Discovery of a novel iota carrageenan sulfatase isolated from the marine bacterium Pseudoalteromonas carrageenovora

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INTRODUCTION

Although sulfated biomolecules are present throughout the tree of life in terrestrial and marine environments, most of the sulfated carbohydrates are found in the marine environment, as illustrated by the structural diversity of sulfated polysaccharides encountered in the cell wall of macroalgae (Lahaye and Robic, 2007; Pomin and Mourao, 2008; Popper et al., 2011; Usov, 2011). As such polysaccharides are absent in the cell wall of fresh water and land plants; their loss has been postulated to be a consequence of the land colonization (Michel et al., 2010). These polysaccharides are thought to be involved in the phenomena of ionic and osmotic regulations. They are supposed to confer to the algae resistance to water currents and, due to their highly soluble nature, they allow water retention thereby slowing the drying of the algae at low tide (Kloareg and Quatrano, 1988).

The cell wall sulfated galactans of the red alga, referred to as agars or carrageenans, consist of a linear backbone of galactose residues linked by alternating β-1,4 and α-1,3 glycosidic bonds. While all the β-linked residues are in the D configuration (G monomer), the α-linked galactose units are in the L configuration in agars (L monomer) and in the D configuration in carrageenans (D monomer). In carrageenans, the repeating disaccharide units and, due to their highly soluble nature, they allow polysaccharides are absent in the cell wall of fresh water and land plants; their loss has been postulated to be a consequence of the land colonization (Michel et al., 2010). These polysaccharides are thought to be involved in the phenomena of ionic and osmotic regulations. They are supposed to confer to the algae resistance to water currents and, due to their highly soluble nature, they allow water retention thereby slowing the drying of the algae at low tide (Kloareg and Quatrano, 1988).

The cell wall sulfated galactans of the red alga, referred to as agars or carrageenans, consist of a linear backbone of galactose residues linked by alternating β-1,4 and α-1,3 glycosidic bonds. While all the β-linked residues are in the D configuration (G monomer), the α-linked galactose units are in the L configuration in agars (L monomer) and in the D configuration in carrageenans (D monomer). In carrageenans, the repeating disaccharide units are classified according to the number and the position of ester sulfate (S) and by the presence of a 3,6-anhydro-bridge (DA) in the 4-linked residue (Knutset et al., 1994). DA units are found in gelling carrageenans such as the kappa (κ; G4S-DA) and the 3,6-anhydro-bridged ε-carrageenan (ε; G4S-DA) and the κ-carrageenan sulfatase was detected in the cell-free lysate of the marine bacterium *Pseudoalteromonas carrageenovora* strain PscT. It was purified through Phenyl Sepharose and Diethylaminoethyl Sepharose chromatography. The pure enzyme, *Psc* ι-CgsA, was characterized. It had a molecular weight of 115.9 kDaltons and exhibited an optimal activity/stability at pH 8.3 and at 40 ± 5°C. It was inactivated by phenylmethylsulfonyl fluoride but not by ethylene diamine tetraacetic acid. *Psc* ι-CgsA specifically catalyzes the hydrolysis of the 4-S sulfate of iota-carrageenan. The purified enzyme could transform iota-carrageenan into hybrid iota-/alpha- or pure alpha-carrageenan under controlled conditions. The gene encoding *Psc* ι-CgsA, a protein of 1038 amino acids, was cloned into *Escherichia coli*, and the sequence analysis revealed that *Psc* ι-CgsA has more than 90% sequence identity with a putative uncharacterized protein Q3IKL4 from the marine strain *Pseudoalteromonas haloplanktis* TAC 125, but besides this did not share any homology to characterized sulfatases. Phylogenetic studies show that *P. carrageenovora* sulfatase thus represents the first characterized member of a new sulfatase family, with a C-terminal domain having strong similarity with the superfamily of amidohydrolases, highlighting the still unexplored diversity of marine polysaccharide modifying enzymes.

**Keywords:** sulfatase, novel family, iota carrageenan, marine bacteria, sulfated polysaccharides, bioconversion
iota (ι; G4S-DA2S)-carrageenan (Figure 1). Other substitutions, such as methyl or pyruvate groups, have also been observed, increasing the diversity of carrageenans which depends also on the algal source, the growth conditions and the extraction procedures (Pereira and Mesquita, 2004; Pereira et al., 2009). The physico-chemical properties of carrageenans, which are extensively used as thickeners and stabilizers in the food and cosmetic industries (de Ruiter and Rudolph, 1997), depend on their molecular weight, the occurrence of anhydrogalactose and their sulfate content and it is well established that higher levels of ester sulfate induce a decrease of the gel strength (Necas and Bartosikova, 2013). The use of specific enzymes to modify the sulfate pattern of carrageenans would therefore offer a biotechnological approach to control their sulfate content and thereby their rheological properties.

The heterogeneous structure of carrageenans, their gelling properties, and their interactions with the other components of red algal cell walls challenge the microorganisms using these polymers as carbon and energy sources. To breakdown the complex polysaccharides, marine bacteria secrete specific glycoside hydrolases (GHs), referred to as agarases and carrageenases, which catalyze the hydrolysis of the β-1,4 glycosidic bond between two galactopyranose units of their respective substrate (Michel et al., 2006). However, these enzymes are not sufficient alone to lead to the complete substrate assimilation. As revealed by the increasing number of sequenced marine microbial genomes, marine bacteria possess a large number of sulfatases. Although their precise function has not been elucidated yet, it is likely that they play an important role in the degradation of algal sulfated polysaccharides (Glöckner et al., 2003).

While genomic and metagenomic approaches offer promising strategies for marine biodiscovery (Ekborg et al., 2006; Shin et al., 2010), there are still some limitations, as screening of such libraries is indeed either sequence based or function based (Kennedy et al., 2008). These limitations sometimes do not allow assigning new functions to proteins annotated as hypothetical, and in such cases, it is still necessary to go through the isolation and purification of a defined activity in order to ascribe a function to a gene.

