

Three-dimensional structures, dynamics and calcium-mediated interactions of the exopolysaccharide, Infernan, produced by the deep-sea hydrothermal bacterium Alteromonas infernus

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Olga Makshakova, Agata Zykwinska, Stephane Cuenot, Sylvia Colliec-Jouault, Serge Perez. Threedimensional structures, dynamics and calcium-mediated interactions of the exopolysaccharide, Infernan, produced by the deep-sea hydrothermal bacterium Alteromonas infernus. Carbohydrate Polymers, 2022, 276, pp.118732. 10.1016/j.carbpol.2021.118732 . hal-03426167

HAL Id: hal-03426167 https://hal.science/hal-03426167

Submitted on 12 Jul2022

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1 Three-dimensional structures and calcium-mediated interactions of the exopolysaccharide, 2 Infernan, produced by *Alteromonas infernus*

3

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21 Highlights

- Complete determination of the 3D structure of the anionic EPS Infernan by MM and MD
 calculations
- Clustering of the conformations into families having either extended two-fold or five-fold
 helical structures
- Identification, geometry and energetics of Ca²⁺ binding cavities via DFT calculations
- Five-fold helical structure as Ca²⁺ a binding reservoir
- Two-fold helices form « junction zones » with Ca²⁺throughout a heterotypic mode of binding
- 29

30 Abstract

31 The exopolysaccharide Infernan, from the bacterial strain GY785, has a complex repeating unit of nine monosaccharides established on a double-layer of sidechains. A cluster of uronic and sulfated 32 33 monosaccharides confers to Infernan functional and biological activities. We characterized the 3-34 dimensional structures and dynamics along Molecular Dynamics trajectories and clustered the 35 conformations in extended two-fold and five-fold helical structures. The electrostatic potential 36 distribution over all the structures revealed negatively charged cavities explored for Ca²⁺ binding 37 through quantum chemistry computation. The transposition of the model of Ca²⁺complexation 38 indicates that the five-fold helices are the most favourable for interactions. The ribbon-like shape of 39 two-fold helices brings neighbouring chains in proximity without steric clashes. The cavity chelating 40 the Ca²⁺ of one chain is completed throughout the interaction of a sulfate group from the neighbouring 41 chain. The resulting is a 'junction zone' based on unique chain-chain interactions governed by a

42 heterotypic binding mode.

43 **Key Words:** ExoPolysaccharides, *Alteromonas infernus;* 3 Dimensional Structures, Gel

44 forming, Molecular Dynamics, Quantum chemistry, Calcium Binding,

45 Introduction

In 1977, discovering the first deep-sea hydrothermal vents on the Galapagos rift changed our view of biology. It raised the question of the ability of vent organisms to survive and thrive in such extreme pressures and temperatures in the presence of toxic mineral plumes. The conversion of mineral-rich hydrothermal fluid into energy is a crucial feature of these environments. Through chemosynthesis, which converts carbon dioxide into sugar in the absence of light, bacteria provide energy and nutrients to vent species without the need for sunlight. (Dick, 2019)

52 In 1991, the submersible « Nautile » collected samples of vent fluids, chimney rocks, 53 invertebrate tissues, sediments and seawater at a depth of 2000 m in a rift system of the Guaymas 54 basin (Guezennec, 2002). The collection took place in the proximity of the active hydrothermal vent 55 named « Claire ». The bacterial strain, labelled as GY785, was isolated from a diluted vent fluid among 56 a dense population of tubeworms Riftia pachyptila (Raguénès et al., 1997). Phylogenetic analysis 57 showed that the new bacterium belongs to the gamma subdivision of the Proteobacterial phylum. The 58 bacterium belongs to the monophyletic unit that defines the true genus Alteromonas. The Gram-59 negative genus Alteromonas belonging to the Alteromonadaceae family (Baumann, Baumann, 60 Bowditch, & Beaman, 1984) was named Alteromonas infernus (Raguénès et al., 1997).

