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Structural studies on the exopolysaccharide from *Erwinia persicina*

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Abstract—The Gram-negative bacterial strain HKI 0380 was isolated from biofilms located on palaeolithic rock paintings in the Cave of Bats in Zuheros, southern Spain. It was identified as the phytopathogenic *Erwinia persicina* and attracted attention due to the production of considerable quantities of slime. The acidic exopolysaccharide produced by the *E. persicina* was studied after O-deacylation by sugar and methylation analyses, along with ^{1}H and ^{13}C NMR spectroscopy. The following structure of the branched pentasaccharide repeating unit of the O-deacylated exopolysaccharide was established:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad\quad \alpha\text{-D-Gal}p^{III}\text{-}(1\rightarrow4)\text{-}\beta\text{-D-Glc}p\text{A} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad\ \ 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad\ \ \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad\ \ 4 \\ \rightarrow\!6)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow\!3)\text{-}\beta\text{-D-Gal}p^{I}\text{-}(1\rightarrow\!3)\text{-}\alpha\text{-D-Gal}p^{II}\text{-}(1\rightarrow \end{array}$$

Keywords: Phytopathogenic bacteria; *Erwinia persicina*; Bacterial polysaccharide; Exopolysaccharide structure

Many subterranean monuments worldwide are endangered by complex microbial biofilms settling particularly on prehistoric wall paintings or frescoes. Biofilm development is advantaged by anthropogenic factors like enhanced humidity and CO_2 concentration as well as intense illumination of the artwork. A promising way for gently removing such microbial mats under preservation of the cultural monuments is to interfere with communication between bacterial cells.[1]

In the present study, a biofilm-associated Gram-negative bacterial strain was isolated from a palaeolithic painting in the Cave of Bats in Zuheros, southern Spain. The isolate produced large quantities of extracellular polysaccharide (EPS) and a number of signaling substances like acylated homoserine lactones. Both characteristics predestine this strain to be involved in early stages of biofilm development. EPS constitute an appropriate matrix for embedding of diverse microorganisms in the course of biofilm formation.

Sequence analysis of HKI 0380 16S rRNA resulted in 98.6% similarity with the published sequence for *Erwinia persicina* LMG 2691 (GenBank accession number AJ001190). The biochemical profile tested with Biolog GN2 MicroPlate™ offered no direct hit due to the fact that *E. persicina* is not included in this identification system. However, the evaluation software stated a 37.8% metabolic similarity to the closely related *Pantoea agglomerans*. Furthermore, the utilization pattern for carbon sources is in good accordance with previously published data.[2] These facts corroborate the affiliation of HKI 0380 to *E. persicina*.

The fermentation system used in this study is optimized for high cell density cultivation. Due to the

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production of a highly viscous EPS the oxygen delivery was restricted at OD_{600} = 24.8 AU already. This absorption does not represent the real cell density since the purified EPS itself gives opaque aqueous solutions. Purification of EPS from 1.5 L fed-batch fermentation yielded 12.33 g polysaccharide (8.22 g L^{-1}). Protein estimation resulted in approximately 6.7% whereas DNA concentration was below 1%. However, measurement of both values may be apparently enhanced by the reactivity of the highly concentrated EPS itself.

The ^{1}H and ^{13}C NMR spectra of the EPS showed irregularity in the polysaccharide structure, tentatively ascribed to non-stoichiometric O-acylation. Therefore, the EPS was O-deacylated with aqueous ammonia, which resulted in a significant simplification of the spectral region for sugar signals (Figs. 1 and 2). However, upfield to the sugar region in the ^{1}H NMR spectrum, a number of minor non-carbohydrate signals were observed, which remained assigned.

Sugar analysis of the O-deacylated EPS by GLC of the alditol acetates revealed glucose and galactose in the ratio 2.5:1. In addition, glucuronic acid (GlcA) was identified by anion-exchange chromatography using a sugar analyzer. Determination of the absolute configurations of the monosaccharides by GLC of the acetylated (+)-2-octyl glycosides showed that glucose and galactose have the D configuration. The absolute configuration of D-GlcA was determined by analysis of glycosylation effects in the ^{13}C NMR spectrum of the O-deacylated EPS (see below).