As both exo- and endo-acting sulfatases have been demonstrated in the case of glycoaminoglycans, we postulate their existence in the case of carrageenans. We therefore screened marine bacteria for endo-sulfatases to specifically modify the sulfate pattern of carrageenans in a polymeric state. In a previous work, a carrageenan sulfatase converting ι- in α-carrageenan was isolated from P. atlantica T6C and recombinantly overexpressed (Préchoux et al., 2013). Analysis of its mode of action confirmed the endo-character of this sulfatase removing the sulfate ester groups, most likely in a random pattern along the polysaccharide chain. Aiming at monitoring the physico-chemical properties of carrageenan, we were looking for further sulfatases potentially having different properties as this would allow to fine tune the rheological properties of these hydrocolloids or to adapt to different industrial conditions. In this context, we isolated and purified to homogeneity an endo-sulfatase from the marine bacterium P. carrageenovora Psc. Despite the fact that this enzyme has the same specificity as the ι-carrageenan sulfatase from P. atlantica (Préchoux et al., 2013), sequence analysis revealed that the P. carrageenovora sulfatase did not share any homology to already known sulfatases. It has, however, more than

![FIGURE 1](https://via.placeholder.com/150) Structure of the idealized repeating units of ι-(G4S-DA2S) (A), α-(G-DA2S) (B), κ-(G4S-DA) (C), and β-(G-DA) (D) carrageenans. The arrow between G4S-DA2S and G-DA2S illustrates the reaction catalyzed by Psc ι-CgsA. Enzymatic production of β-carrageenan (D) from κ-carrageenan (C) has not been yet demonstrated and is represented by a dashed arrow.
90% sequence identity with a putative uncharacterized protein, Q3IK4, from the marine strain *Pseudoalteromonas haloplanktis* TAC 125. Phylogenetic studies show that *P. carrageenovora* sulfatase thus represents the first characterized member of a new sulfatase family, with a C-terminal domain having strong similarity with the superfamily of amidohydrolases, highlighting the still unexplored diversity of marine polysaccharide modifying enzymes.

**MATERIALS AND METHODS**

**MATERIALS**

λ-carrageenan (GENU X-7055) was extracted from tetrasporophytic plants of *Gigartina skottsbergii*, κ-carrageenan (GENU X-6913) was extracted from *Eucheuma cottonii* and τ-carrageenan (GENU X-6908) was extracted from *Eucheuma spinosum*. All of these samples were kindly provided by CP Kelco (Copenhagen). The marine bacteria *P. carrageenovora* strain Psc° (ATCC 35555°) (Bellion et al., 1982) and *Pseudoalteromonas haloplanktis* 545° (DSMZ 6060°) were obtained from the American Type Culture Collection and the Deutsche Sammlung von Mikroorganismen und Zellkulturen collection respectively.

**PRODUCTION AND PURIFICATION OF THE λ-CARRAGEENAN SULFATASE FROM P. CARRAGEENOVORA (PSC-ι-CgsA)**

Unless otherwise stated, all purification steps were performed at 4°C. Hydrophobic interaction chromatography and anion exchange chromatography were carried out at 18°C. *P. carrageenovora* strain Psc° was grown in 5 L of sulfate free ZoBell medium (ZoBell, 1941) containing 1g L−1 of λ-carrageenan. After 36 h of incubation at 15°C, the culture medium was centrifuged for 60 min at 1400–1800 g. The supernatant was discarded and the cells were slowly suspended for about 1 h in 30 mL of buffer A (50 mM Tris-HCl, pH 8.3) before lysis with a French Press. The lysate was centrifuged for 1 h at 29000 g. The cell-free supernatant was brought to 30% (NH₄)₂SO₄ saturation by slow addition of (NH₄)₂SO₄ (16.4 g (NH₄)₂SO₄ per 100 mL of extract) by slow addition of buffer A. The desalted sample (about 140 mL) was loaded at a flow rate of 1 mL min⁻¹ during 20 column volumes (CVs). Fractions of 6.5 mL were collected. Elution of bound proteins was achieved at a flow rate of 2 mL min⁻¹ with a linear increasing gradient from 0 to 1 M NaCl in buffer A. The final concentration of NaCl was reached after 20 CVs and 5.5 mL fractions were collected. The fractions containing pure Psc—CgsA were pooled and stored at 4°C in buffer A.

At the different steps of purification, the fractions were tested for sulfatase activity using standard τ-carrageenan as substrate. The standard reaction mixture contained 100 μL of protein fraction and 100 μL of 1.2% (w/v) τ-carrageenan both in 50 mM Tris-HCl buffer, pH 8.3. Incubation was performed for 12 h at 35°C. The reaction was stopped by diluting the incubation medium 2-fold and by centrifuging the samples in a Microcon-10 (Millipore) to remove the carrageenan. For each sample, the corresponding blank was performed in the same conditions but using the sample previously boiled for 10 min. The amount of free sulfate present in the filtrate was assayed by high pressure anion exchange chromatography (HPAEC) using a Dionex 500 chromatography system as described previously (Genicot-Joncour et al., 2009). Briefly, the anions present in the reaction medium were separated on an AS11 anion-exchange column (4 × 200 mm, Dionex) equipped with an AG11 guard column (4 × 50 mm, Dionex). Elution was performed with an isocratic gradient of 12 mM NaOH at a flow rate of 1 mL min⁻¹ using a GP40 gradient pump (Dionex). Detection of the anions was carried out with an ED40 electrochemical detector in the conductivity mode. The peak of sulfate eluted separately from the other ions at 3 min and the concentration of sulfate was deduced from the signal intensity and calculated from a standard sulfate calibration curve. The active fractions were analyzed by SDS-PAGE (Laemmli and Favre, 1973) using 12% Criterion precast Bis-Tris gels (Bio-Rad). Gels were stained routinely with Coomassie blue R-250 and colloidal Coomassie blue staining when subjected to mass spectrometry analyses (Candiano et al., 2004). Protein quantification was performed according to Bradford (1976) using the Bio-Rad protein assay. Bovine serum albumin was used as a standard. Protein concentration of pure sulfatase was also estimated at 280 nm using a Nanodrop 2000 Spectrophotometer (Thermofisher). A molar extinction coefficient of 172.120 M⁻¹ cm⁻¹ and a molecular weight of 115.916 kDaltons (kDa), both deduced from the protein sequence (see below), were used to calculate the concentration of the enzyme.