61 This mesophilic gram-negative bacterium GY785 secretes two different exopolysaccharides 62 (EPS), a water-soluble EPS (EPS1) and a non-soluble EPS (EPS2) embedded in a gelatinous matrix 63 containing bacterial cells (Raguénès et al., 1997). The GY785 soluble EPS is called Infernan. Infernan comes from the contraction of 'Infern' (from infernus) followed by the suffix 'an', which refers to its 64 65 nature as glycan. Infernan in its native form is a high-molecular-weight, highly-branched anionic 66 heteropolysaccharide. Structural analyses revealed that its repeating unit consists of nine osidic 67 residues assembled in a complex structure (Figure 1; Roger et al., 2004). Three osidic residues compose 68 the EPS main chain, glucose (Glcp), galacturonic acid (GalpA-2S) and galactose (Galp), which are 69 covalently linked in the following sequence: \rightarrow 4)- β -D-Glcp-(1 \rightarrow 4)- α -D-GalpA-2S-(1 \rightarrow 4)- α -D-Galp-(1 \rightarrow . The GalpA residue of the main chain carries at O-2, one sulfate group. It is substituted at O-3 by a short 70 71 side chain constituted of two glucuronic acids (GlcpA), Galp and Glcp linked in the sequence: β-D-Glcp-72 $(1\rightarrow 6)-\alpha$ -D-Galp- $(1\rightarrow 4)-\beta$ -D-GlcpA- $(1\rightarrow 4)-\beta$ -D-GlcpA- $(1\rightarrow . The two GlcpA of the side chain are each$ 73 substituted by a terminal Glcp.

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75

76

Figure 1. The repeating unit of Infernan (GY785 EPS) (Roger et al., 2004).

77 The chemical composition of Infernan, with uronic acids and sulfate groups and its particular 78 structure, is unique in the occurrence of voluminous side chains linked to a backbone made up of a 79 monosulfated trisaccharide repeat. Among the soluble and capsular EPS structures established from 80 marine bacteria and archaebacteria, Infernan displays the most complex sequence of 81 monosaccharides (Toukach & Egorova, 2016; Casillo, Lanzetta, Parrilli, & Corsaro, 2018; Birch, Van 82 Calsteren, Perez, & Svensson, 2019). The occurrence of a cluster of negatively charged 83 monosaccharides confers to this EPS several potential functional properties, such as 84 glycosaminoglycan (GAG) activities, explored in tissue engineering and cancer treatment (Colliec-85 Jouault et al., 2001; Merceron et al., 2012; Heymann et al., 2016; Rederstorff et al., 2017; Ruiz Velasco, et al., (2011)) as well as gelling properties with calcium (Ca²⁺), exploited to develop growth factor 86 87 delivery systems (Zykwinska et al., 2019). The formation of gel from Internan chains in the presence 88 of calcium has been observed and further characterized by AFM (Zykwinska et al., 2019). Also, the high 89 content of uronic acids correlated to the metal-binding ability makes this EPS a good candidate for 90 waste-water treatment, metal recovery and strontium therapy.

Recent advances in the computational modelling of polysaccharides have reached a point where highly complex structures can be investigated through a series of complementary methods (Perez, Fadda, & Makshakova, 2021). They allow establishing structures and dynamics as well as intermacromolecular interactions. As for polysaccharides, it is challenging to sample the conformational state and transitions in solution experimentally because of their high flexibility. The computational methods offer a way to sample both long-living and short-living conformations via molecular dynamic

97 simulations. It enables the description of the processes of chain interactions, which may result in 98 polymeric entanglements and other soft matter state formation. The interactions of linear anionic 99 polysaccharides containing uronic residues, such as pectins or alginates, with Ca²⁺ and other ions with 100 the eventual chain assembly in the form of "egg-box" have been a topic several computational studies. 101 (Braccini & Pérez, 2001; Hecht & Srebnik, 2016; Plazinski, 2011; Plazinski & Rudzinski, 2012; 102 Stewart, Gray, Vasiljevic, & Orbell, 2014; Hecht & Srebnik, 2016; Maire du Poset, Zitolo, Cousin, 103 Assifaoui, & Lerbret, 2020; Cao, Lu, Mata, Nishinari, & Fang, 2020). Another type of anionic 104 polysaccharide, carrageenans, carry sulfate groups. The carrageenans have a distinct type of chain 105 complexation. Counter-ions mediate them by cross-linking helical chains as molecular dynamic 106 simulations resolve (Perez & Claudio, 2020). However, as reported for chondroitin sulfate, a linear 107 polysaccharide from the GAG family that bears both carboxylate and sulfate functional groups, the 108 subtle differences in force field parametrization may have a drastic effect on counter-ion binding and 109 the consequent interactions mediated by them as seen in molecular dynamic simulations (Guvench & 110 Whitmore, 2021).

111 The present article describes for the first time the conformational flexibility of the highly-112 branched anionic EPS, Infernan, which is characterized by diverse monosaccharides in the backbone 113 and sidechains and bears both carboxylate and sulfate groups. It establishes the calcium-binding sites 114 and the ability for Infernan to form gels in the presence of divalent cations such as Ca²⁺, which is the 115 key to biological functions and technological applications.

