OH-stretch, Amide A	(Talari et al., 2017)

3.4. Enzymatic digestion of enriched sulfated glycosaminoglycans-like polymer

To further confirm the structural similarity between DMMB positive fraction and chondroitin sulfate, Chondroitinase ABC digestion was conducted, which resulted in a 26.3 ± 7.9% signal decrease after measurement with Blyscan™ sGAG kit. Thus chondroitinase could effectively digest part of the DMMB positive fraction. This implies that there is a similar structure with chondroitin-sulfate. If there is DNA in the sample, it can potentially bind to DMMB, resulting in an overestimation of sGAG-like polymers (Zheng and Levenston, 2015). However, DNase treatment of the samples resulted in a 3.3 ± 1.9% signal decrease of the DMMB positive fraction only, meaning a minor interference of DNA in the estimation of the total sGAG-like polymers.

3.5. Mining prokaryotic protein databases for sulfotransferases

Sulfotransferases are enzymes that transfer sulfo- groups to polysaccharides (Kusche-Gullberg and Kjellén, 2003) and are an important factor in the biosynthesis of sGAGs. Knowledge on the occurrence of sulfotransferases in prokaryotes is limited; here we mined protein databases of microorganisms belonging to the genera *Mycobacterium*, *Acidomyces* and *Acidithiobacillus* for sulfotransferases. These genera were predominantly found in samples for the Sulfur Cave analyzed previously by Sarbu et al. (2018). Two reference proteins were used for protein Blast (BlastP): chondroitin 4-O sulfotransferase from *Pseudomonas fluorescens* and heparan sulfate glucosamine 3-O-sulfotransferase

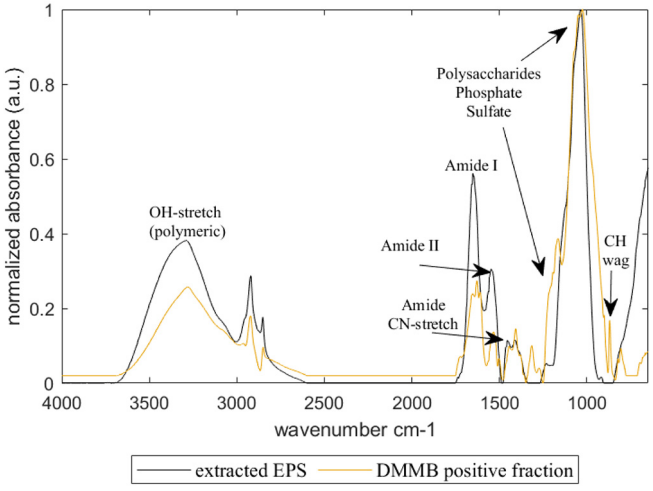

Fig. 3. Normalized FT-IR spectra of extracted EPS and complexed sulfated polymers. Spectra were recorded from 4000 to 650 cm^{-1} and normalized by rescaling to maximum absorbance, absorbance is in arbitrary units (a.u).

Table 2
List of significant BlastP search matches against chondroitin 4-O sulfotransferase (accession number: WP_014336261.1).

Accession number	Name protein and species	Identity (%)	Bitscore	E-value
WP_207065469.1	sulfotransferase family 2 domain-containing protein [unclassified mycobacterium]	31.016	225	1.31e-15
WP_192722148.1	sulfotransferase family 2 domain-containing protein [Mycobacterium sp. OAS707]	31.056	236	2.68e-08

from *Sinorhizobium fredii* (Boleij et al., 2020). For chondroitin 4-O sulfotransferase, two matches were obtained, both occurring in the *Mycobacterium* genus (Table 2). For the second enzyme, heparan sulfate glucosamine 3-O-sulfotransferase, forty protein matches were found in the *Acidithiobacillus* and *Mycobacterium* genera (Table 3). These results suggest that at least two of the microbial genera reported to inhabit the Sulfur Cave have the potential to synthesize sulfated polysaccharides.

4. Discussion

Currently, the microbial production of glycosaminoglycan-like polymers is mostly studied in pathogenic bacteria, e.g. *Pseudomonas aeruginosa* and model organisms like *Escherichia coli*. These bacteria produce non-sulfated glycosaminoglycans, or closely related molecules, which mimic the backbone of sulfated glycosaminoglycans produced by the host, supposedly as a camouflage to avoid detection by the host immune system (Badri et al., 2021). Recently, sGAG-like polymers have been reported in prokaryotic ecosystems, such as marine microbial mats (Decho and Gutierrez, 2017) and granular biofilms (including aerobic, anaerobic and anammox granular sludge) collected from municipal wastewater treatment plants (Felz et al., 2020; Bourven et al., 2015; Boleij et al., 2020). This study targets another type of biofilm ecosystems, i.e., extremely acidophilic biofilms.