**PROTEIN SEQUENCE DETERMINATION OF THE PSC-ι-CGS A PROTEIN**

The band corresponding to the Psc—CgsA sulfatase was excised from Coomassie blue stained SDS-PAGE and was subjected to in-gel tryptic digestion as described in Larré et al. (2010). Briefly, the gel slice was washed with 100 μL of 25 mM NH₄HCO₃, followed by dehydration with 100 μL of 50% (v/v) acetonitrile in 25 mM NH₄HCO₃. Proteins were reduced and alkylated by incubation for 1 h at 57°C in the presence of 10 mM Dithiothreitol (DTT), followed by 45 min of incubation at room temperature with 55 mM iodoacetamide. The gel slice was further washed with 25 mM NH₄HCO₃ and dehydrated as described before. The band was then incubated overnight at 37°C with 10 μL of trypsin (sequencing grade, Promega) solubilized at 12.5 ng μL⁻¹ in 25 mM NH₄HCO₃. The supernatant was collected and the tryptic fragments were analyzed by Matrix Assisted Laser Desorption Ionization coupled to a Time-of-Flight analyzer (MALDI-TOF) and nanoscale capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS). MALDI-TOF mass spectrometry was
performed on a M@LDI LR instrument (Waters). One microliter of the sample was mixed with 1 μL of the matrix preparation (2.5 g L⁻¹ α-cyano-4-hydroxycinnamic, 2.5 g L⁻¹ 2,5-dihydroxybenzoic acid, 70% (v/v) acetonitrile, and 0.1% (w/v) trifluoroacetic acid) and deposited onto the MALDI sample probe. Mass spectra were acquired on the mass-to-charge ratio range from 800 to 3000. LC-MS/MS analysis was performed using a nanoflow high pressure liquid chromatography (HPLC) system (Switchos-Ultimate II, Dionex) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Waters). Chromatographic separations were conducted on a reverse-phase capillary column (75 μm i.d., Pepmap C18, Dionex) at a flow rate of 200 nL min⁻¹ using a gradient from 2 to 50% of 0.08% (w/v) formic acid in acetonitrile. Mass data were recorded in “data dependent” mode: one MS spectrum was recorded on the mass-to-charge ratio range 400 to 1500 within 1 s, after which the three most intense ions were selected and fragmented in the collision cell. Raw data obtained by MALDI-TOF or LC-MS/MS were processed by means of the Protein Lynx Global Server v. 2.1. Software (Waters).

Protein identification was carried out by comparing the collected LC-MS/MS data against Uniprot databank. Databank searches were performed through the use of the Mascot server v. 2.2. program (Matrix Science). The mass tolerance was set to 120 parts per million (ppm) for parent ions (MS mode) and 0.3 Da for fragment ions (MS/MS mode), and one missed cut per peptide was allowed. Some MS/MS spectra were de-novo sequenced using the Protein Lynx Global Server v. 2.1. Software. This procedure was facilitated by the use of the OVNlp program (Tessier et al., 2010).

**CLONING, HETEROLOGOUS EXPRESSION AND PURIFICATION OF THE Psc T-CgsA**

Genomic DNA from *P. carrageenovora* PscT was prepared as previously described (Barbeyron et al., 1984). The primers forward (5'-CCCCCGGACTTCATTGTGTGTTCATAAAATAGTGTTTAC-3'; EcoRI restriction site is underlined) and reverse (5'-GGGGGGGGATCCCAACAGCATGGAGCGAACAAAATGG-3'; BamHI restriction site is underlined), deduced from the gene PSHAa1171 of *P. haloplanktis* TAC 125 (Uniprot accession number Q3IKL4), were used to amplify the Psc t-cgsA gene. The SignalP 3.0 program (Bendtsen et al., 2004) predicted the presence of a signal peptide with a cleavage site between residues Ala22 and Gln23 in the Q3IKL4 protein. Here, the gene without the signal peptide was cloned in between the BamHI/ EcoRI sites of the expression vector pFO4 (Grosillier et al., 2010) which encompass an N-terminal fused six-histidine-tag (6 His-tag). The sequence of the gene was checked using a genetic analyzer ABI 3130xl (Applied Biosystems) equipped with 50 cm capillaries and POP7™ polymer. The amplified and verified gene sequence of Psc t-cgsA was deposited at GenBank with accession number JN228253. The Psc t-cgsA gene was also optimized for *Escherichia coli* codon use by GENEART (Life Technologies), amplified using the forward primer (5'-CCCGGGATCCGAGCCGATGTGAACCG-3'; BamHI restriction site is underlined) and the reverse primer (5'-GGGGGGATCCATTGTGTGTTCATAAAATAGTGTTTAC-3'; EcoRI restriction site is underlined) and cloned into the same expression vector.