116

117 Material and Methods

118 **Conformational descriptors of the constituting monosaccharides.** The conformational descriptors of 119 the monosaccharides include the shape of the rings and the orientations of chemical groups. The Φ 120 and Ψ torsion angles dictate the orientation of the two successive monosaccharides linked by a 121 glycosidic bond in a disaccharide. In crystallography, Φ is defined as $\Phi = 05$ -C1-O1- Cx, and Ψ as $\Psi =$ 122 C1-O1-Cx-Cx+1, where x is the number of the carbon atom of the second monosaccharide with which 123 the 1-x glycosidic bond is formed. An alternate definition used in NMR spectroscopy refers to the 124 budgesen stars with $\Phi^{H} = 141$ C1 O1. Gr and $W^{H} = C1$ O1 Gr LW

124 hydrogen atoms with Φ^{H} = H1-C1-O1- Cx and Ψ^{H} = C1-O1-Cx-Hx

125 Molecular mechanics calculations and potential energy surfaces (PES) The geometry optimization of 126 the monosaccharides and the eight disaccharides was performed with the MM3 force field with proper 127 parametrization for carbohydrates containing sulfate groups (Allinger & Yan, 1993; Le Questel, Cros, 128 Mackie, & Perez, 1995). The calculations and plots of the conformational maps (PHI/PSI maps) were 129 performed using the Conformational Analysis Tool (CAT) software (http://www.md-130 simulations.de/CAT/, Martin Frank, martin@glycosciences.org, May 2012) with the TINKER (Harger et 131 al., 2017) program to minimize, optimize and run molecular dynamics calculation usually for a 50 ns 132 simulation at 700 K.

- 133 Initial models of oligosaccharide models. Four types of oligosaccharides were constructed:
- 134 1. The linear backbone of two repeats of the elementary block $(\rightarrow 4)$ - β -D-Glcp- $(1\rightarrow 4)$ - α -D-GalpA-135 $(1\rightarrow 4)$ - α -D-Galp- $(1\rightarrow)_2$;
- 1362. The backbone of two repeats, branched with one linear side chain α-D-Galp-(1→4)-β-D-GlcpA-137(1→4)-β-D-GlcpA-(1→ attached to the O-3 of GalpA ;
- 1383. The side chain branched with terminal glucose residues α-D-Galp-(1→4)-β-D-GlcpA[2 α-D-139Glcp](1→4)-β-D-GlcpA[3 α-D-Glcp]-(1→3)-α-D-GalpA-2S ;

- 4. Branching point extended with branched side chain β-D-Glcp- α -D-GalpA-2S[α -D-Galp-(1 \rightarrow 4)-β-D-
- 141 GlcpA[2 α-D-Glcp](1 \rightarrow 4)-β-D-GlcpA[3 α-D-Glcp]-(1 \rightarrow 3)]-α-D-Galp. Such a variety of initial structures is

aims at unravelling the long-distance conformational influence of branching.



143

Figure 2. Schematic representation of the repeating unit of Infernan. The structural elements
considered in the present study use the Symbol Nomenclature for Graphical Representations of Glycans
(Varki et al., 2015).

147

148 Molecular Dynamics and Mechanics calculations. Molecular Dynamics (M.D.) simulations started 149 from the initial structures immersed in a water box with periodic boundary conditions. Na⁺ ions were 150 added to the system to neutralize the negative charges of Infernan. The simulations were carried out 151 using program package AMBER12 (Case et al., 2012) in the isotherm isobar thermodynamic ensemble 152 at 300K, using the force-field parameters for GLYCAM06 for saccharides (Kirschner et al., 2008). The 153 AMBER force field was used for ions and the TIP3 model for water molecules. A time step of 2 fs was 154 applied with the SHAKE algorithm (Ryckaert, Ciccotti, & Berendsen, 1997) constrained bonds involving hydrogen atoms. Particle Mesh Ewald (PME) (Essmann et al., 1995) was used to handle long-range 155 156 electrostatic interactions. A cut-off value for electrostatic interactions was used 8Å in simulations; a 157 rectangular water box was selected, having dimensions commensurate to the size of the molecule under investigation. The temperature and the pressure were kept constant using a Langevin 158 thermostat with a collision frequency of 2 ps⁻¹ and a weak coupling anisotropic algorithm with a 159 relaxation time of 2 ps⁻¹, respectively. The system was optimized stepwise, with restraints on all atoms 160 of the complex gradually decreasing from 20 to 5 kcal mol⁻¹ Å⁻². Each minimization step was followed 161 162 by 20 ps of molecular dynamics equilibration (NVT, 100K) with the same restraints. The system was then heated from 100 to 300K using a Langevin thermostat with a collision frequency of 5 ps⁻¹. Initial 163 164 velocities were derived from a Maxwellian distribution at 100K using a random seed for each simulation. Then, ten ns equilibration phases in the NVT and NPT ensemble were then run. Finally, the 165 166 production phases were performed on totally relaxed systems in the NPT ensemble at 300K with 167 trajectories in the range of 100 ns and output frequency of 10 ps. The Schrödinger Release 2021-3: 168 Maestro, Schrödinger, LLC, New York, NY, 2021, was used for Molecular Mechanics calculations.

