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The marine bacteria *Cobetia marina* DSMZ 4741 synthesizes an unexpected K-antigen-like exopolysaccharide

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Abstract :

We have studied the exopolysaccharide produced by *Cobetia marina* DSMZ 4741, a marine bacterium isolated from coastal seawater. This strain is able to produce a polysaccharide in presence of carbon sources as glucose, mannitol and alginate. The maximum production occurs in aerobic condition, during the end of the exponential phase. The polymer is a non-viscous, acidic heteropolysaccharide of 270 kDa constituted of a repeating unit of:

→2)-β-D-Ribf-(1→4)-[7,8-O-(Pyr)]-α-D-KDOp-(2→

This kind of chemical structure is generally related to K-antigen polysaccharide of pathogenic *Escherichia coli* strains. This is the first time this type of EPS is described from a marine bacterium. Moreover the polysaccharide exhibits a pyruvate substitution on its 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) residue never encountered before. The discovery of such an unexpected EPS with high biotechnological potential is a new incentive for a better exploration of bioactive marine resources.

Highlights

►The marine bacteria *Cobetia marina* DSMZ 17117 synthesize an exopolysaccharide (named L6); The repetitive unit of the L6 exopolysaccharide is a disaccharide constituted of ribose and pyruvated KDO; This type of structure is generally encountered in antigen-K of uropathogenic E. coli strains.

Keywords : bacterial exopolysaccharides, K-antigen, Cobetia marina, KDO, marine polysaccharide

1. Introduction

Exopolysaccharides (EPSs) are polymers of sugars whose synthesis has been identified in the three domains of life (Eukarya, Prokarya and Archeae) (Sutherland, 2001). Bacterial EPSs display a wide range of chemical structure with homopolymeric or heteropolymeric composition, linear or branched backbones. Organic or inorganic substituents are also frequently observed on their structures (Freitas, Alves, & Reis, 2011). EPSs occur in two different forms as a capsular polysaccharide where the polysaccharides is closely associated with the cell surface and may be covalently bound, and as a high molecular weight slime polysaccharide that can be excreted in very high amount (Sutherland, 2001) and may be loosely bound at a limited number of discrete sites of the cell (Vanhooren & Vandamme, 1998). EPS-producing bacteria have been isolated in a wide range of ecosystems, from human digestive tracts (Hidalgo-Cantabrana *et al*., 2013) to the deep hydrothermal vents (Poli, Anzelmo, & Nicolaus, 2010), but also in oil wells (Sen, 2008) or Antarctic floe (Van der Merwe et al., 2009). Since their first descriptive studies in the middle of the last century, the biological and physicochemical unique properties of these polymers make them unavoidable in the fields of biotechnology (Sutherland, 2001; Kumar *et al*., 2009; Freitas et al., 2011). Because of their remarkable characteristics, they can also represent a real problem through their role in biofouling phenomenon, protecting EPS-producing bacteria against biocide and antibiotherapy, or by increasing corrosion (Stewart & William Costerton, 2001). In nature EPS are usually found in the form of a biogel framing the bacterial biofilm. Beyond this 'architectural' function, from an ecophysiological point of view, EPS can have multiple purposes. Its defensive roles against desiccation or grazing by protozoa as well as phages predation are the most documented (Ophir & Gutnick, 1994). By preventing the entry of exogenous macromolecules in the biofilm, EPSs can make some pathogenic strains 1000 times more

resistant against antibiotics (Stewart & Costerton, 2001). Moreover, the natural chelating properties of EPS can help bacteria to enhance nutrients bioavailability and trap toxins like heavy metals (Ordax *et al*., 2010). Finally, their implications in intra or inter specific allelopathy are well established (Valepyn *et al*., 2013).

Cobetia marina (DSMZ 4741), previously described as *Arthobacter marinus*, *Pseudomonas marina*, *Pseudomonas halodurans*, *Deleya marina*, *Halomonas halodurans* as well as *Halomonas marina* (Arahal *et al*., 2002) was first isolated from littoral seawater in Woodshole (USA) in 1970 (Cobet *et al*., 1970). It is a Gram negative aerobic bacteria, slightly halophilic, (from 0.5% to 20% (w/v)). This bacteria is often associated with macroalgae environment (Kraiwattanapong *et al*., 1998 ; Ivanova *et al*., 2002 ; Ivanova *et al*., 2005). *C. marina* is also well known to form biofilm and was studied as biologic model in the biofouling phenomenon (Maréchal *et al*., 2004). Its ability to synthetize and excrete exopolysaccharides and accumulate intracellular Poly-β-hydroxyalkanoate were already described on a different strain than DSMZ 4741 (Shea *et al*., 1991; Kokoulin *et al*., 2014).

This study presents the detailed description of a new EPS (designated as L_6) excreted by the marine bacteria *Cobetia marina* strain DSMZ 4741 from the production by fermentation to its fine structural elucidation. This EPS shows chemical similarities with some K-antigen from the group II.

2. Material and method

2.1 EPS production

Cobetia marina EPS production was performed in a 3 l fermenter (INFORS®) containing sterilized Zobell medium composed of yeast extract (1 $g.l^{-1}$), peptone (5 $g.l^{-1}$), Tris-base (1.5 $g.l^{-1}$) and 30 $g.l^{-1}$ of glucose, diluted in 80% filtered seawater and 20% MilliQ. The pH medium was adjusted to 7.6 and maintained this value by addition of H_2SO_4 or NaOH. The fermenter was inoculated with a 10 % (v/v) suspension of cells in exponential phase. Bacteria were grown at 25 °C until the late exponential phase to obtain a high cell density. During the stationary growth phase, the temperature was decreased to 20 °C for 48 hours in order to enhance the EPS production. The oxygenation was monitored and regulated by stirring and air flux. The consumption of glucose was monitored with the enzyplus kit (St Gobain, France). Alternative carbon source were tested to optimize EPS biosynthesis. A production was performed in batch for mannitol (2.5 l Erlenmeyer culture, Minitron, INFORS®) and for

alginic acid in fermenter (10 l, INFORS®) and in batch (2.5 l Erlenmeyer culture, Minitron, INFORS®) with the parameters used with glucose induction.