Methylation analysis of the O-deacylated EPS revealed 2,3,4,6-tetra-*O*-methylgalactose, 2,3,4-tri-*O*-methylglucose, 2,4,6-tri-*O*-methylgalactose and 2,6-di-*O*-methylgalactose in approximately equal amounts (data of GLC detector response). When the methylated polysaccharide was carboxyl-reduced prior to hydrolysis, 2,3-di-*O*-methylglucose was additionally identified, which was evidently derived from GlcA. Therefore, the EPS has a branched pentasaccharide repeating unit with terminal Gal, 3-substituted Gal, 6-substituted Glc, 4-substituted GlcA and 3,4-disubstituted Gal at the branching point. An underestimation of the content of Gal in sugar analysis could be accounted for by its partial retention in a GlcA-Gal disaccharide owing to the known resistance of the glycosidic linkage of uronic acids towards acid hydrolysis (see the structure of the EPS below).

The ^{13}C NMR spectrum of the O-deacylated EPS (Fig. 2) contained signals for five anomeric carbons at δ 99.3–105.4, one O-substituted and three non-substituted $HOCH_2$–C groups (C-6 of Glc and Gal) at δ 66.6 and 61.8–62.2, respectively, HOOC group (C-6 of GlcA) at δ 171.6 and other sugar carbons in the region δ 68.9–83.4. The ^{1}H NMR spectrum (Fig. 1) contained signals for five anomeric protons at δ 4.65–5.45, and other major sugar protons in the region δ 3.40–4.44.

The ^{1}H NMR spectrum of the O-deacylated EPS was assigned using 2D COSY, TOCSY and ROESY experiments (Table 1). In the TOCSY spectrum, the H-1 signals of Glc and all three Gal residues (Gal^{I}–Gal^{III})

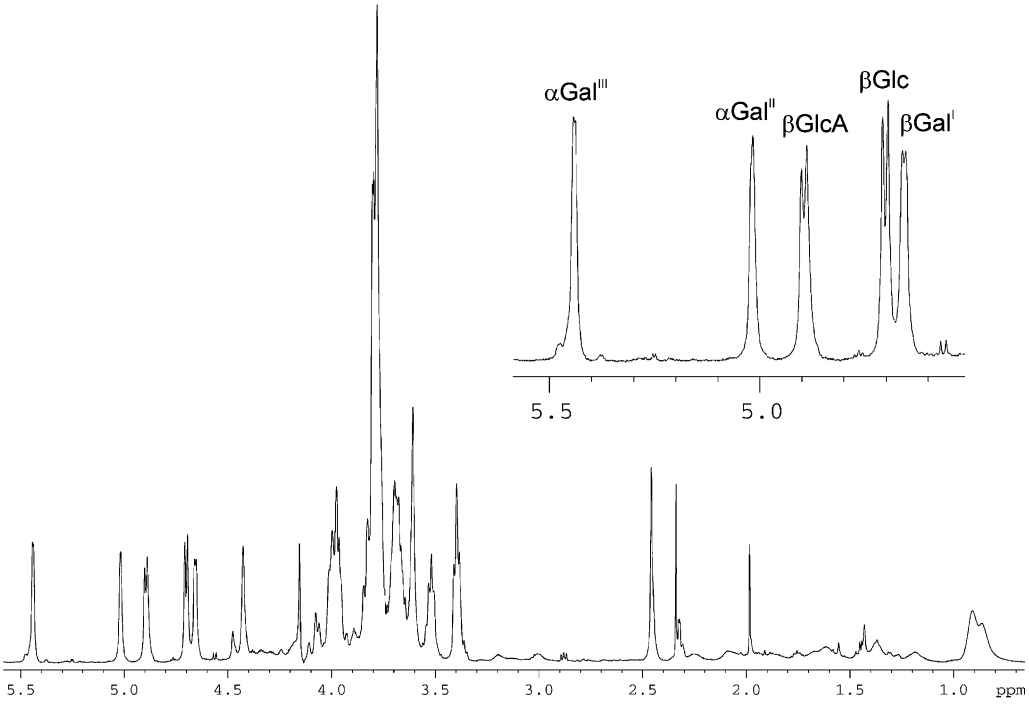

Figure 1. ^{1}H NMR spectrum of the O-deacylated exopolysaccharide from *Erwinia persicina*. The region for H-1 signals is shown in the extension.