DFT calculations. The complexes of Infernan with Ca²⁺ ions were calculated to estimate the location of 169 the binding site with a maximum calcium coordination number. The location of putative Ca²⁺ binding 170 171 sites derived from the examination of the electrostatic potential of Infernan was calculated using the Avogadro program (Hanwell et al. 2012) (see Figure Supplemental Material). Then, one Ca²⁺ ion was 172 173 placed in the vicinity of the sulfate group or carboxylate group of α GalpA-2S residue in tetrasaccharide 174 and further optimized. The calculations were carried out at the DFT level using B3LYP hybrid density 175 functional (Becke, 1983; Lee, Yang, & Parr, 1988), widely employed for the description of complexes 176 of biological molecules (Makshakova, Faizullin, & Zuev, 2020; Bekri, Zouaoui-Rabah, Springborg, & 177 Sekkal Rahal, 2018; Makshakova, Chachkov, & Ermakova, 2011; Yousfi, Sekkal-Rahal, Sayede, & 178 Springborg, 2010; Cao, Lu, Mata, Nishinari, & Fang, 2020). Two types of calculations were implemented 179 for all the systems studied: 1. with the 6-31G(d,p) basis set in vacuum and 2. 6-31G+(d,p) with the 180 polarized continuum conditions with SMD variation of integral equation formalism variant (IEFPCM) 181 was used to take the solvation effects into account implicitly (Marenich, Cramer, & Truhlar, 2009). All 182 calculations were carried out using the Gaussian09 program (Frisch et al., 2009)

183 Results

184 Building and displaying the three-dimensional components of Infernan. A prerequisite to explore and 185 build the three-dimensional structures of Infernan requires the computation of the potential energy 186 surfaces of each constituting disaccharide component. The determination of conformational 187 preferences of the constituting disaccharides was first performed by characterizing their preferred 188 conformations on potential energy surfaces as a function of their glycosidic torsional angles Φ and Ψ . Due to its occurrence as a terminal residue of the side chain, we did not consider the β 1-6 linked Glcp. 189 190 Its location at the periphery of the repeating unit does not impact the 3D features of Infernan. 191 The potential energy surfaces of the eight disaccharides occurring in Infernan were built. A typical

conformational map displaying the shape of the low energy region (up to 10 kcal/mol concerning the lowest energy minimum), the positions of energy minima, the routes for interconversion between conformers and the heights of the transitional barriers were obtained for each disaccharide. These maps display similar features, i.e., the occurrence of a low energy basin embedding one or two low-

196 energy conformations (Figure 3).



197

Figure 3. Potential Energy Surfaces of the constituting disaccharides of Infernan, (a) α -D-Galp-(1 \rightarrow 4)- β -D-Glcp; (b) β -D-Glcp-(1 \rightarrow 4)- α -D-GalpA-2S; (c) α -D-GalpA-2S-(1 \rightarrow 4)- α -D-Galp; (d) β -D-GlcpA-(1 \rightarrow 3)- α -D-GalpA-2S; (e) α -D-Galp-(1 \rightarrow 4)- β -D-GlcpA; (f) β -D-GlcpA-(1 \rightarrow 4)- β -D-GlcpA; (g) β -D-Glcp-(1 \rightarrow 2)- α -D-GalpA; (h) β -D-Glcp-(1 \rightarrow 3)- α -D-GalpA. The iso-contours are drawn at 1 kcal intervals with respect to the energy minimum of the corresponding map. The stars indicate the location of the low energy conformers as determined from Molecular Dynamics performed on the whole repeating unit of Infernan (see text for explanation).