Our results show the presence of sGAG-like polymers in the acidophilic biofilm growing in Sulfur Cave. Firstly, the results from the DMMB assay indicated the presence of anionic polymers. Since the Blyscan kit is designed for quantification of sGAG, it is selective for strong acidic polymers. Secondly, the enriched polymer was sensitive to the digestion with chondroitinase ABC, implying a similar but not necessary identical structure as chondroitin sulfate is present in the biofilm. It is known that the cleavage sites of chondroitinase are: (1 → 3) glycosidic linkage between β-D-glucuronic acid and N-Acetyl-β-D-galactosamine-4-sulfate; (1 → 3) glycosidic linkage between β-D-glucuronic acid and N-Acetyl-β-D-galactosamine-6-sulfate; and (1 → 3) and (1 → 4) glycosidic linkages between β-D-glucuronic acid and N-Acetyl-β-D-glucosamine (Sigma-Aldrich, 2007; Felz et al., 2020). So the molecular structure of the enriched polymer should have at least one of those linkages. Thirdly, the FT-IR spectrum of the DMMB positive fraction is dominated by absorbance bands in the polysaccharide region (1200–900 cm^{-1}). Two bands in particular were more pronounced in the spectrum of the DMMB positive fraction relative to that of EPS. These two bands suggest the possible presence of organosulfate, and/or the C–O–C stretch of uronic acids, respectively (Talari et al., 2017; Devlin et al., 2019).

Table 3
List of significant BLAST search matches against heparan sulfate glucosamine 3-O sulfotransferase (accession number: WP_015887312.1).

Accession number	Name protein and [species]	Identity (%)	Bitscore	E-value
WP_142086737.1	Sulfotransferase [*Acidithiobacillus thiooxidans*]	28.767	291	4.21e-20
WP_163098766.1	Sulfotransferase [Acidithiobacillus ferrianus]	32.161	216	1.62e-19
WP_163055154.1	Sulfotransferase [*Acidithiobacillus ferrooxidans*]	29.825	291	2.99e-19
WP_192722155.1	Sulfotransferase domain-containing protein [Mycobacterium sp. OAS707]	31.944	290	3.18e-19
WP_126605524.1	MULTISPECIES: sulfotransferase [Acidithiobacillus]	28.772	291	7.68e-19
WP_012536779.1	MULTISPECIES: sulfotransferase [Acidithiobacillus]	28.772	291	8.24e-19
WP_113525668.1	Sulfotransferase [Acidithiobacillus ferridurans]	28.772	291	1.06e-18
WP_014029380.1	sulfotransferase [*Acidithiobacillus ferrivorans*]	29.123	291	1.25e-18
WP_035190410.1	sulfotransferase [*Acidithiobacillus ferrivorans*]	29.123	291	1.43e-18
WP_193650373.1	MULTISPECIES: sulfotransferase [unclassified Acidithiobacillus]	28.814	303	1.58e-18
WP_101537278.1	Sulfotransferase [Acidithiobacillus sp. SH]	28.239	301	1.79e-18
WP_010638122.1	Sulfotransferase [*Acidithiobacillus thiooxidans*]	31.088	200	3.20e-18
WP_065973527.1	Sulfotransferase [*Acidithiobacillus thiooxidans*]	31.088	200	4.11e-18
WP_031568475.1	MULTISPECIES: sulfotransferase [Acidithiobacillus]	31.088	200	4.40e-18
QFX96614.1	sulfotransferase [*Acidithiobacillus thiooxidans* ATCC 19377]	31.088	202	5.20e-18
WP_206385958.1	sulfotransferase [Acidithiobacillus sp. MC6.1]	28.772	291	6.58e-18
WP_176562256.1	sulfotransferase domain-containing protein [Mycobacterium palauense]	34.254	187	6.58e-18
WP_163098168.1	sulfotransferase [Acidithiobacillus ferrianus]	28.772	291	8.35e-18
TQN51840.1	hypothetical protein DLNHIDIE_01720 [*Acidithiobacillus thiooxidans* ATCC 19377]	28.571	303	8.50e-18
WP_193870059.1	sulfotransferase [Acidithiobacillus sulfuriphilus]	30.964	204	9.58e-18
WP_064219950.1		28.070	291	1.52e-17
WP_140390866.1	MULTISPECIES: sulfotransferase [Acidithiobacillus]	28.231	303	1.61e-17
WP_024895070.1	sulfotransferase [*Acidithiobacillus thiooxidans*]	28.475	303	1.91e-17
WP_024892770.1	sulfotransferase [*Acidithiobacillus thiooxidans*]	30.570	200	2.09e-17
WP_193650115.1	MULTISPECIES: sulfotransferase [unclassified Acidithiobacillus]	30.570	200	4.60e-17
WP_075323453.1	Sulfotransferase [*Acidithiobacillus albertensis*]	27.891	303	5.26e-17
WP_010639455.1	sulfotransferase [*Acidithiobacillus thiooxidans*]	30.808	216	1.42e-16
WP_193640087.1	Sulfotransferase [Acidithiobacillus sp. HP-11]	29.851	215	3.06e-16
HGE68600.1	TPA: sulfotransferase [Acidithiobacillus sp.]	32.768	182	6.24e-16
WP_142089850.1	Sulfotransferase [*Acidithiobacillus thiooxidans*]	29.353	215	6.75e-16
WP_142087470.1	Sulfotransferase [*Acidithiobacillus thiooxidans*]	29.353	215	7.30e-16
WP_193651055.1	MULTISPECIES: Sulfotransferase [unclassified Acidithiobacillus]	29.353	215	7.45e-16
WP_004871655.1	Sulfotransferase [*Acidithiobacillus caldus*]	33.696	201	1.50e-15
WP_014002827.1	Sulfotransferase [*Acidithiobacillus caldus*]	32.609	201	1.93e-15
WP_070114154.1	Sulfotransferase [*Acidithiobacillus caldus*]	32.609	201	2.19e-15
WP_198926362.1	Sulfotransferase [*Acidithiobacillus thiooxidans*]	27.972	278	1.66e-14
MBO0863535.1	Sulfotransferase domain-containing protein [Mycobacterium sp.]	28.936	265	2.90e-14
WP_066916256.1	sulfotransferase [*Mycobacterium interjectum*]	30.769	186	1.69e-10
SPM30343.1	Putative sulfotransferase, partial [Mycobacterium terramassiliense]	30.220	186	1.94e-10
WP_077101318.1	Sulfotransferase [Mycobacterium terramassiliense]	30.220	186	2.07e-10