2.2 EPS extraction and purification

At the end of the fermentation, the culture medium was centrifuged (1 h, 14000 g, 4 °C) and the supernatant was filtered with a Buchner through a 0.45 µM glass filter (Whatman®). Sodium azide (NaN_3, 0.4 $g.l^{-1}$ final concentration) was added to the filtrate to prevent bacterial regrowth. The filtered culture supernatant containing the soluble EPS was then purified by tangential ultrafiltration with a 100 kDa cut-off cartridge (Millipore®) against mQ water. The purified EPS was then frozen, freeze dried and stored away from light and moisture.

2.3 EPS analysis

Before analysis, EPS was resuspended in MilliQ water and it was centrifuged (12000 g, 10 min, 20°C) to remove insoluble materials. The supernatant was used for further analysis.

2.3.1 Fourier Transform Infra Red Spectroscopy

About 20 mg of a lyophilized sample was placed under the beam of a FT-IR spectrometer (Nicolet sI10, ThermoScientific®) in attenuated total reflectance mode (ATR, diamond/ZnSe crystal) at ambient temperature. Spectra were acquired with 16 scans and data were processed with the Omnic spectra software (thermoscientific®).

2.3.2 Quantification of DNA and RNA in EPS samples

DNA and/or RNA content of the EPS was evaluated by spectrophotometry. OD of a 0.1% EPS solution (w/v) in MilliQ water measured at 260 nm (Hitachi U-1800®) was converted in DNA or RNA equivalent (1 OD unity = 50 $\mu g.ml^{-1}$ of DNA or 40 $\mu g.ml^{-1}$ for RNA).

2.3.3 Total sugar determination

The Dubois colorimetric method (Dubois, 1956) takes into account all types of sugars such as aldoses, ketoses and uronic acids. This assay provides a relative percentage of total sugar content expressed in ribose equivalent used for the standard curve. A 200 µl EPS sample solution (10 $mg.ml^{-1}$) was treated with 200 µl of a 5% phenol solution and 1 ml of pure H_2SO_4. The mixture was cooled to room temperature for 30 min. Absorbance at 492 nm of samples as well as standard sugar (10 to 100 $\mu g.ml^{-1}$ ribose) was read by spectrophotometry (Hitachi U-1800®).

2.3.4 Protein concentration measurement

The Bradford method (Bradford, 1976) using Coomassie blue is based on the difference in color depending on its complexation with some amino acids present in proteins. 1 ml of reagent (Biorad) was added to 20 µl of sample (10 $mg.ml^{-1}$) and incubated 5 min at room temperature. OD of samples as well as the standard protein (0 to 0.75 $mg.ml^{-1}$ of Bovine Serum Albumin (BSA)) was measured at 595 nm (Hitachi U-1800®).

2.3.5 Observation of degradation products

Thin Layer Chromatography (TLC) of oligosaccharides was carried out by deposit 2 µl of polysaccharide solution (10 $mg.ml^{-1}$) hydrolyzed with trifluoroacetic acid (2%) at the basis of Silica Gel-60 TLC plates (E. Merck®, Darmstadt, Germany) and by migrating with a solvent composed of 2:1:1 1-BuOH/AcOH/H_2O in a chromatography tank. Oligosaccharides and monosaccharides were visualized by spraying 10% (v/v) H_2SO_4 in EtOH, and then heating at 120 °C for 15 min. In order to specifically detect unsaturated/deoxy sugars, thiobarbituric acid (TBA) staining was performed after the periodic acid degradation (Lanning & Cohen, 1951). Namely, the plate was sprayed with a 0.02 M sodium periodate solution and left for 15 min for the degradation of sugars on the plate. The plate was then sprayed with an Ethylen Glycol/Acetone/H_2SO_4 solution (50:50:0.3) and dried for 10 min. Finally, the plate was sprayed with a 6% (v/v) solution of 2-thiobarbituric acid and heated at 150 °C for 10 min to detect malondialdehyde produced from unsaturated/deoxy sugars as bright red spots.

2.3.6 Fractionation of EPS

About 300 mg of native EPS were diluted in 30 ml of 5 mM ammonium acetate buffer, pH 6. The sample was applied to a Toyopearl DEAE-650M anion exchange (Tosoh, Tokyo, Japan, 20 x 2.2 cm) chromatographic column. Elution was performed with 600 ml of a NaCl linear gradient from 0 to 1 M with a collected volume of 10 ml per fraction. For each fraction, total sugar content and uronic acid content were determined with Dubois method and carbazole/sulphuric acid method respectively. EPS fractions were analyzed in a Native Polyacrylamide Gel electrophoresis to observe potential difference in the migration patterns. 12 µl of fractions samples were mixed with 12 µl of loading buffer (25% (v/v) glycerol, 75 mM Tris-HCl (pH 8.9), 1 mM EDTA) and 5 µl of 0.02 % (w/v) Bromophenol Blue Buffer. 20 µl of samples were loaded in wells of a precasted 7.5% acrylamide gel (Biorad®). Electrophoresis was achieved at a constant current of 20 mA, for 50 min in a migration buffer

comprising 25 mM Tris HCl / 192 mM Glycine, pH 8.3. The native gel was then stained with Stain All (Cosmo Bio C;., ltd, Tokyo, Japan) for 30 min and destained with distilled water under the room light.

2.3.7 EPS molecular weight determination

The EPS samples were dissolved at a concentration of 0.5 $g.l^{-1}$ in water and injected on two Shodex® columns 805 and 806 placed in series (fractionation range: 805: 4.10^5 Da ; 806: $2,10^7$ Da) . Elution was made with 0.1 M sodium nitrate + 0.2% sodium azide at a flow rate of 0.5 $ml.min^{-1}$. Detection was performed using a refractometer (Waters®), an 18 angles light scattering detector (Wyatt®). For the series of measurements, the dn/dc was taken equal to 0.150 $ml.g^{-1}$.