205 Building and display of the three-dimensional structural models of Infernan. The construction of 206 three-dimensional structures of Infernan was performed using the polysaccharide builder program 207 POLYS (Engelsen, Hansen, & Perez, 2014; Perez & Rivet, 2022). It is based on an algorithm designed to 208 use optimized monosaccharide structures and connect them using established energetically 209 favourable geometries. This approach was previously used and efficiently built reliable complex 210 carbohydrate structures (Perez & Tvaroska, 2014). Steric conflicts rarely occur when using energetic 211 minima derived from conformational analysis of disaccharides. To construct the initial structure of 212 Infernan's fragments, the values corresponding to the global minima on the were Potential Energy 213 Surfaces were assigned to the glycosidic linkages.

214 **Conformational variability of polysaccharides.** The results confirm that two of the glycosidic junctions: 215 α GalpA-2S(1 \rightarrow 4) α Galp and α Galp(1 \rightarrow 4) β Glcp), within the backbone, do not significantly change during the length of the trajectories. The oscillations around their average values do not exceed 10-216 217 20°. In contrast, the β Glcp(1 \rightarrow 4) α GalpA-2S linkage oscillates between two conformational states 218 (Figure 4 and Figures S1; S2). The transitions occur in a concerted way, with the correlation coefficient 219 of 0.7 (the correlation coefficient may vary from -1 to +1, the larger the value of the modulus of 220 magnitude is, the more concerted two events are). Therefore, we will consider two conformational 221 The most populated state (-74±16; 153±13) has a longer lifetime of about a dozen states. 222 nanoseconds, occurring 70±15% of the time. The less populated state (-148±12; 114±12) lives for 2-3 223 ns and a 30±15% occupancy. They will be referred to as Model 1 and Model 2.



Figure 4. Evolution of the β Glcp(1-4) α GalpA-2S and β GlcpA(1-3) α GalpA-2S linkages over a 100 ns trajectory.

The conformations of two of the glycosidic junctions within the side chain $(\alpha \text{Galp}(1 \rightarrow 4)\beta \text{GlcpA} \text{ and} \beta \text{GlcpA}(1 \rightarrow 4)\beta \text{GlcpA})$ remain stable and oscillate around their conformation, no more than 10-20°. The conformation of the glycosidic linkage at the branching point $(\beta \text{GlcpA}(1-3)\alpha \text{GalpA}-2S)$ experiences transitions between two states (Φ, Ψ) =(-75±12; 133±13) and (Φ, Ψ) = (-149±16; 85±19) that occur with the correlation coefficient 0.7; the respective lifetime being 10ns and 2-3ns (Figure 4). They are referred to as Model A and Model B.

The conformations of the two α Glcp in side chains α Glcp $(1 \rightarrow 2)\beta$ GlcpA and α Glcp $(1 \rightarrow 3)\beta$ GlcpA 233 oscillate between two low energy regions (Figure 3g, 3h), without any influence on the conformational 234 equilibrium of the backbone. Each of them can adopt two sets of Φ, Ψ torsion angles (deduced from 235 236 correlated changes of Φ and Ψ angles. The population of the two states for both linkages relates as 237 70±15% to 30±15% (Table S1). The α Glcp(1 \rightarrow 3) β GlcpA linkage demonstrates the highest lifetimes of 238 each state compare to other linkages in this polysaccharide. The lifetime of these conformations 239 amounts up to 10 ns. It results from competitive hydrogen bonds between OH-2 and OH-4 groups of 240 α Glcp with one of the O6 of the neighbouring β GlcpA. As the conformational transitions in the backbone and the branching points occur independently, the overall 3D structures of Infernan can be 241 242 described based on only four models from Models 1 and 2, namely M1A, M1B, M2A and M2B.

Further propagation of the polysaccharide backbone in a repetitive fashion reveals two types of helical conformations. These are two-fold and five-fold helices (Figure 5) based on the results derived from the molecular dynamics simulation. The former is more populated, resulting in 70±15% occupancy over the trajectory. The latter is less occupied presenting only in 30±15% of simulation time. Overall the side chains are oriented, either in the direction of the backbone or perpendicular to it.