For protein expression, transformed *E. coli* strains BL21(DE3) (Novagen®) were either grown at 20°C for 72 h in ZYP 5052 medium containing 200 μg mL⁻¹ ampicillin (Studier, 2005) or in Luria Bertani (LB) medium. In the latter case, the recombinant *E. coli* BL21 (DE3) cells were grown at 37°C in LB medium containing 100 μg mL⁻¹ ampicillin and 0.025% (w/v) glucose until the optical density at 600 nm reached ~1.2–1.5. The culture medium was then diluted twice with an equal volume of cold LB medium and buffered with HEPS buffer pH 7 to a 20 mM final concentration. Induction was performed by addition of lactose (0.6%) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (2 mM) (Korf et al., 2005). The cultivation was further continued for ~18 h at 20°C until the optical density at 600 nm reached ~5–8. *E. coli* BL21 (DE3) bearing pFO4 without insert was used as the negative control. Culture was stopped by centrifugation at 1400–1800 g for 60 min. The pellet was then suspended in 50 mM Tris-HCl (pH 8.3) buffer containing 200 mM NaCl and 15 mM imidazole (buffer C) before lysis with a French Press. The lysate was centrifuged for 1 h at 29,000 g. The cell-free supernatant was then 0.2 μM filtered before being loaded onto a HisPrep FF 16/10 column (1.6 × 10 cm, GE Healthcare) equilibrated in buffer C. Elution of the protein was performed in buffer C using a linear gradient, increasing from 15 mM to 500 mM imidazole. The final concentration of imidazole was reached after 10 CVs and 2 mL fractions were collected. Fractions containing the recombinant tagged enzyme were estimated by SDS-PAGE analysis and by Western blot. Transfer from SDS gel onto ready to use 0.2 μM nitrocellulose membrane (BioRad) was performed using a Trans Blot Turbo system in the conditions specified by the manufacturer (BioRad). Monoclonal anti-polyhistidine peroxidase conjugate (Sigma) was used at a final concentration of 1/10,000 to specifically recognize the his-tagged fusion proteins. Immuno-detection was performed by chemiluminescence using the Clarity Western ECL Substrate kit (BioRad) and visualization was achieved using the Chemi-Capt 50001 software. Recombinant enzyme activity was tested using 4- methylumbeliferyl sulfate (potassium salt, Sigma), further on called MUS, as substrate. Unless explicitly indicated in the text, the standard conditions included 8–20 μg sulfatase, 50 mM Tris-HCl pH 8.3, 200 mM NaCl, 850 μM MUS in a 150 μL reaction volume. The reaction was carried out for up to 120 min at 35°C and the increase of absorbance was measured as a function of time at 360 nm using a Saphire2 microplate reader (Tecan, Männedorf, Switzerland). For each reaction, a blank was made using the negative control at the same protein concentration than that of the sample.

**BIOCHEMICAL CHARACTERIZATION OF THE NATIVE Psc T-CgsA**

Sulfatase activity of the native enzyme was characterized using t-carrageenan as substrate and the amount of sulfate released was determined by HPAEC as described above. pH optimum determination was performed at 35°C in a pH range of 5.8–9.5. Assays were carried out by incubating 50 μL of t-carrageenan (1.8% w/v in water) with 80 μL of 500 mM buffer and 40 μL of sulfatase solution (600 μg mL⁻¹ in 50 mM Tris-HCl pH8). The solutions used to buffer the reactions were as follows: 500 mM...
Nuclear Magnetic Resonance (NMR) Spectroscopy

The c-carrageenan incubated with pure native Psc t-CgsA was freeze dried, exchanged twice with 99.97 atom % 2H2O and then dissolved in 2H2O at a final concentration of 10 mg mL−1. The product was then transferred into a 5 mm NMR tube and 1H-NMR spectra were recorded at 70°C, using a BRUKER Advance DRX 500 spectrometer equipped with an indirect 5 mm gradient probehead TXI H13C/31P. Chemical shifts are expressed in ppm in reference to trimethylsilylpropionic acid (TSP), which was used as an external standard. No suppression of the HOD signal was performed.

Sequence and Phylogenetic Analysis

Searches for protein sequence similarities were performed in Uniprot database using the BlastP program (Altschul et al., 1997). Protein structure prediction was performed with the Phyre2 server (Kelley and Sternberg, 2009). A multiple alignment was generated with 421 sequences using the MAFFT program and L-INS-i algorithm (Katoh et al., 2005) and manually refined. From the multiple alignments, 171 positions were used to build a phylogenetic tree by the Maximum Likelihood method using the Whelan and Goldman evolution model (Whelan and Goldman, 2001). Reconstruction of the tree and bootstrap analysis (resampling of 100) were conducted with the MEGA v. 5.05. Software (Tamura et al., 2011).

Results

Screening and Purification of the Psc t-CgsA

The marine bacterium P. carrageenovora was screened for potential sulfatase activity capable to catalyze the hydrolysis of sulfate from carrageenans. As a consequence the use of carrageenans as substrate was mandatory during the screening and the sulfate assay was performed by HPAEC as described previously (Genicot-Joncour et al., 2009). To avoid interference of ions with the detection of sulfate groups during chromatography analysis, P. carrageenovora strain PscT was grown in ZoBell medium.

Carrageenan-sulfatase activities were detected only when the culture medium was supplemented with κ-, τ- or λ-carrageenan. As reported in Table 1, carrageenan-sulfatase activities were measured both in the bacterial pellet and in the culture supernatant. τ- and λ-carrageenan proved to be good inducers of carrageenansulfatases that were active on κ-, τ- and λ-carrageenan. In this study, we focus our attention on the τ-carrageenan sulfatase activity detected in the pellet of P. carrageenovora cultures induced by the λ-carrageenan.