2.3.8 ABEE labelled monosaccharide analysis

This method allows to link a 4-aminobenzoate ethyl ether residue (ABEE) to the anomeric hydroxyl of a monosaccharide in acidic condition. The labelled monosaccharide can be detected with UV spectrometry. 10 µl of 8 M TFA was added to 10 µl of a 0.1% (w/v) EPS solution. Hydrolysis was carried out for 1 h at 100 °C. After cooling down, the sample was dehydrated with a centrifugal concentrator (30 min, 500 g). Once dried, 40 µl of 2-propanol was added and the sample was dehydrated again. Then, 10 µl of mQ water and 40 µl of ABEE-labeling reagent (Cosmo Bio Co., Ltd) were added to the sample and the mixture was incubated at 80 °C for 60 min. Dropped at room temperature, 200 µl of mQ water and 200 µl of chloroform were added to the mixture, which was agitated for 1 min. After a short centrifugation, the supernatant was recovered and filtered through a 0.2 µm membrane before injection. 20 µl of ABEE-derivative sample was injected to an HPLC (Prominence system, Shimadzu®, Tokyo, Japan) equipped with a Honenpak® C18 column (7.5x0.46 cm, special ABEE-labeling, Cosmo Bio C., Ltd). HPLC run was performed at 1 $ml.min^{-1}$ for 60 min at 30 °C with 0.2 M sodium borate, pH 8.9 / acetonitrile (93/7 v/v). Detection was performed with UV absorbance at 305 nm.

2.3.9 Alditol acetates derivatives monosaccharide analysis

Alditol acetates derivatives of the EPS were prepared as follows: 4 mg of EPS were mixed with 125 µl of 72% of H_2SO_4 solution. The mixture was vortexed and placed for 1 hour at ambient temperature. Then, acid was diluted to 2 N and left for 6 hours at 110 °C. Tubes were cooled and 200 µl of hydrolyzed sample was taken to perform alditol acetates derivatives.

After neutralization by ammonia, 50 µg of myo-inositol were added to the sample as internal standard. Monosaccharides released from hydrolysis were reduced with 1 ml of a $NaBH_4$/DMSO solution for 90 min at 40 °C. After reduction, excess of $NaBH_4$ was decomposed by 200 µl of 18 M acetic acid. Then, 200 µl of 1-methylimidazole and 2 ml of acetic anhydride were added to the samples. After 10 min of acetylation at room temperature, 5 ml of distilled water were added to decompose the excess of acetic anhydride. Once the tubes cooled, alditol acetates were extracted by the addition of 1 ml of dichloromethane, mixture agitation and recovery of the organic phase after a short centrifugation. The organic phase was filtered through a 0.2 µm syringe filter. Monosaccharides were analyzed by gas chromatography (Agilent®) and injected on a SP 2380 column with nitrogen as carrier gas (1.5 $ml.min^{-1}$). The temperature program was the following: by 3 min at 190 °C, 5 $°C.min^{-1}$ up to 250 °C.

2.3.10 Substituents determination

Organic and inorganic substituents content was determined by High Performance Anion Exchange Chromatography/ Conductivity Detection (HPAEC/CD;GP 50 pump and ED 50 detector, Dionex®) equipped with a AG11HC Ionpac precolumn (Dionex® 4x50 mm), a AS11HC Ionpac Analytical column (4x250 mm Dionex®), a ATC-HC Ionpac-Trap ion trap column (9x75 mm, Dionex®) and a ASRS 300 suppressor (4 mm, Dionex®). A 2 $mg.ml^{-1}$ EPS solution was hydrolyzed with 0.8 N HCl for 3 h at 110 °C. The solution was then diluted with 2 ml of H_2O and 20 µl were injected into the column. Elution was carried out with a NaOH linear gradient from 0.15 mM to 6 mM. Substituents were identified by comparison with a standard mixture (lactate, acetate, succinate, pyruvate, sulfate, phosphate) and concentrations were calculated with reference to an internal standard (nitrate, absent from the EPS).

2.4 Structural elucidation of the EPS

2.4.1 NMR

About 10 mg of native and depolymerized L_6 fractions were analyzed by NMR after three exchanges/dehydration cycles in deuterated water (99.9%). EPS were resuspended in 700 µl D_2O. 1D and 2D NMR spectra were recorded at room temperature in the Laboratory of Nuclear Magnetic Resonance Spectroscopy (University of Western Brittany) on a 500 MHz Bruker® spectrometer .

2.4.2 Glycosidic linkage analysis

Glycosyl-linkage positions were determined with the method described by Doares *et al*., (1991). Hydroxyl groups of the native EPS were first methylated as follows. About 2 mg of EPS was dissolved in 500 µl of DMSO and 500 µl of Butyl Lithium was added (2.5 M, in hexane, Sigma-Aldrich). Reaction was performed during 3 h at room temperature. Then, 500 µl of iodomethane were added and the mixture was kept under agitation overnight. Methylation was stopped by addition of 1 ml of ultrapure water and the exceeding methylation reagent was evaporated under nitrogen stream. The methylated EPS was extracted with 1 ml of dichloromethane and centrifuged (1 min, 1500 rpm). The organic phase was recovered, washed twice with 4 ml of ultrapure water and evaporated under nitrogen stream. Methylated EPS was hydrolyzed with 500 µl of trifluoroacetic acid (2 N) and 20 µl of myo-inositol was introduced (1 $mg.ml^{-1}$) as internal standard. The mixture was heated to 120 °C for 2 h and finally, acid was evaporated under nitrogen stream. EPS was reduced with $NaBD_4$ at 80 °C during 30 min. The excess of $NaBD_4$ was eliminated with glacial AcOH. Reducing agents were eliminated by 3 co-distillations with a methanol / acetic acid (9 : 1 ; v/v) mixture followed by 3 co-distillations with pure methanol. Finally, *O*-acetylation of the EPS was performed with 200 µl of 1-methyl-imidazole and 2 ml of Ac_2O during 10 min at room temperature. *O*-acetylation was stopped by addition of 5 ml of ultrapure water and the O-acetylated product was extracted twice with 1 ml of dichloromethane. The organic phases were pooled and washed 5 times with 1 ml of ultrapure water and evaporated under nitrogen stream.

3. Results

3.1 EPS production

Production of EPS was carried out in unbalanced C/N culture medium. The maximum production of exopolysaccharide generally occurred at the end of the log phase. Two other carbon sources other than glucose were tested. Indeed, *C. marina* (DSMZ 4741) exhibits alginate lyase activity, suggesting macroalgae thalli as its natural environment. We chose to test 2 carbon sources related to macroalgae environment (alginic acid and mannitol). 3 carbon sources (glucose, mannitol and alginates) were added to the same culture medium to test different potential induction conditions: the average L_6 EPS production rate was 0.4 $g.l^{-1}$ in a glucose induced medium, 0.3 $g.l^{-1}$ in a mannitol induced medium and 1 $g.l^{-1}$ in an alginate induced medium. Despite the better production rate obtained with alginic acid, glucose was

chosen in order to avoid contamination by non-degraded alginic acid chains in the final product. For *Cobetia marina*, the maximum amount of EPS was obtained at 20 °C, pH 7.6 after 50 h of culture. Over this time, the bacteria began to depolymerize its own EPS, the average molecular mass of L_6 decreased (seen on size exclusion chromatography, data not shown) and seemed to synthesize PHA (according to inclusions observation by optic microscopy, data not shown). The consumption of glucose was about 17 $g.l^{-1}$ after 50 h of fermentation.