The end-to-end distance in the backbone extended in the five-fold helix motif of the five-mer is 248 67.5±2.2 Å. It gives an axial rise of 13.6±0.4 Å per repeating unit (α Galp(1 \rightarrow 4) β GlcpA-2S(1 \rightarrow 4) β GlcpA) 249 or 4.5±0.14 Å per monosaccharide residue. It indicates an almost total elongation. The overall 250 backbone extension of 67.5±2.2 Å does not change when the conformational transition brings the 5-251 252 fold helix to the 2-fold helix. In the 2-fold conformation, the overall structure has the shape of a brush. 253 The side chains alternate their orientation by 180°, separated by a distance of 27.7±0.7 Å. The height 254 of such polysaccharide calculated as a distance on the projection between distant α -Galp residues is 255 around 33 Å. In this conformation, the width of the polysaccharide is about 8 Å.

256 Without influencing the conformations of Model 1 to 4, the four possible combinations resulting from

the two α linked terminal Glcp monosaccharides enhance a volume of 20 Å diameter the space occupied at the branching point β GlcpA(1 \rightarrow 3)αGalpA-2S (Figure S3).



259

260

Figure 5. Three-dimensional representation of the four structures of Infernan.

261 **Ca²⁺ binding at the branching point.** The computation of the electric potential of each low energy 262 model, as calculated from the Avogadro program, gives clear indications of the occurrence of a 263 negatively charged region around the branching points (Figure S4). Such a feature results from the 264 vicinity of sulfate and several carboxylate groups of Infernan and indicates regions for the location of Ca^{2+} . The calcium ion was placed next to COO⁻ or SO3⁻ fragments of α GalpA-2S of tetramer in the 265 backbone branching point. The detailed structural organization of Ca²⁺ in the combining site was 266 optimized throughout quantum mechanics methods following the protocol described in the Material 267 268 and Methods. To avoid potential effects of the monosaccharide units at extremities, we restained the Φ, Ψ angles of the backbone linkages β Glcp $(1 \rightarrow 4)\alpha$ GalpA-2S and α GalpA-2S $(1 \rightarrow 4)\alpha$ Galp to those in 269 two-fold and five-fold conformation (which are respectively (-55, 153); (84, 131) and (-148, 114); (84, 270 271 131)). Only β GlcpA(1 \rightarrow 3) α GalpA-2S linkage connecting the side chain and the backbone was allowed 272 to adjust to accommodate fully Ca²⁺ binding. The results show that Ca²⁺ can interact, either with the

- $\label{eq:carboxylate} 273 \qquad \mbox{two carboxylate groups belonging to $$\beta$GlcpA and αGalpA-2S or with the carboxylate group from $$\beta$GlcpA$}$
- and a sulfate group from α GalpA-2S (Figure 6).



Figure 6. Depiction of the complexation schemes of Ca²⁺, combining sites of the four low energy
 conformations of Infernan; as results of quantum chemistry computation using B3LYP/6-31(d,p). Colour
 coding: Red: oxygen; Yellow: sulfur; Purple: calcium. The arrow indicates the direction of the backbone,

279 from non-reducing to reducing end.

280

The B3LYP functional with the Pople's split-valence basis sets has been extensively used for the calculation of related systems, including uronic acids (Plazinski & Drach, 2013; Stewart, Gray, Vasiljevic, & Orbell, 2014; Zhao et al., 2018; Ardiles & Rodriguez, 2021). In the current study, the minimization of system has been performed both in the conditions of vacuum and accounting water implicitly. These give slightly different results (Table S2 and S3). The use of 6-31(d,p) in vacuum provides Ca2+...O

- distances comparable with those reported for relative systems experimentally (Sommer et al., 2015;
 Novoa et al. 2014; Goldberg et al., 2018; Goldberg et al., 2021). Interestingly, the use of diffuse function
 on heavy atoms shortened the distances. In contrast, the implementation of implicit water makes the
 Ca²⁺...O distances longer than experimentally observed (Table S2, S3).
- 290 In the four types of complexes, the Ca^{2+} ion interacts with up to five oxygen atoms at distances ranging 291 from 2.2 to 2.6 Å (Figure 6) β GlcpA, α GalpA-2S and β Glcp form the binding site. In three complexes, 292 O2, belonging to β Glcp from the backbone, interacts with Ca²⁺. In contrast, in the first M1A complex, 293 the β Glc offers its O6 and O5 atoms. For all the optimized complexes, the Φ,Ψ values of 294 β GlcpA(1 \rightarrow 3) α GalpA-2S remain in the low energy region. They slightly deviate from those populated 295 in the course of molecular dynamics simulations. In all models, the coordination sphere of the Ca²⁺ 296 could be completed by interaction with other oxygen atoms to form a seven-fold up to nine-fold 297 coordination shell (Dheu & Perez, 1983). This is where the oxygen atoms of a sulfate group can 298 complete the coordination to six; (Figure 7), leaving room for water molecules. (Figure 7). The atomic 299 coordinates of the four models are given in Table S3.