The newly detected enzyme, further on named Psc t-CgsA, was purified by a combination of ammonium sulfate fractionation, hydrophobic interaction chromatography and ion exchange chromatography. The first chromatographic step using hydrophobic interaction chromatography on phenyl-Sepharose was efficient in purifying the sulfatase as this one elutes between 91 and 78 mS cm−1, just before the majority of the proteins (Figure 2A). All the active fractions were pooled and dialyzed before being loaded on top of the anion exchange chromatography column (DEAE Sepharose). At this stage, the sulfatase eluted between 300 and 370 mM NaCl i.e., 29.5 and 32.3 mS cm−1 respectively (Figure 2B). The purified enzyme gave a single band with an apparent molecular mass of 110 kDa in SDS-PAGE under reducing conditions (Figure 2C).
IDENTIFICATION OF THE Psc ι-CgsA PROTEIN, A NOVEL SULFATASE

Following digestion of the pure Psc ι-CgsA protein by trypsin, the peptide fragments were analyzed by LC-MS/MS and compared to the Uniprot database. Eight peptides (YVEPTFSPDGK, VIENGVIITDGK, DLGEMFSPDGGR, SLGAGEWLYHK, YYYSHDATPGK, FTQNLDTDEFDVK, LLNSPAWSPDGDYLVAR, VSPDGQYLAFAER) were identical to peptides found in a putative uncharacterized protein Q3IKL4 (117.873 kDa), encoded in the genome of the marine strain P. haloplanktis TAC 125 (Medigue et al., 2005). In depth sequencing by MALDI-MS and by de novo allowed to cover 14 additional sequence stretches (LYSEHATEFRR, QQVIEAGR, TDVWNNHPR, AGENLS, IVYTTW, VLDG PL, WSLNPYSV, LTSGLA—QPR, QPQFG—DR, YELF-QYSR, ELF-QYSR, VTPFVE—LNSP, LF-A-TM—VGKK, ELETVL). Altogether, peptide sequencing of the Psc ι-CgsA provided coverage of 23% of the 1060 amino acids of the P. haloplanktis ι-CgsA protein (Figure 3). The sequence of the here described novel Psc ι-CgsA was further confirmed as described in the following section.

SEQUENCE DETERMINATION, CLONING AND OVEREXPRESSION OF THE ι-CgsA SULFATASE

The nucleotide sequence encoding the Q3IKL4 protein without the peptide signal was used to design specific oligonucleotide primers that were used to clone and sequence the gene encoding the Psc ι-CgsA (ι-cgsA) from P. carrageenovora. After successful expression and sequencing, the translation of the ι-cgsA gene yielded a protein of 1037 amino acids (GenBank accession number JN228253) with a theoretical isoelectric point (pI) value of 6.78 and a molecular weight of 115.9 kDa. This mass corresponds to the apparent molecular mass of the purified native and expressed Psc ι-CgsA (~110 kDa), deduced from SDS-PAGE. As expected, all the peptide sequences identified by mass spectrometry analysis were present in the protein sequence. The Psc ι-CgsA shares 92.6% sequence identity with the putative uncharacterized protein Q3IKL4 of P. haloplanktis TAC 125 (Figure 3).

Psc ι-CgsA was expressed as a soluble protein at about 6.5 mg L\(^{-1}\) in E. coli BL21 (DE3) when induced with 0.2% lactose (ZYP 5052 medium). The yield was more than doubled (16.9 mg L\(^{-1}\)) when induction was carried out with both lactose (0.6%) and IPTG (2 mM). After affinity chromatography on a HisPrep sepharose column, the enzyme was pure (Figure 2D). As shown on Figure 2D, the use of anti-histidine antibody allows recognizing the his-tagged protein which migrates as a single band at the expected size.

BIOCHEMICAL CHARACTERIZATION OF THE NATIVE Psc ι-CgsA PROTEIN

The purified sulfatase was kinetically evaluated using carrageenan as substrates. The amount of sulfate released after incubation of the pure sulfatase using ι-, κ- or λ-carrageenan was monitored by 1H NMR spectroscopy. The desulfation reaction of ι-carrageenan could be kept for several months at 4°C. The desulfation reaction of ι-carrageenan was slow, in the range 10–20% of the optimal activity, observed at 200 mM NaCl and pH optimum was measured at 8.3 (Table 2).

As shown in Figure 4, the desulfation reaction of ι-carrageenan by the Psc-ι sulfatase was slow, in the range of hours. The rate of sulfate release increased with the concentration of ι-carrageenan up to 0.6% (w/v). At higher concentrations the medium became very viscous interfering with the enzyme diffusion. When 0.5% (w/v) ι-carrageenan was used, the rate of desulfation was linear for the first 15 h. Under these experimental conditions the temperature optimum was determined to be 40 ± 5°C and the pH optimum was measured at 8.3 (Table 2). The Psc ι-CgsA could be kept for several months at 4°C at this pH. Addition of sodium chloride at high concentrations affects the enzyme activity. Indeed, at 500 mM NaCl, the activity drops to 17.5% of the optimal activity, observed at 200 mM NaCl (Table 2).

With the aim to determine the specificity of the ι-carrageenan sulfatase more precisely, the structural modification of ι-carrageenan was monitored, as a function of time, by 1H NMR (Figure 4). The signals of 1H NMR spectrum of the standard ι-carrageenan (Figure 4 top spectrum: 0 h) were attributed on the basis of previously reported analyses (van de Velde et al., 2002). The signal observed at 5.32 ppm corresponds...
to the anomic proton of the 4-linked anhydrogalactose unit (DA2S-H1). The G4S-H4 and DA2S-H3 protons, which signals are at 4.91 ppm and 4.85 ppm respectively, are also indicated on the spectrum. These signals decreased after 15 h of incubation of 41.25 mg of ι-carrageenan with 1.12 mg of ι-CgsA whilst two other signals, at 5.26 ppm and 4.80 ppm, appeared (Figure 4, middle spectrum: 15 h). To overcome heat inactivation of the enzyme, 300 μg of Psc ι-CgsA were added after 60 h. This addition, combined with prolonged enzymatic incubation (up to 120 h), allowed the complete modification of the ι-carrageenan (Figure 4, bottom spectrum: 120 h). Based on the analyses reported by Falshaw et al. (1996), this spectrum is characteristic of alpha (α)-carrageenan. Signals assignable to κ- (5.1 ppm) or beta (β)-carrabiose units (5.09 ppm) or to the production of reducing and non-reducing ends were not observed. This suggests that Psc ι-CgsA specifically catalyzes the conversion of ι-carrabiose into α-carrabiose units (Figure 1) within a polysaccharide chain and is devoid of glycoside hydrolase activity.