3.2 EPS Characterization

The purity of the purified EPS was estimated with colorimetric dosages. Total sugar content was measured by Dubois assay. Protein content was calculated by Bradford method and genetic material content was measured at 260 nm. The lyophilized EPS is characterized by a high content of sugars ($78_{+/-6.1}$ % w/w) and a low amount of proteins ($6_{+/-0.6}$ % w/w) and genomic contaminants ($3_{+/-0.5}$ % w/w).

The Fourier transform infrared spectroscopy was used as a preliminary analysis to assess the 'quality' of the sample before colorimetric dosages, osidic composition characterization and structural elucidation. It provides information about the purity of the polymer and helps to discriminate exopolysaccharides of protein or dna/rna made exopolymeric substances. This technique was also suitable to detect functional group as e.g. carboxylic function or sulfate ester function. The Fourier transform infrared spectroscopy (FTIR) performed on L_6 shows a typical polysaccharide-fingerprint spectrum characterized by a broad band beyond 3000 cm^{-1}, resulting from O-H and C-H stretching bands at 3420 cm^{-1} and 2900 cm^{-1} respectively, and an intense absorption band at 1600 cm^{-1} with a small shoulder around 1730 cm^{-1} due to the presence of carboxylic groups.

The L_6 EPS was hydrolysed with TFA and the corresponding degradation products of L_6 were analyzed by TLC. Sulphate and TBA staining were used to reveal total and unsaturated/deoxy sugars, respectively (**Fig. 1.**). The sulphate stainings indicated 2 prominent bands as well as faint bands in upper and lower positions of the two prominent bands, respectively. TBA staining indicated that the lower prominent band was unsaturated/deoxy sugar, while the upper prominent band showed no such sugar. Weak bands detected below the unsaturated/deoxy sugar correspond to incompletely hydrolyzed material comprising the unsaturated/deoxy sugar and other constituents. The trace band in the top of TBA-stained TLC may be another incompletely hydrolyzed material comprising of the unsaturated/deoxy

sugar and of another constituent. These results indicate that repetitive unit of the L_6 EPS consists of at least 2 kinds of sugars which one is unsaturated/deoxy sugar.

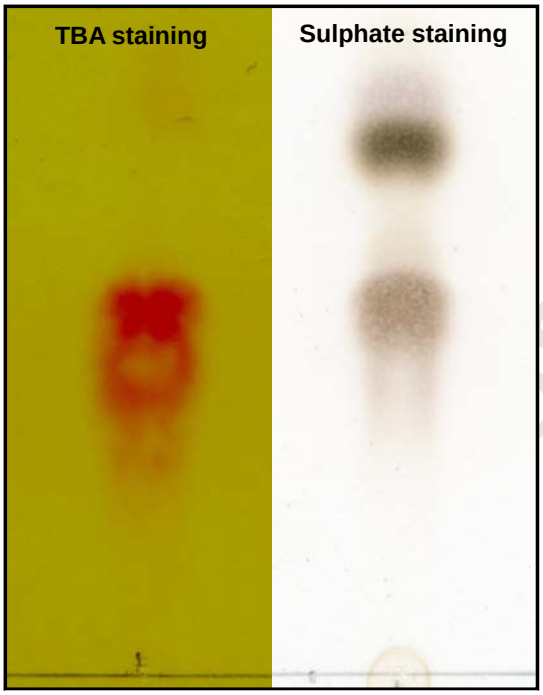

Fig. 1 : TBA and sulphate staining of L_6 after TLC.

3.3 Fractionation of the EPS

L_6 was fractionated on a DEAE column and eluted with NaCl gradient. The presence of polysaccharide in the different fractions was measured with Dubois assay. Only one peak was obtained corresponding to a 0.3 M concentration of NaCl and suggesting a negatively charged polymer which is in accordance with the carboxylic groups observed with FTIR. The different fractions were analyzed with a native-polyacrylamide gel electrophoresis. The migration patterns showed an increase of the molecular weight during the peak elution suggesting an increase in the affinity toward DEAE resin with the increase in the chain length of EPS.

The L_6 EPS is a high molecular weight polysaccharide with an average value of $2.7.10^5$ Da and a polydispersity index of about 3.75, it represents some heterogeneous populations in terms of polysaccharide chains size.

3.4 Chemical composition of L_6

Alditol acetates of the L_6 EPS were prepared and analyzed with gas chromatography and ABEE labeled EPS by liquid chromatography. The ribose was the only residue that could clearly be identified in both technics. Moreover, EPS substituents determination based on conductimetry using Dionex-HPLC after mild acidic degradation revealed the presence of pyruvate.

3.5 Glycosidic linkage analysis

A structural information about ribose linkage was obtained after analysis of the partially methylated alditols acetates of the L_6 EPS . The presence of 1.2.4-tri-acetyl-1-deuterio-3.5-di-O-methyl-D-ribitol was assessed by the observation of 2 majors signals at m/z 161 and m/z 190, respectively. The *O*-acetylation of the C2 of the ribitol demonstrates the attachment of another glycosidic residue to the C2 of ribose under its native form and also determines the furanoid form of the ribose.