Therefore, the DMMB positive fraction is considered enriched with sGAG-like polymer. Lastly, when we screened the NCBI database, chondroitin 4-O sulfotransferase showed two hits in the *Mycobacterium* genus. As this enzyme determines the biosynthesis of chondroitin sulfate, this suggests the possibility of chondroitin sulfate (probably chondroitin 4-O sulfate) synthesis in the acidophilic biofilm. Additionally, the large number of microorganisms encoding heparan sulfate glucosamine 3-O-sulfotransferase implies that there might be sGAGs other than chondroitin sulfate produced in the biofilm. Unlike the granular sludge collected from wastewater treatment plants, the biofilms growing in Sulfur Cave are not exposed to input of other organic substances. The detection of sGAGs-like polymers in this biofilm therefore provides strong support for the hypothesis that sGAGs-like polymers can be directly produced by prokaryotes.

Currently, the EPS sulfation process in microorganisms is still unclear. However, in a mammalian cell, the sulfation process of sGAG begins with uptake of inorganic sulfate from the extracellular milieu (Soares da Costa et al., 2017). Thus to produce sGAGs, sulfate compounds should be readily available in the environment surrounding the biofilm. In this regard, sulfated extracellular carbohydrate-rich polymers are found in various marine biofilms. In fact, highly sulfated exopolysaccharides containing up to 27% (wt/wt) in sulfate were isolated from marine microbial mats (Moppert et al., 2009). The availability of sulfate might explain the prevalence of sulfated polymers in marine biofilms; sulfate is the second most abundant anion in the surrounding seawater, with an average concentration of 28 mM (Canfield and Farquhar, 2009). In biofilms used for treating wastewater (including activated sludge and granular sludge), the content of sulfate polysaccharides-containing EPS varies. For example, 418 mg sulfated EPS/g biomass was found in the sludge collected from full-scale wastewater treatment plant in Hong Kong, which contains 5.2 mM sulfate in the influent. Oppositely, no sulfated EPS was detected in the sludge collected from a wastewater treatment plant with no sulfate and/or sulfide in the influent (Xue et al., 2019). A lower content of sulfated EPS (24-31 mg sGAG/g biomass) (Felz et al., 2020; Boleij et al., 2020) was further observed in biofilms used for treating non-saline domestic wastewater that had a lower sulfate concentration (0.28–0.62 mM; Van den Brand et al., 2015). Sulfur Cave is a high sulfate environment due to the oxidation of hydrogen sulfide to sulfuric acid (Sarbu et al., 2018). Assuming sulfuric acid is the only acid present, the maximum sulfate concentration in the cave can be estimated from the pH values ~0.5–1 as high as 100 mM. These high concentrations of sulfate may favor the biosynthesis of sGAGs. The Sulfur Cave biofilm however, contained sGAG-like polymers at 11 mg/g organic dry weight (estimated from biomass minus the pellet mineral fraction), which is rather low compared to the wastewater and marine microbial mat data previously published. The difference can be explained by a lower actual concentration of sulfate than estimated. Alternatively, in the wastewater and marine biofilms competition for sulfate is present in the form of sulfate reducing bacteria; which couple sulfate reduction to organic carbon oxidation. In these biofilms, charged modification, by e.g. sialic acids, of extracellular polymers provide protection from degradation (Traving and Schauer, 1998). Less competition of this kind is present in the Sulfur Cave due to a lower microbial diversity in this extreme environment (Sarbu et al., 2018). How microbial diversity and sulfate presence are connected to sulfated polymer production is yet unknown and could provide interesting insight in microbial interactions inside biofilms.