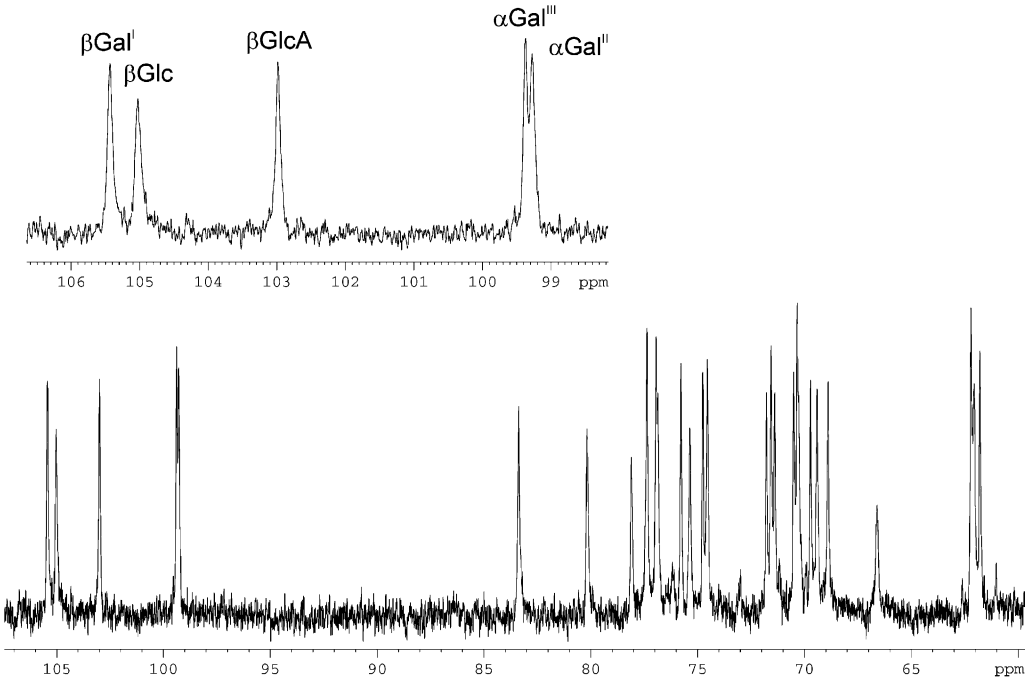

Figure 2. ^{13}C NMR spectrum of the O-deacylated exopolysaccharide from *Erwinia persicina*. The region for C-1 signals is shown in the extension; the region for CO resonances is not shown.

Table 1. ^{1}H and ^{13}C NMR data of the O-deacylated exopolysaccharide from *Erwinia persicina* (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→6)-β-D-Glc*p*-(1→	4.71	3.40	3.52	3.62	3.62	3.77	4.01
→3)-β-D-Gal*p*^{I}-(1→	4.65	3.80	3.82	4.17	3.69	3.78	3.78
→3,4)-α-D-Gal*p*^{II}-(1→	5.02	4.12	4.07	4.44	3.97	3.67	3.77
α-D-Gal*p*^{III}-(1→	5.45	3.79	3.84	3.98	3.99	3.39	3.77
→4)-β-D-Glc*p*A-(1→	4.90	3.40	3.78	3.78	3.78		
	C-1	C-2	C-3	C-4	C-5	C-6	
→6)-β-D-Glc*p*-(1→	105.0	74.5	76.9	70.3	75.4	66.6	
→3)-β-D-Gal*p*^{I}-(1→	105.4	71.6	83.4	69.4	75.8	62.2	
→3,4)-α-D-Gal*p*^{II}-(1→	99.3	68.9	80.2	76.9	71.4	61.8	
α-D-Gal*p*^{III}-(1→	99.4	69.7	70.5	70.3	71.8	62.1	
→4)-β-D-Glc*p*A-(1→	103.0	74.7	77.4	78.1	77.4	171.6	

showed correlations with H-2,3,4 signals, which were assigned within each spin system using tracing connectivities in the COSY spectrum. The ROESY spectrum enabled the assignment of H-5 signals of Gal^{I} and Gal^{II} by H-4, H-5 correlations, and those of β-linked Gal^{I}, Glc and GlcA by H-1, H-5 correlations. ^{1}H NMR signals for H-4, 5 of Gal^{III}, H-4,5 of Glc and H-3,4,5 of GlcA coincided at δ 3.98–3.99, 3.62 and 3.78, respectively. Finally, all H-6 signals were assigned by H-5, H-6 correlations in the COSY spectrum.

The three Gal spin systems were distinguished from those of Glc and GlcA by typically small $J_{3,4}$ and $J_{4,5}$ values of <3 Hz, which were estimated either directly from the ^{1}H NMR spectrum or from the 2D NMR spectra. Relatively small $J_{1,2}$ values of ~3 Hz indicated that Gal^{II} and Gal^{III} are α-linked, whereas significantly higher $J_{1,2}$ values of >6 Hz and higher-field positions of the H-1 signals at δ 4.65–4.90 showed the β-configuration of Glc, GlcA and Gal^{I}. These and methylation analysis data (see above) also confirmed that all monosaccharides are in the pyranose form.