3.6 NMR spectroscopy

The ^{1}H and ^{13}C-NMR study of L_6 confirmed the high degree of purity of the exopolysaccharide and was in accordance with the presence of 2 osidic residues and one organic substituent. A total of 15 carbon peaks were observed on the ^{13}C-NMR spectrum, and, from theses first data, EPS repetitive unit seems to be constituted by ribose (named spin system A) and KDO (3-deoxy-D-manno-oct-2-ulosonic acid, named spin-system B). The spin system-A shows a proton in the anomeric region (5.3 ppm) that is characteristic of ribose (Kocharova *et al*., 1989). Its furanoid form and β anomeric configuration was determined by the ^{13}C chemical shift of the C1 (107,1 ppm) and C4 (85 ppm) (Jennings *et al*., 1982 ; Van *et al*., 1983 ; Leone *et al*., 2007). The KDO residue was determined to be α based on the chemical shift difference between the axial and equatorial methylene proton signals (δH_3-H_3' = 0.52 ppm). The signal at δ 34.9 ppm is characteristic of C3 of a KDO*p* and indicative of a pyranoid form (Lenter *et al*., 1990). The presence of pyruvate (spin system C) with an R configuration was also assessed by the chemical shift of the methyl protons (1.5 ppm) (Garreg *et al*., 1980). Integration of characteristic peaks (H1-A, H3-B, H3-C) suggests a Rib/KDO/Pyr 1:1:1 molar ratio (**Fig. 2**). All these data are strongly correlated with the data preliminary obtained by the chemical analysis (negative charge of the EPS, presence of carboxylic groups, two kinds of sugar constituents one of which is a deoxy sugar).

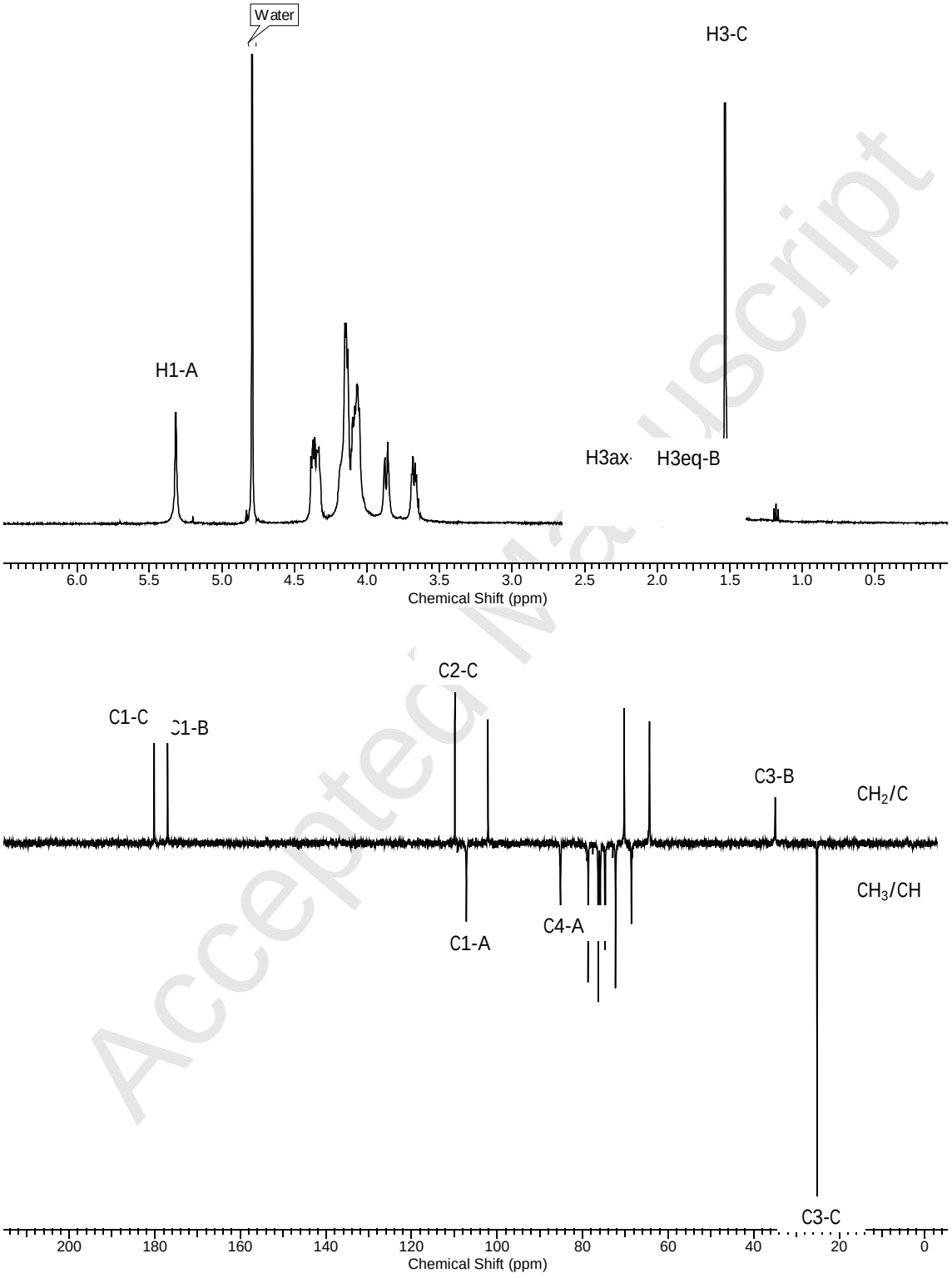

Fig. 2 : ^{1}H-NMR and ^{13}C-NMR (Jmod) spectra of L_6.

Additional data were provided by 2D NMR experiments. Assignment of each peaks are summarized in the **Table 1.** Accurate assignments of nucleus constituting the three spin-systems (A, B and C) were obtained by COSY and HMQC experiments (**Fig. 4 ; Fig. 5 & Table 1**). Based on these results, the link between the spin-system A and B was determined by HMBC experiment (**Fig. 6**). Spectrum analysis showed a clear evidence for a (1→4) glycosidic linkage as proven by a strong correlation between the H1-A and the C4-B. This data was comforted by the downfield chemical shift of the C4-B compared with a similar polysaccharide from the literature (Lenter *et al*., 1990a). In addition, no correlation with the other potential link (C5-B/H1-A) was observable. The position of the pyruvate on the KDO was first established by the correlation between the C2-C and the H7-B/H8-B (**Fig. 6**). According to literature data, the important downfield chemical shift of the C7-B and C8-B (**Table 1**) suggested a substitution and, therefore, confirmed the second position between KDO and pyruvate (Jennings *et al*., 1982 ; Vann *et al*., 1983 ; Dengler *et al*., 1985 ; Lenter *et al*., 1990). From all these data, the L_6 EPS proves to be constituted by a sequence of →2)-β-D-Rib*f*-(1→4)-[7,8-*O*-(Pyr)]-α-D-KDO*p*-(2→ (**Fig. 3**).