**BIOCHEMICAL CHARACTERIZATION OF THE RECOMBINANT ι-CgsA PROTEIN**

The purified recombinant ι-CgsA was able to specifically remove sulfate from ι-carrageenan. After 24 h of incubation at 35°C and pH 8.3, the amount of sulfate released represents roughly 3% of the total sulfate of the polymer. Although this is not sufficient to detect the conversion of ι-carrageenan into α-carrageenan by 1H NMR, this low but significant activity confirmed that the
**FIGURE 3** | Sequence alignment of Pscι-CgsA and Q3IKL4_PSEHT from *P. haloplanktis* TAC 125 using the program MEGA 5. The ESPript 3 program (Robert and Gouet, 2014) was used to enhance the conserved amino acids which are shown in white letters on a red background.
DISCUSSION

DISCOVERY OF A t-CARRAGEEAN SULFATASE, t-CgsA, SPECIFICALLY RELEASING SULFATE FROM THE D-GALACTOSE-4-SULFATE UNITS IN t-CARRAGEEAN

Many genes encoding sulfatases have been cloned from all kingdoms of life (Sasaki et al., 1988; de Hostos et al., 1989; Paietta, 1989; Yang et al., 1989; Hallmann and Sumper, 1994; Ferrante et al., 2002; Medigue et al., 2005; Sardiello et al., 2005; Frese et al., 2008), but the number of characterized sulfatases remains limited and does not reflect the huge chemical diversity of sulfated biomolecules. The recent discovery that a vast number of sulfatases are present in marine bacteria (Glöckner et al., 2003; Barbeyron, personal communication) highlights the untapped resource of this type of activity in the marine environment. Indeed, most of the characterized sulfatases are specific of metabolizing glycosaminoglycans (Buono and Cosma, 2010), due to their importance in pathogenicity or human health. Nevertheless, it has been shown that the sulfatase from Sphingomonas sp. AS6330 (Kim et al., 2004) and the arylsulfatase AtsA from P. carrageenovora Psc T6c (Lim et al., 2004; Kim et al., 2005) catalyze the desulfation of agar. Recently, a formylglycine-dependent endo-4S-t-carrageenan sulfatase from the marine bacterium P. atlantica T6c has been purified and characterized (Préchoux et al., 2013). In the present study we have identified, sequenced

| Characteristics | Native Psc t-CgsA | P. atlantica sulfatase | P. carrageenovora arylsulfatase |
|-----------------|------------------|------------------------|-----------------------------|
| Sulfatase family| New sulfatase family | Formylglycine family | β-lactamase superfamily New sulfatase family |
| Polysaccharide substrate | t-carrageenan | t-carrageenan | Agar |
| Molecular mass | 115.9 kDa | 55.7 kDa | 35.8 kDa |
| Theoretical pl | 6.8 | 7.1 | 5.4 |
| Optimal temperature | 35–45°C | 35°C | 45°C |
| Optimal pH | 8.3 | 7.5 | 7.0–8.5 |
| Optimal (NaCl) | 200 mM | ~25 mM | ~500 mM |
| EDTA 1 mM | 0% inhibition | Data not available | 53.2% inhibition |
| PMSF 1 mM | 91% inhibition | Data not available | 26% inhibition |
| Phosphate buffer | 82% inhibition | Data not available | Inhibition |

Both the native Psc t-CgsA and the t-carrageenan sulfatase from P. atlantica (Préchoux et al., 2013) were characterized using t-carrageenan as substrate. The data are compared to those of the arylsulfatase isolated from P. carrageenovora (Barbeyron et al., 1995; Lim et al., 2004; Kim et al., 2005).
and characterized a novel t-carrageenan sulfatase, Psc t-CgsA, first member of a new family of sulfatases. NMR studies of the reaction products unambiguously demonstrate that Psc t-CgsA catalyzes the removal of the sulfate ester group localized on the position C4 of the G4S moieties from the t-carrabiose units, leading to the formation of α-carrabiose units. Despite the complete absence of sequence homology, this activity appears to be the same as that already described for the formylglycine-dependent sulfatase from the marine bacterium P. atlantica T6c, indicating that this catabolic activity plays an important role in carrageenan metabolism. The desulfation is t-carrageenan specific, since neither the P. atlantica 4S-t-carrageenan sulfatase nor the here described Psc t-CgsA sulfatase are able to catalyze the removal of the 4S sulfate group from t-carrabiose units. In κ-carrageenan, the G4S residues are located between two neutral anhydrogalactose residues whilst in t-carrageenan the G4S moieties are positioned between two sulfated anhydrogalactose in their substrate binding site to display the observed activity.

### Table 3 | Characterization of the native and the recombinant Psc t-CgsA.

#### (A) BIOCHEMICAL CHARACTERIZATION

| Characteristics | Native Psc t-CgsA | Recombinant Psc t-CgsA |
|-----------------|-------------------|-----------------------|
| Optimal temperature (°C) | 35–45 | 35 |
| Optimal pH | 8.3 | 8.4 ± 0.3 |
| Optimal [NaCl] (mM) | 200 | 100 |
| EDTA 10 mM | 132.8% ± 12.9 | 93.6% ± 3.3 |
| EGTA 10 mM | 125.5% ± 9.2 | 100.9% ± 8.5 |
| MgCl2 up to 2 mM | 378% ± 2.8 | 81.7% ± 2.5 |
| DTT 1 mM | 46.8% ± 4.2 | 74.8% ± 6.9 |
| PMSF 2 mM | 0% | 34.1% ± 13.4 |

Characterization of the native enzyme has been performed using t-carrageenan whereas MUFS was used for the recombinant enzyme. As stated in material and methods, incubation with t-carrageenan was performed during 12 h whilst kinetics with MUFS were carried out for 2 h. Unless otherwise stated, the activity is expressed as the percentage of the initial activity observed without addition of reagents.