With regard to the function of sGAGs in the Sulfur Cave biofilm, one has to consider the low pH value (<1 pH) of the system. Under such extreme conditions, weakly acidic groups (carboxylic acids) become protonated and lose their charge (Boleij et al., 2020; Felz et al., 2020), while highly acidic polymers, like sGAGs, may maintain a negatively charged EPS (Thornton et al., 2007; Gutierrez et al., 2009). A highly negative charged EPS contributes to the ability of the EPS to bind water or cations (Varki, 2017; Gagliano et al., 2018) and form a hydrogel. This hydrogel matrix not only helps microorganisms to compete and survive under extreme environmental conditions, but also maintain the stability of the biofilm. Protein to carbohydrate ratio from this unique biofilm (1.28) was lower than observed in granular sludge (2.61) (Felz et al., 2019). This difference might be due to differences in hydrophobicity. Granular sludge is typically hydrophobic, which is the result of a high protein content (Gao et al., 2011; Santschi et al., 2020). Inside Sulfur Cave, water is only provided by condensation, thus it would be advantageous for the microbes living in this environment to evolve a mechanism to retain water. Remarkably, the function of the microbial chondroitin sulfate in this acidophilic biofilm is likely similar as in animal cartilage where chondroitin sulfate is responsible for the water retention (Jerosch, 2011). In addition, chondroitin sulfate (sGAG) is stable at pH 1, only when it is heated at this pH at high temperature (>30 °C) for more than 960 h, a slight hydrolysis starts (Volpi et al., 1999). Thus the sGAG-like polymers found in the biofilm of Sulfur Cave would be stable at the low pH and may serve as a hydration mechanism. However, the exact function of sGAGs in biofilms requires targeted experiments and the extreme environment of Sulfur Cave offers a unique opportunity for in depth research on the topic.

5. Conclusion

This research set out to investigate the possible presence of sGAGs in a unique microbial biofilm isolated from any external organic carbon source. The acidophilic biofilm growing at the non-aquatic gas-gas interface of Sulfur Cave showed a clear presence of sulfated glycosaminoglycan-like polymers. Furthermore, the microbial taxa (*Mycobacterium* and *Acidithiobacillus*) present in the biofilm appear to have the metabolic potential to synthesize these compounds (chondroitin and heparan sulfate). The possibility of de novo microbial production of such polymers provides support for the widespread prevalence of sulfated glycosaminoglycan-like polymers in biofilms. The negatively charged nature of these polymers in fact suggests a potential functional role in water retention for the biofilm in Sulfur Cave, but more targeted research needs to be conducted to elucidate its true function.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154472.

CRediT authorship contribution statement

SdB: Methodology, Investigation, Writing-Original Draft **DV-C**: Conceptualization, Investigation, Writing-Reviewing and Editing **SS**: Conceptualization, Writing-Reviewing and Editing **FM**: Writing-Reviewing and Editing **DS**: Writing-Reviewing and Editing **MvL**: Writing-Reviewing and Editing **YL**: Writing-Reviewing and Editing, supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Jure Zlopasa for fruitful discussions and insight on negative charge compounds in biofilms. Additionally the authors thank the members of the Vinca Minor association for their efforts for maintaining and protecting the mofettes on Puturosu Mountain, and the GESS team for providing the protective equipment used during field work in the extreme conditions encountered at sites such as Sulfur Cave. This research was funded by the SIAM Gravitation Grant 024.002.002, The Netherlands Organization for Scientific Research. DVC and FJRM was financially supported by the Research Foundation Flanders via FWO grant G031416N, and the Netherlands Organization for Scientific Research (VICI grant 016.VICI.170.072).

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