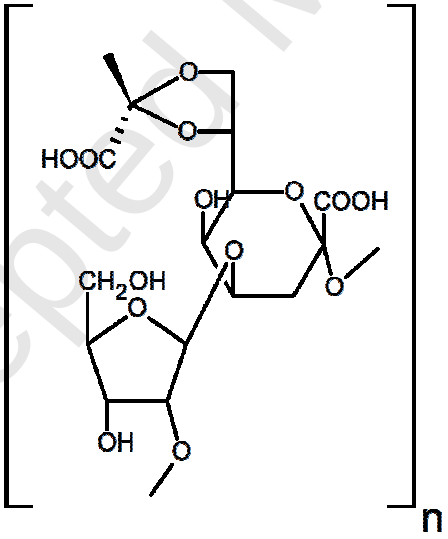

Fig. 3 : Chemical structure of repetitive unit of the EPS L_6

Table 1 : ^{1}H-NMR and ^{13}C-NMR spectral data for the L_6 EPS.

Residue	Nucleus	Chemical shift (ppm)							
		1	2	3	4	5	6	7	8

A	^{1}H	5.3	4.1	4.3		4.0	3.8	3.6					
	^{13}C	107.1	78.6	72.3		85.1	64.3						
B	^{1}H			1.7_{eq}	2.3_{ax}	4.19	4.14	4.06	4.33	4.15	4.06		
	^{13}C	176.9	102.1	34.9		75.8	68.5	74.7	76.3	70.2			
C	^{1}H			1.5									
	^{13}C	180.2	109.8	25.1									

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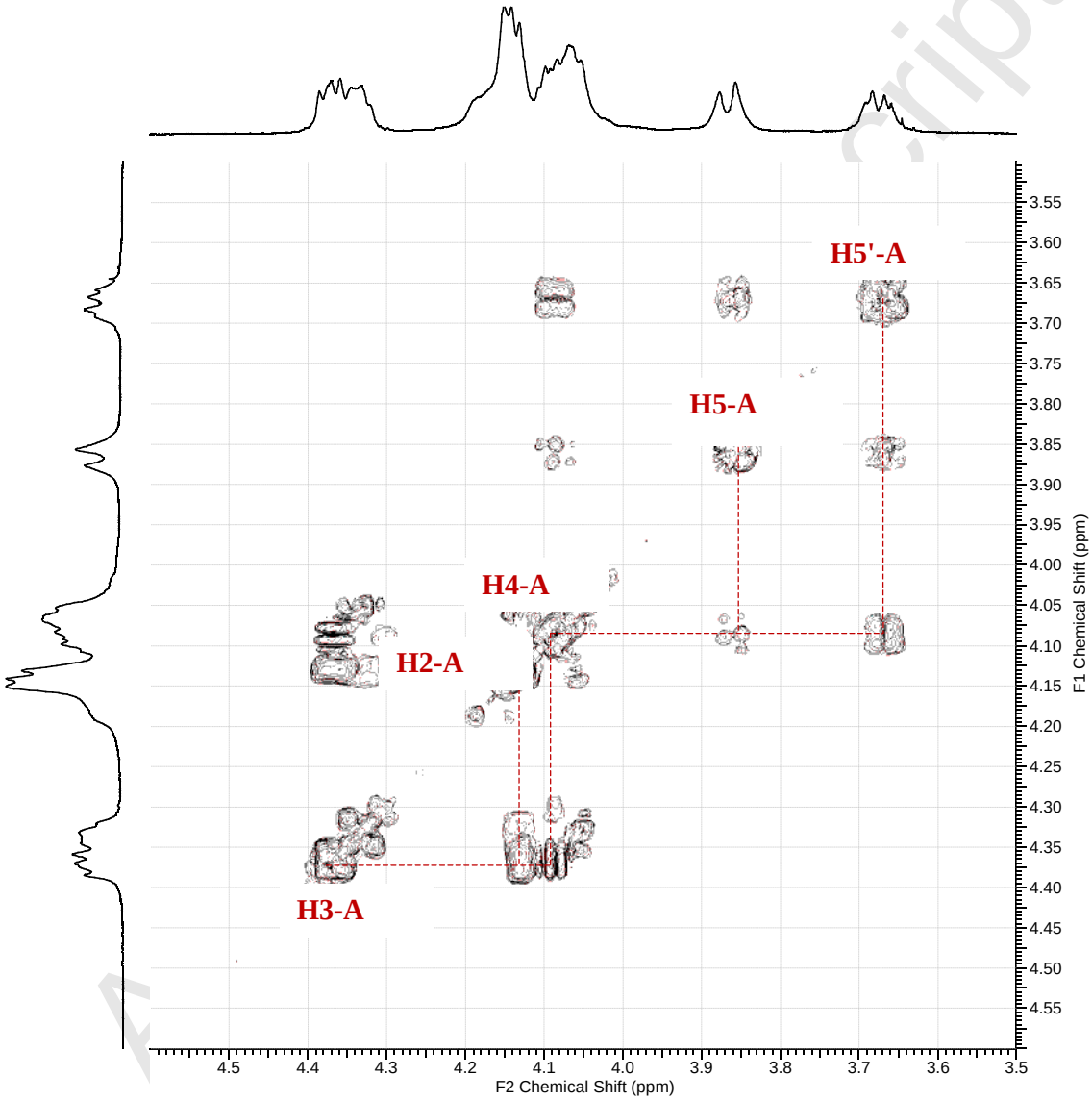

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360 **Fig. 4 :** COSY spectrum of L_6.