With the ^{1}H NMR spectrum assigned, the ^{13}C NMR spectrum of the O-deacylated EPS was interpreted using an H-detected ^{1}H, ^{13}C HMQC experiment (Table 1). The ^{13}C NMR chemical shifts for C-2,3,4,5,6 of Gal^{III} in the EPS were close to those of unsubstituted α-galactopyranose,[3] thus demonstrating the terminal position of Gal^{III}. Downfield displacements of the ^{13}C NMR signals for C-3 of Gal^{I}, C-4 of GlcA, C-6 of Glc, C-3 and C-4 of Gal^{II} to δ 83.4, 78.1, 66.6, 80.2 and 76.9, that is, by 5–10 ppm as compared with the values in the corresponding non-substituted monosaccharides,[3,4] were

due to α-effects of glycosylation and confirmed the substitution pattern of the monosaccharides.

The ROESY spectrum of the polysaccharide showed correlations between the following anomeric protons and protons at the linkage carbons: Glc H-1, Gal^{I} H-3; Gal^{I} H-1, Gal^{II} H-3; Gal^{II} H-1, Glc H-6a and H-6b; Gal^{III} H-1, GlcA H-4; and GlcA H-1, Gal^{II} H-4 at δ 4.71/3.82; 4.65/4.07; 5.02/3.77 and 5.02/4.01; 5.45/3.78; and 4.90/4.44, respectively. These data defined the monosaccharides sequence in the repeating unit. A positive effect on the ^{13}C NMR chemical shift of GlcA C-3 caused by glycosylation of GlcA by α-D-Gal at position 4 indicated the same absolute configuration of the linked monosaccharides,[3] that is, the D-configuration of GlcA.

Therefore, the O-deacylated exopolysaccharide of *E. persicina* has the structure shown in Figure 3. An attempt to determine the nature and the position of the O-acyl group(s) by NMR spectroscopic studies of the initial polysaccharide failed.

The basic structure of *E. persicina* EPS is similar to that of stewartan isolated from *E. stewartii*[5] as well as to FF1 and FF2, two EPS subtypes produced by *E. fututulu*.[6] In particular, the backbone of all four polysaccharides is identical and contains β-glucose instead of β-galactose as found for *E. amylovora*[7] and *E. pyrifoliae*[8] In contrast to hexasaccharide repeating units of *E. stewartii* and *E. fututulu* polysaccharides, the repeating unit of *E. persicina* EPS is a pentasaccharide with only one branching. The side chain consists of α-D-Gal*p*-(1→4)-β-D-Glc*p*A, whereas the same side chain in stewartan and FF1 is terminated with a (1→6)-bound β-D-Glc*p* and the α-D-Gal*p* side chain in FF2 carries a pyruvic acid acetal.

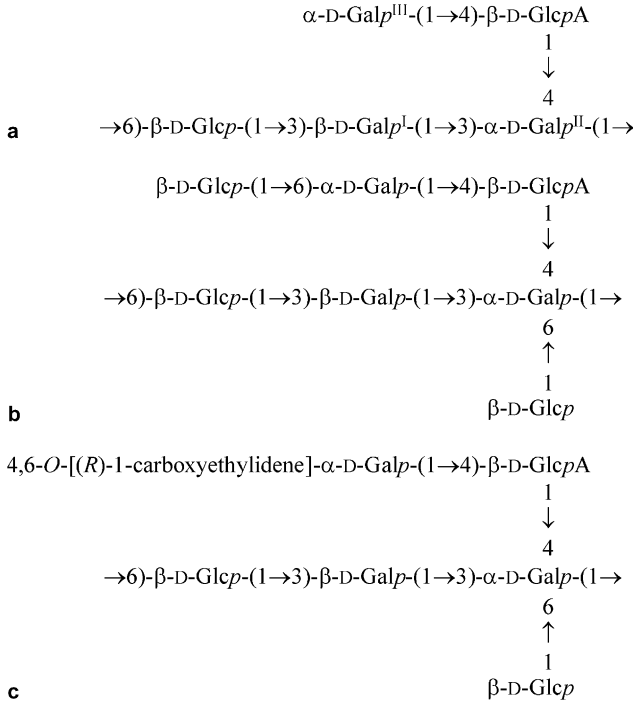

Figure 3. Structure of the O-deacylated exopolysaccharide from (a) *Erwinia persicina* in comparison with structures of (b) stewartan from *E. stewartii*[5] and identical FF1 from *E. fututulu* as well as (c) FF2 from *E. fututulu*.[6]

In addition to 16S rRNA sequencing and the metabolic pattern the high degree of similarity of EPS structures substantiates the affiliation of strain HKI 0380 to the genus *Erwinia*. The ability to synthesize significant quantities of EPS may be advantageous for biofilm development. A possible correlation between quorum sensing signaling and formation of EPS is currently under investigation.