#### (B) KINETIC PARAMETERS

| Enzyme | Apparent Km (μM) | kcat (min⁻¹) | Kcat/Km (μM⁻¹ min⁻¹) |
|--------|------------------|--------------|-----------------------|
| Native Psc t-CgsA | 21.9 ± 8.2 | 6.32 ± 0.38 | 0.280 ± 0.05 |
| Recombinant t-CgsA | 13.4 ± 4.9 | 1.64 ± 0.23 | 0.12 ± 0.04 |
| Arylsulfatase from P. carrageenovora | 68 | ND | ND |

Preliminary kinetic parameters of the native and recombinant Psc t-CgsA were measured using the optimal reaction conditions of the enzymes as denoted above and the artificial substrate MUFS at concentrations ranging from 5.2 to 206.5 μM. Data are compared to that of the P. carrageenovora arylsulfatase (Barbeyron et al., 1995) using the same substrate. N.D. means not determined.

### THE t-CARRAGEENAN SULFATASE t-CgsA IS A MODULAR ENZYME BELONGING TO A NOVEL CLASS OF SULFATASES

Despite their identical substrate specificity, the t-carrageenan sulfatases of P. carrageenovora PscT and P. atlantica T6c have very different biochemical characteristics (Table 2) and can also be distinguished by their primary sequence that do not share enough similarity to allow an accurate sequence alignment. The t-carrageenan sulfatase from P. atlantica T6c is indeed a formylglycine sulfatase and in this respect it has the characteristic signatures of family 1 formylglycine-dependent sulfatases, namely [SAPG]-[LIVMST]₁-[CS]₁-[STACG]₁-[P]₁-[STA]-R-x(2)-[LIVMFYW]₁(2)-[TAR]-G and G-[YV]-x₁-[ST]-x(2)-[IVAS]-G-[K]-x(0,1)-[FYWMK]₁-[HL]₁ (Prosite signatures PS00523 and PS00149 respectively), which are well conserved in this family of enzymes. These signatures are not present in the Psc t-CgsA sulfatase, thus indicating that this latter enzyme does not belong to the well-defined and represented family of formylglycine-dependent sulfatases. Sequence alignment of the Psc-t sulfatase with the protein Q3IKL4 from P. haloplanktis and 9 other proteins chosen amongst the ones representing the new sulfatase family revealed that no cystein amino acids are conserved (Supplementary Figure 1). However, several conserved serine could potentially be subject to post-translational modification. Therefore, and based on sequence data alone, it cannot be completely excluded (although improbable) that this sulfatase potentially reveals the existence of a new formylglycine-dependent family. It would however involve a serine modification. Interestingly, a t-carrageenan sulfatase was revealed to be very active in P. haloplanktis 545T extracts. This enzyme was induced in similar conditions to those observed for induction of the Psc t-CgsA (Table 1, data in brackets). Since the genomes of both sequenced strains of P. haloplanktis TAC 125 (Medigue et al., 2005) and P. haloplanktis ANT/505 (GenBank ADOP00000000.1) do not contain any formylglycine-dependent sulfatase genes, it is tempting to assume that the sulfatase activity detected in P. haloplanktis 545T is due to a t-CgsA-type enzyme, such as in P. carrageenovora. Indeed, as described above, proteins orthologous to Psc t-CgsA are present both in P. haloplanktis TAC 125 and ANT/505.

A BlastP sequence similarity search with the Psc t-CgsA used as query sequence against the Uniprot database revealed that Psc t-CgsA showed more than 90% identity with putative uncharacterized proteins from several marine species of Pseudoalteromonas. Moreover, many proteins from different marine strains such as Colwellia psychrerythraea 34H (Methe et al., 2005) and Shewanella sediminis HAW-EB3, belonging to the amidohydrolase superfamily, exhibited more than 60% of identity with the Psc t-CgsA sulfatase (Supplementary Figure 1). Three-dimensional structure modeling of Psc t-CgsA using the Phyre2 (Kelley and Sternberg, 2009) tool reveals that Psc t-CgsA most likely has a multi-modular arrangement that covers 94% of the residues and are modeled with more than 90% of confidence. From this model it is hypothesized that Psc t-CgsA consists of an N-terminal module (from residues 24 to 618 approximately) featuring a first α-propeller fold composed of six blades (from residues 24 to 300 approximately), and a second β-propeller fold composed of seven blades (from residues 310 to 618 approximately). This type of organization has already been observed among carbohydrate processing
enzymes, such as glycoside hydrolases from the clans GHE, GHF, GHJ, and for κ-carrageenases (Guibet et al., 2007), but also in the PL11 family of polysaccharide lyases involved in the degradation of anionic polysaccharides. The C-terminal module comprising amino acids 663–1060 in Psc ι-CgsA displays a typical TIM-barrel fold found in the amidohydrolase superfamily. This superfamily includes an outstanding set of enzymes that catalyze the hydrolysis of a wide range of substrate having amide or ester groups. For example, urease, amidohydrolase, guanine deaminases and phosphoesterase share the same three-dimensional fold describing this superfamily (Seibert and Raushel, 2005).

A phylogenetic analysis of the C-terminal module found in Psc ι-CgsA and in 420 sequences of enzymes classified as belonging to the amidohydrolases superfamily showed that Psc ι-CgsA was not related to any known activity in this superfamily (Figure 6). In contrast, it appears that Psc ι-CgsA belongs to a clade composed of uncharacterized proteins only. In this cluster, a sub-clade supported by a node with a relatively good bootstrap value (73%) is composed of 122 sequences displaying the same modular organization as Psc ι-CgsA (i.e., the presence of β-propeller modules at the N-terminal extremity) (Supplementary Table 1). The topology of the phylogenetic tree suggests that Psc ι-CgsA and these 122 sequences represent a novel family of sulfatases, which might have different substrate specificities, as supported by the low percentage of sequence identity between some proteins within this clade.