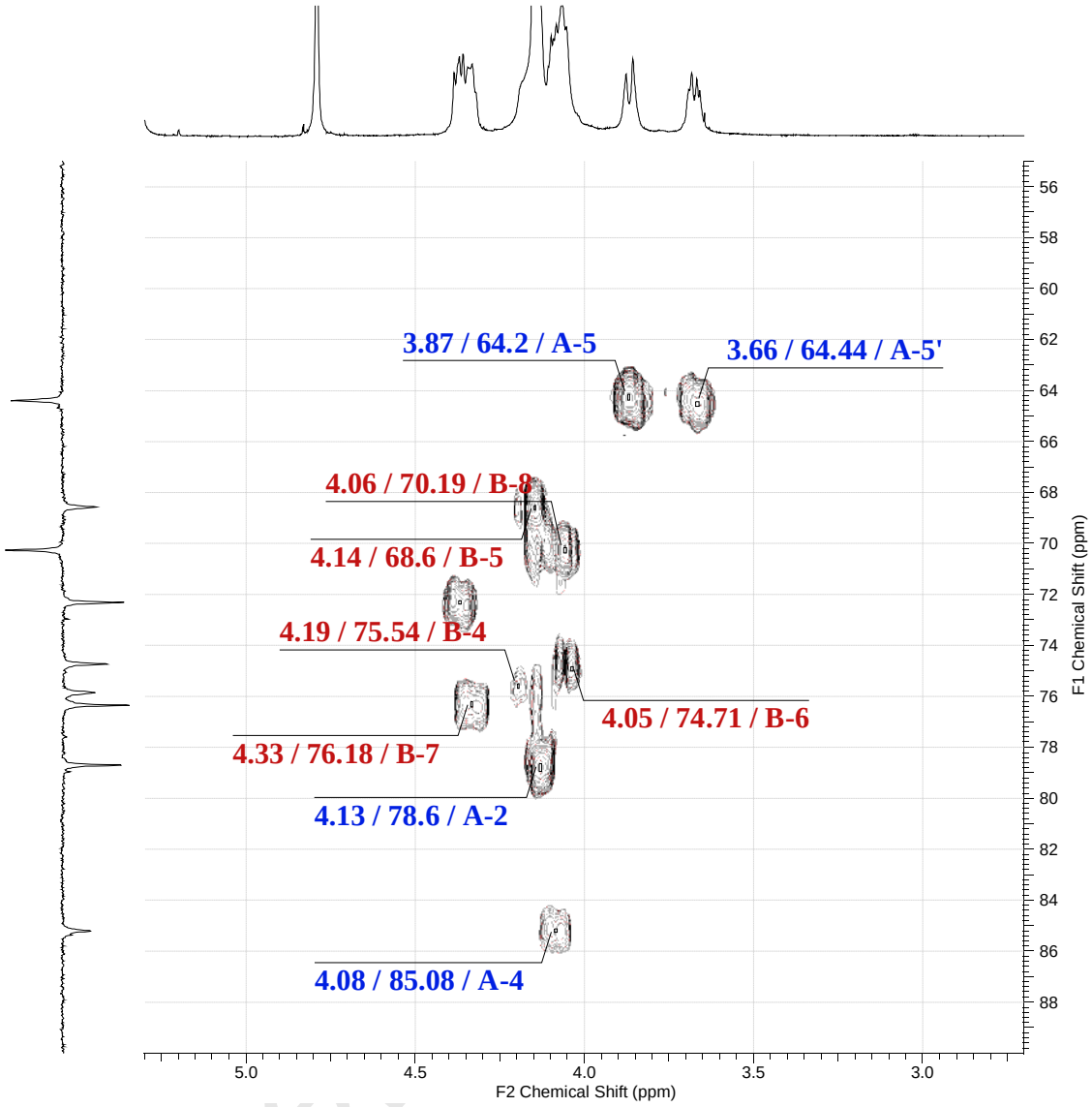

Fig. 5 : HMQC spectrum of L_6 (Blue : Ribose, red : KDO).

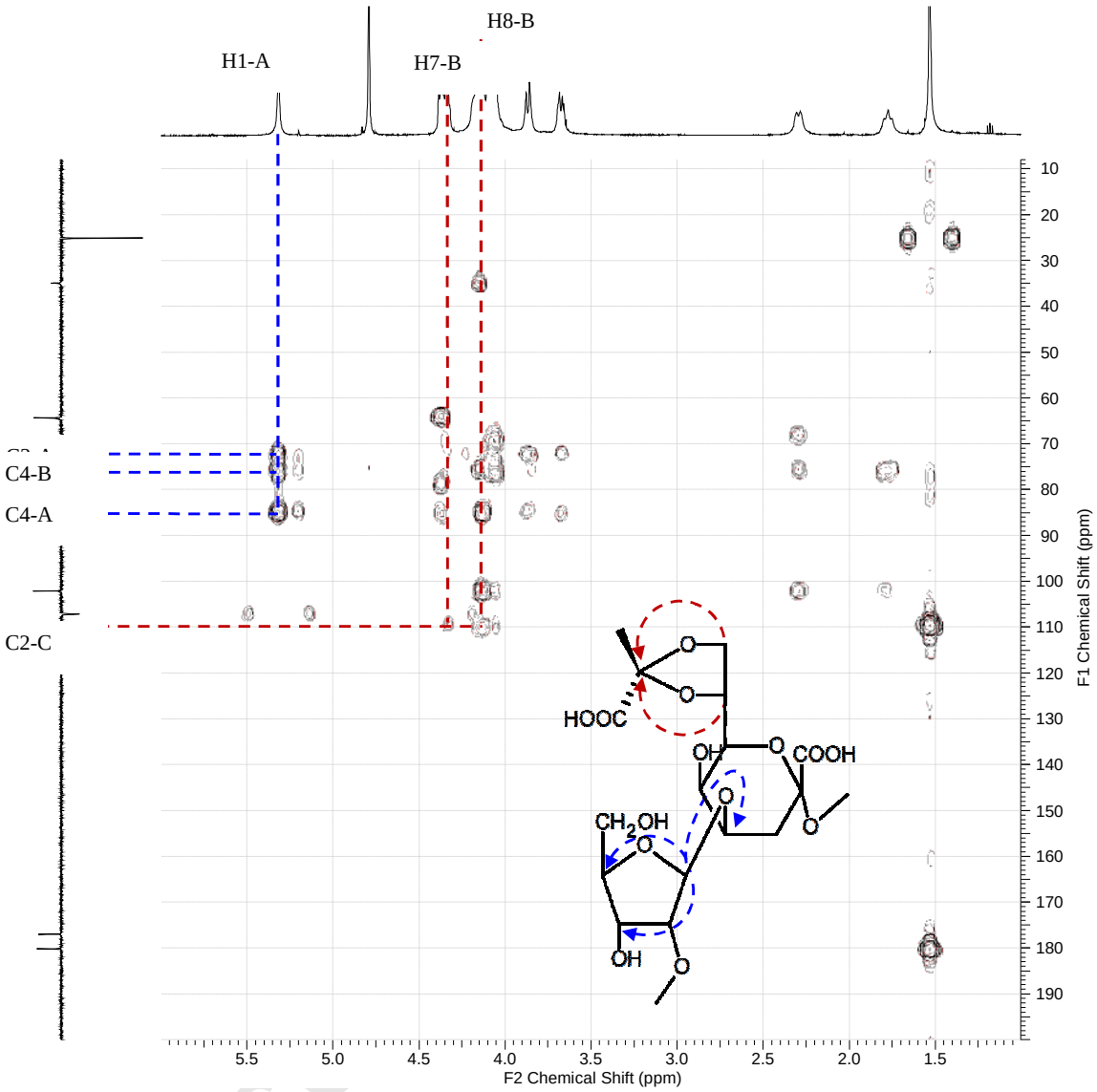

Fig. 6 : HMBC spectrum (partial contour plot) of L_6.