1. Experimental

1.1. Bacterial strain

E. persicina strain HKI 0380 was isolated from the Cueva de los Murcielagos (Cave of bats) located near Zuheros in southern Spain. Metabolic characterization was carried out in Biolog GN2 MicroPlates™ according to the instructions of the manufacturer (Biolog, Hayward CA, USA) and evaluated with MicroLog3 Software. 16S rRNA sequencing was done by Amodia Bioservice GmbH (Braunschweig, Germany).

1.2. Isolation and deacetylation of EPS

E. persicina strain HKI 0380 was cultivated at 30 °C in 1.5 L M9 minimal medium[9] pH 6.6 using a Biostat B fermenter (Braun, Germany). After consumption of the initial provided 4 g L^{-1} glucose, a feeding was started with 2.9 g h^{-1}. It was subsequently adjusted to the growth rate leaving glucose the limiting factor. After 10 h the cells were removed by centrifugation and the supernatant was cooled down to 2 °C. EPS was precipitated by addition of 0.75 vol ice cold ethanol and pelleted by centrifugation (9.000*g*, 30 min, 2 °C). Subsequently, the precipitate was dissolved in 450 mL MilliQ water and dialyzed against 5 L water with six changes in 64 h. Dialysis tubings with a MWCO of 12–14 kD were from regenerated cellulose (Serva, Heidelberg, Germany). The white fluffy powder resulting from lyophilization was analyzed for the contaminating protein using the Coomassie Protein Assay (Pierce, Rockford, USA) and measured for DNA specific absorption at 260 nm. The purified EPS was kept dry and dark and used for further experiments.

The EPS was heated with 12% aq ammonia at 37 °C for 60 h, after flushing off the ammonia, the O-deacylated polysaccharide was isolated by GPC on a 40 × 2.5 cm column of Sephadex G-50 (Amersham Biosciences, Sweden) in 0.1 M NH_4HCO_3 buffer (7.91 g NH_4HCO_3 and 10 mg NaN_3 in 1 L water) at 40 mL h^{-1}.

1.3. Sugar analysis

The O-deacylated EPS (0.5 mg) was hydrolyzed with 2 M CF_3CO_2H at 120 °C for 2 h, and neutral sugars were analyzed as the alditol acetates[10] by GLC on a Hewlett–Packard model 5890 Series II instrument equipped with a 30 m capillary column of SPB-5 (Supelco), using a temperature gradient of 150–320 °C at 5 °C min^{-1}. Glucuronic acid was identified using a Biotronik LC-2000 sugar analyzer (Germany) as described[11] after treatment of the hydrolysate with 0.2 M NaOH at 20 °C for 1 min followed by acidification with 2 M H_3PO_4 to pH 2. The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (+)-2-octanol[12] under the same chromatographic conditions as above.

1.4. Methylation analysis

The polysaccharide was methylated with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinyl methanide.[13] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, conventionally reduced with $NaBD_4$, acetylated and analyzed by GLC–MS on a Hewlett–Packard HP 5989A instrument equipped with a 30 m HP-5 ms column (Hewlett–Packard) under the same chromatographic conditions as in GLC. Part of the polysaccharide after methylation was reduced with $LiBH_4$ in 70% 2-propanol (20 °C, 16 h) and then treated as described above.

1.5. NMR spectroscopy

NMR spectra were obtained on a Bruker Avance DRX-600 spectrometer (Germany) at 30 °C and pD 6 (uncorrected) in D_2O (99.96% D; Cambridge Isotope Laboratories, UK). Prior to the measurements, the samples were lyophilized twice from D_2O (~0.5 mL, 99.6% D). Chemical shifts are referenced to internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.0) or external aq 85% H_3PO_4 (δ_P 0.0). Bruker software XWINNMR 3.5 was used to acquire and process the data. A mixing time of 100 and 200 ms was used in 2D TOCSY and ROESY experiments, respectively.

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