**PRODUCTION AND ANALYSIS OF THE RECOMBINANT ι-CARRAGEENAN SULFATASE**

In addition to the identification of the native Psc ι-CgsA enzyme, the gene coding for Psc ι-CgsA was successfully cloned and heterologously expressed in E. coli, although the purified recombinant enzyme displayed lower activity on the natural substrate than the native form. The biochemical characteristics of the native and the recombinant enzymes, measured on the artificial substrate MUFS, are very similar (Table 3A), but the kcat of the native enzyme is roughly about 3 times that of the recombinant enzyme (Table 3B). The slightly different biochemical behavior of native vs. recombinant enzyme might be due to intrinsic elements in the native protein that E. coli does not supply, such as potential post-translational modifications and/or the requirement of a cofactor or a chaperone during protein folding, preventing the production of a fully active recombinant enzyme. Future work and a more detailed biochemical depiction of different recombinant homologs of this novel sulfatase family are necessary, and will shed more light on the precise catalytic mechanism and mode of action of this newly discovered enzyme family.

**DECIPHERING A NEW PATHWAY FOR CARRAGEENAN BIODEGRADATION**

The degradation pathway of κ-carrageenan by P. carrageenovora PscT has been partially determined and involves a κ-carrageenase (Weigl and Yaphé, 1966a; McLean and Williamson, 1979a; Barbeyron et al., 1994; Michel et al., 2001) to produce oligosaccharides which are readily degraded into neocarrabiose through the concerted action of a glycosulfatase (Weigl and Yaphé, 1966b; McLean and Williamson, 1979b) and a neocarratetraose monosulfate hydrolase (McLean and Williamson, 1981). For the catabolism of ι-carrageenan a similar pathway might be assumed as a low ι-carrageenase activity has been detected in the crude extract of P. carrageenovora PscT (Hñares et al., 2010). However, based on our results with Psc ι-CgsA, we propose an alternative mechanism in which ι-carrageenan is first desulfated and converted into α-carrageenan. The latter likely constitutes a metabolic intermediate probably subject to further degradation by an α-carrageenase and/or desulfation by other sulfatases, leading to the end-product of galactose residues. Except for the conversion of ι into α-carrageenan, these steps are speculative and need to be corroborated.

**BIOCONVERSION OF RED ALGAL POLYSACCHARIDES**

α-Carrageenan has been observed in the cell wall of several red algae such as Catenella nipae Zanjardini (Zablackis and Santos, 1986), Sarconema filiforme (Sonder) Kylin (Chiovitti et al., 1998; Kumar et al., 2011) and in some Solieria spp. (Chiovitti et al., 1997; Bondu et al., 2010). Although structural studies revealed
FIGURE 6 | Phylogenetic tree of the amidohydrolase module present in Psc i-CgsA and in 420 sequences of enzymes classified as amidohydrolases. The maximum likelihood tree was built with the MEGA v. 5.05. Software, using the substitution model WAG. Evolutionary rate differences among sites (5 categories) were modeled using a discrete Gamma distribution, from an alignment produced by the MAFFT program with the Li-INSi algorithm (Katoh et al., 2005). Phylogenetic analysis was performed using 171 positions, defined from the multiple sequence alignment. All ambiguous positions were removed for each sequence pair. Only the bootstrap values higher than 40 are shown. The potential new sulfatase family (in red) was delimited on the basis of sequence similarities to the here identified sulfatase and the bootstrap value (73) of the best node (the deepest, the first red node) in the tree. Numbers in brackets indicate the number of sequences involved in each group.
that this carrageenan referred to as hybrids of α-1/κ-carrageenan in which the α-carrabiose content does not exceed 30–40% (mol/mol) (Falshaw et al., 1996), it has been shown that the sodium salt of such hybrids exhibit twice the capacity to suspend cacao particles in milk compared to κ-carrageenan which is commonly used for that purpose (Zabrackis and Santos, 1986). As suggested by Figure 4, the Psc-1-CgsA sulfatase could be used to produce carrageenan with controlled ratio of 1/α-carrabiose and even pure α-carrageenan. It is therefore more than likely that new hybrids of α-1/κ-carrageenans or pure α-carrageenan will harbor new and interesting functional properties.

Recent marine genomic projects have shown that marine bacteria are a potential source of large sulfate diversity, with the presence of huge multigenic sulfate families, as exemplified by the formylglycine-dependent sulfatase family (up to 300 genes in the Lentinus araneus HTCC2155T genome) (Thrash et al., 2010). Since the origin of these bacteria is marine, it is tempting to assume that a large portion of these enzymes are involved in the degradation of sulfated polysaccharides from marine algae (Glöckner et al., 2003). In addition to these sulfatases accessible through genome mining, our work demonstrates that the sulfate diversity extends even beyond those that have already characterized members. By screening a marine bacterium for sulfate activity on carrageenan substrate, we have successfully identified and characterized a new sulfatase family, members of which were annotated “hypothetical proteins with unknown function” before hand. Altogether, these data open up a large field for future work in knowing respectively. We also thank Dr. Fanny Gaillard for her help with the mass spectrometry analyses. Special thanks also to Nelly Kervarec for excellent technical assistance in mass spectrometry analyses. Special thanks also to Nelly Kervarec for excellent technical assistance in mass spectrometry analyses. Special thanks also to Nelly Kervarec for excellent technical assistance in mass spectrometry analyses. Special thanks also to Nelly Kervarec for excellent technical assistance in mass spectrometry analyses. Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fchem.2014.00067/abstract

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