4. Discussion

L_6 is a soluble, non-viscous, non-branched acidic heteropolysaccharide of high molecular weight (270 kDa) constituted of a repeating unit of →2)-β-D-Rib*f*-(1→4)-[7,8-*O*-(Pyr)]-α-D-KDO*p*-(2→. In comparison with others marine bacterial EPS, L_6 exhibits lowest polysaccharide chains sizes (Nichols *et al*., 2005 ; Satpute *et al*., 2010) . This is maybe due to the natural lability of the KDO residue which would be consistent with the important polydispersity index measured in the study. Previous studies about the ability of *Cobetia marina* to produce EPS do not match with present results (Shea *et al*., 1991 ; Kokoulin *et al*., 2014). Indeed, this polymer showed, in its composition and structure, strong analogies with some *Escherichia coli* K-antigen polysaccharides from the group II (**Table 2**). K-antigens (for

kepsular antigen) are a class of polysaccharides (LPS or free CPS) synthesized by pathogenic *E. coli*. They are separated in 2 major groups (I and II) and a minor one (III). The group II is characterized by its small molecular size (less than 50 kDa) and its acidic component (presence of ulosonic acid residues) (Jann *et al*., 1990). If KDO residues have already been reported in EPS (Nimtz *et al*., 1997 ; Steinmetz *et al*., 2000 ; Vanhaverbeke *et al*., 2001 ; Cescutti *et al*., 2003), its occurrence remains still rare in such type of polysaccharide. Most notably, this is the first time we described a 'true' EPS (understanding different from a LPS or capsular polysaccharide) from marine origin constituted by this type of chemical structure. . EPS L_6 also exhibits other unique properties, e.g. the presence of a stoichiometric pyruvate in position 7 and 8 on the KDO residue, a high molecular weight compared to K-antigens and an important yield of production regarding previously described K-antigen producing strains. The 7,8-pyruvilated KDO is the more intriguing of these three features. This unusual case of substitution never reported before is maybe an adaptation to the ionic environment linked to the oceanic way of life of *Cobetia marina* DSMZ 4741. Another hypothesis could be a mechanism of resistance against phage-borne polysaccharidases. Indeed, viral KDO-hydrolases generally act on non-substituted KDO residue (Sutherland, 1999, Labrie *et al*., 2010). This is even more surprising if we consider both the differences in terms of ecology and phylogeny between *Cobetia marina* and *E. coli* K-trains. *Cobetia marina* is a marine bacteria, often associated with marine algae (Kraiwatanapong *et al*., 1999 ; Ivanova *et al*., 2002 ; Ivanova *et al*., 2005). For example, the strain DSMZ 4741 exhibits an inducible alginate lyase and can produce L_6 from the fermentation algae alginate, while the K-strains of *E. coli* are always observed in human urinary tracts. Additionally, the K-strains of *E. coli* naturally synthesize a large amount of K-antigen whereas *C. marina* requires the presence of a high concentration of carbohydrate substrate (glucose or alginic acid). Previous studies have shown that KDO-containing K-antigen are susceptible to be hydrolyzed by phages borne polysaccharidases, allowing the implementation of enzybiotics (Altmann *et al*., 1987 ; Altmann *et al*., 1990 ; Nimmich, 1997). We also isolated, from seawater samples, bacteriophages able to depolymerize the EPS L_6. The characterization of these phage enzymes is currently in progress (Lelchat *et al*., in prep). This study provides new evidences of the potential of marine plankton as a quasi-unexplored reservoir of novel bioactive compounds of medical or biotechnological interests.

Table 2 : Structural comparison between L_6 and related Antigen-K (red : homologies with L_6, green : structural singularity of L_6).

Antigen	Chemical structure of the repeating unit	reference
K6	→3)-β-D-Rib*f*-(1→7)-α-D-KDO*p*-(2→3)-β-D-Rib*f*-(1→ 2 2 ↑ ↑ 1 1 β-D-Rib*f* β-D-Rib*f*	Jennings *et al*., 1982
K16	→2)-β-D-Rib*f*-(1→3)-β-D-Rib*f*-(1→5)-α-D-KDO*p*-(2→ 3 ↑ OAc	Lenter *et al*., 1990a
K13	→3)-β-D-Rib*f*-(1→7)-β-D-KDO*p*-(2→ 4 ↑ OAc	Vann & Jann, 1979
K20	→3)-β-D-Rib*f*-(1→7)-β-D-KDO*p*-(2→ 4 ↑ OAc	Vann *et al*., 1983
K19	→3)-β-D-Rib*f*-(1→4)-β-D-KDO*p*-(2→	Jann *et al*., 1988
K23	→3)-β-D-Rib*f*-(1→7)-β-D-KDO*p*-(2→	Vann *et al*., 1983
K74	→3)-β-D-Rib*f*-(1→2)-β-D-Rib*f*-(1→6)-β-D-KDO*f*-(2→ 2 ↑ OAc (65%)	Ahrens *et al*., 1988
K95	→3)-β-D-Rib*f*-(1→8)-β-D-KDO*f*-(2→	Dengler *et al*., 1985
L_6	→2)-β-D-Rib*f*-(1→4)-α-D-KDO*p*-(2→. 7,8 ↑ Pyr	present study

5. Conclusion

New molecules with antigenic properties are generally isolated from pathogenic organisms. Here, we describe a new K-antigen-like EPS named L_6 and synthesized by the non-pathogenic marine bacterium *Cobetia marina* DSMZ 4741. This EPS exhibits an unusual and original structure whose bioactivity potential is still to be studied. Additional works are currently carried out in our lab with bacterioviruses infecting *C. marina* and able to depolymerize L_6

with the aim of generate low molecular weight oligosaccharides consistent with biomedical developments.

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