Figure 7. Depiction of the complexation schemes of Ca²⁺, (M1A) showing as the oxygen atoms of a
 neighbouring sulfate group complete the chelating site of Ca²⁺. Colour coding: Red: oxygen; Yellow:
 sulfur; Purple: calcium. The arrow indicates the direction of the backbone, from non-reducing to
 reducing end.

305

Extension to the polysaccharide chains. The transposition of these four models of calcium complexation to the four models of Infernan does not alter the overall chain conformation for the previously established models. Figure 8 displays the four polysaccharide models in their interactions with Ca²⁺ ions. The five-fold helical model 2B is the most favourable for interactions with Ca²⁺. The two-fold helical models, M1A and M1B offer two types of Ca²⁺ sites of similar binding energy.



Figure 8. The calcium-binding models within the helical structures of Infernan. The lowest energy
 arrangement occurs for model 2B. Model 1A and 1B display similar binding energy.

314 Junctions zones. The occurrence of sulfate groups and uronic acid residues confers to ionic 315 polysaccharides' ability to form gels in the presence of divalent cations. The molecular basis of Ca²⁺ induced gelation in alginates and pectins, among others, are well established and documented 316 317 (Braccini & Perez, 2001). As Infernan forms a gel in the presence of Ca^{2+} (Zykwinska et al., 2019), we 318 explored the modes of interactions that would lead to form junction zones and propose some gelation 319 models. Only those corresponding to a two-fold helical structure (M1A and M1B) must be considered among the four existing structural models. Their ribbon-like structures allow bringing neighbouring 320 321 chains in proximity without steric clashes. Moreover, the orientation of the side chains in model M1A, 322 would not prevent two polymer chains from coming together.

- 323 To evaluate the favourable Inferans chain arrangements in the presence of calcium ions, we used the chain-pairing procedure developed previously to probe the Ca²⁺ induced gelation in alginates and 324 325 pectins (Braccini & Perez, 2001). In this procedure, an inter-helical distance is computed for all the 326 relative translational and rotational positions of one chain with respect to the other. Not surprisingly, 327 and because of the structural features of the infernan chain described above, there is a single way for 328 two infernan chains in the 2-fold conformation to interact in a parallel arrangement. The results of 329 the chain-pairing procedure define a spatial organization of the interacting molecular partners. The 330 ensemble was submitted to an energy optimization at the molecular mechanics level, using the OPLS
- 331 force-field available in the Schrodinger Maestro 11.0 version.
- The resulting is a 'junction zone' based on chain-chain interactions that lead to strong dimer associations with essential contributions from the van der Waals and hydrogen bonding interactions. Calcium ions and sulfate groups, and uronic acids have specific positions in well-adapted cavities. The feature of the interaction is the heterotypic mode of binding. Its nature contrasts with the so-called « egg-box » feature that most frequently characterizes the binding mode of ionic polysaccharides.
- The present model is established following a logical succession of the structural elements of increasing complexity, with a limited amount of experimental data: calcium induced formation of gel, parallel arrangements of chain assemblies of polysaccharides as seen from AFM. Needless to state that further confrontations with available experimental data are needed to ascertain the model's validity. Nevertheless, the model makes it possible to represent some grossly observed features and to make predictions. The availability of a structural model of complex associations constitutes a provisional and partial structural elucidations, represents a tool for thinking and guidelining new experiments.



- 344
- **Figure 9.** Schematic representation of two parallel chains of Infernan (M1A) throughout Ca²⁺
- 346 complexation, illustrating the formation of junction zones of the physical gel.
- 347

348 **Discussion & Conclusion**

- 349 Infernan, the EPS produced by the deep-sea hydrothermal bacterium *Alteromonas infernus*, exhibits
- an unusually complex primary structure, with the main backbone wearing two levels of sidechains. The

351 nature of the monosaccharides and their glycosidic linkages give rise to a linear backbone that 352 propagates with an almost fully extended elongation. Nevertheless, some conformational flexibility 353 about glycosidic linkages exists. The flexibility of β Glcp(1-4) α GalpA-2S linkage determines the conformation of the Infernan's backbone between a 2-fold or a 5-fold helical structure. The 354 355 conformational transition in the polysaccharide backbone occurs through a low energy barrier. The side chains do not influence the overall backbone conformation, unlike other branched 356 357 polysaccharides such as rhamnogalacturonan I (Makshakova, Gorshkova, Mikshina, Zuev, & Perez, 358 2017). However, the side chains of Infernan are essential for creating the Ca²⁺ chelating sites in the 359 region of the branching point. They involve the three adjacent residues: β Glcp and α GalpA-2S from the backbone and β GlcpA from the side chain. Two types of chelating sites can be formed depending 360 361 on the orientation of the side chain to the backbone. This holds true for both conformations of the 362 backbone, thus, resulting in four types of binding sites in total.

The configurations of the binding sites within one Infernan branching point have different binding 363 energy values for Ca²⁺ complexation. The difference of energy of calcium complexation is the 364 determinant factor to the polysaccharide backbone transition. In other words, Ca²⁺ binding may induce 365 366 and stabilize a particular conformational state of the polysaccharide. Even though the two-fold helix being the most populated state of the backbone, as revealed in molecular dynamics in the absence of 367 368 specifically bound counter-ions, the binding of Ca²⁺ may contribute to stabilizing the five-fold helix 369 conformation. As such, the five-fold helix may be considered as a "reservoir" for calcium ions. Then, 370 the transition from five-fold to two-fold helices occurs due to a rotation over the β Glcp $(1\rightarrow 4)\alpha$ GalpA-2S linkage. The Ca²⁺ binding sites are located at a distance of 13.6 ± 0.4 Å from each other along the 371 372 backbone. The two-fold helical conformation of the backbone, presenting the appropriate Ca^{2+} cavity, 373 may induce the formation of the polysaccharide chain pairing, providing that the chains are favourably oriented. The coordination of Ca²⁺ within one chain reaches completion with the oxygen atoms of a 374 375 sulfate group belonging to the neighbouring chain, with a significant gain in enthalpy. Such chain pairing would result in the formation of "junction zones", yielding to gel formation. A parallel 376 377 arrangement of the Infernan chains is energetically more favourable than an antiparallel one. Such a 378 feature is not favourable in terms of entropy. An eventual contribution of the side chain, which also 379 may play a role in the extensive gel network formation (Mikshina et al., 2017; Makshakova, Faizullin, 380 Mikshina, Gorshkova, & Zuev, 2018), is still to be unravelled. For a physical gel to occur, both junction 381 zones and regions that act as "gel breakers" to stop the propagation of the junction zones are required. 382 The five-fold conformation of the backbone where the side chains are fully deployed is likely to act as 383 such.

384 The present results suggest the occurrence of a novel ionic chelating mechanism of polysaccharides. 385 Previously characterized mechanisms involve a monotypic type of interactions underlying the « egg-386 box » binding (Braccini & Perez, 2000). Another type of complexation occurs in the case of κ -387 carrageenan gelation. The κ-carrageenan chains form thermo-reversible gel based on double helices 388 stabilized by a counter-ion binding in junction zones. It was experimentally proven that ion binding 389 precedes helix formation. Upon cooling, the counter-ions being unbound start to occupy, first, the 390 weaker binding site and the stronger binding sites. When the strong binding sites are occupied, the 391 transition from random coil to helix starts, and gel forms (Makshakova et al., 2020). It remains to be 392 seen whether the heterotypic mode of pairing Infernan chains can occur in other polysaccharides 393 interactions. The methodology developed in the present work could provide a way how to understand 394 or predict the formation of physical gels in complex polysaccharide structures.

395 **Acknowledgements.** The calculations were carried out using the equipment of the High 396 Performance Computing centre of University Grenoble Alpes, Grenoble, France and the shared research facilities of high-performance computing resources at Lomonosov Moscow State University,
 Moscow, Russian Federation. The CrossDisciplinary Program Glyco@Alps supported this work within
 the framework 'Investissement d'Avenir' program [ANR-15IDEX-02]. The study was partly funded by
 RFBR & CNRS according to the research project № 21-54-15008 (in part of DFT calculations). O.M.
 acknowledges (in part of M.D. simulations of branched oligosaccharide fragments) funding from the
 government assignment for FRC Kazan Scientific Center of (RAS).

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405 Author Contributions

406 ONM and S.P. coordinated the paper. ONM performed all M.D. calculations, analysis of trajectories

- and writing the paper. S.P. general leading of work, PES calculations, generation of initial structures,
 writing, and revising the manuscript. AZ formulated the task in terms of Infernan structure, S.C. and
 SC-J consulted about previously obtained experimental data.
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