J Ind Microbiol Biotechnol (2006) 33: 359–367
DOI 10.1007/s10295-005-0064-1

ORIGINAL PAPER

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Colanic acid is an exopolysaccharide common to many enterobacteria isolated from paper-machine slimes

Received: 21 February 2005 / Accepted: 30 October 2005 / Published online: 18 January 2006

Abstract In this study, polysaccharide-producing bacteria were isolated from slimes collected from two Finnish and one Spanish paper mill and the exopolysaccharides (EPSs) produced by 18 isolates were characterised. Most of the isolates, selected on the bases of slimy colony morphology, were members of the family *Enterobacteriaceae* most frequently belonging to the genera *Enterobacter* and *Klebsiella* including *Raoultella*. All of the EPSs analysed showed the presence of charged groups in the form of uronic acid or pyruvate revealing the polyanionic nature of these polysaccharides. Further results of the carbohydrate analysis showed that the EPS produced by nine of the enterobacteria was colanic acid.

Keywords Paper mill · Slime · Bacteria · Exopolysaccharides · Colanic acid

Introduction

Slime deposit formation in paper machines can have negative impact on the paper making process and product quality and cause significant economic losses. Slime build-up is initiated by attachment of micro-organisms on process surfaces and development of surface-attached microbial communities, biofilms, which can further entrap other organic and inorganic deposits, fines, fillers and fibres from process waters. Characteristic to microbial biofilms is the formation of an extensive network of highly hydrated exopolysaccharides (EPSs) [5]. In gram-negative bacteria, proteins (flagella, pili) are often involved in the initial attachment of cells to surfaces, in some cases the early attachment is stabilised by EPS. EPS formation is needed in later stages for development of mature three-dimensional biofilm architecture [7].

The bacterial EPSs are heterogeneous, often species- or even strain-specific polymers, the EPSs in mixed-species biofilms can show enormous variability [4]. Bacterial polysaccharides consisting mainly of fucose, rhamnose, galactose, glucose, mannose and glucuronic acid have been detected in paper machine slimes [18, 22, 46] but very little data on chemical structures has been published.

A variety of bacteria species, including *Bacillus*, *Citrobacter*, *Deinococcus*, *Enterobacter*, *Flectobacillus*, *Klebsiella*, *Methylobacterium* and *Pseudomonas*, have been isolated from paper machine slimes [8, 19–21, 24, 29, 30, 34, 47]. Some of the isolates have been characterised based on pigment production, filamentous growth characteristics or initial attachment ability but the contribution of different bacteria to the biofilm matrix formation is not known.

This study forms a part of a project aiming at novel enzymatic slime control methods targeted towards matrix polysaccharides in paper machine slimes. Here, isolation of exopolysaccharide-producing bacteria from slimes and characterisation of the polysaccharides produced by the isolates is reported with special attention to colanic acid produced by several enterobacterial isolates from different mills.

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Materials and methods

Isolation of polysaccharide-producing bacteria

Slime samples were collected from submerged and unsubmerged wet end surfaces of two Finnish (Fin A,

Fin B) and one Spanish (Spa) paper mill. Ten grams of the slime sample was homogenised with 90 ml of sterile 0.9% NaCl solution. Spread plate cultures were made of the dilutions 10^{-2}–10^{-6} on agar plates containing: glucose (20 g/l), yeast extract (0.5 g/l), $(NH_4)_2SO_4$ (0.6 g/l), KH_2PO_4 (3.18 g/l), K_2HPO_4 (5.2 g/l), $MgSO_4{\times}7H_2O$ (0.3 g/l), $CaCl_2$ (0.05 g/l), $ZnSO_4{\times}7H_2O$ (0.2 mg/l), $CuSO_4{\times}5H_2O$ (0.2 mg/l), $MnSO_4{\times}H_2O$ (0.2 mg/l), $CoCl_2$ (0.2 mg/l), $FeSO_4{\times}7H_2O$ (0.6 mg/l) agar (20 g/l). The plates were incubated inverted at 30°C and at 50°C. Slimy colonies were picked from the plates and twice purified on same agar medium.

Identification of isolates

The isolates were ribotyped using the standard method of the automated RiboPrinter® System (DuPont Qualicon, Wilmington, DE, USA) and *Eco*RI restriction enzyme as described by Bruce [3]. The similarity of the generated patterns was compared with the identification patterns existing in the RiboPrint databases of Oualicon (Release12.2 c 2000) and VTT Biotechnology [33]. In order to form a dendrogram the generated ribogroups were transferred to the BioNumerics programme (Applied Maths, Sint-Martens-Latem, Belgium), where clustering was carried out using Pearson correlation and a Ward dendrogram type. In addition, the patterns of 13 relevant type strains (T) and of *Serratia fonticola* E-991258 were included from the database of VTT as references. The isolates were also subjected to partial 16S rDNA sequencing (around 450 nucleotides between 1 and 600 bp of the 16S rRNA gene according to *Escherichia coli* numbering) as described by Suihko and Stackebrandt [39] or Saarela et al. [35]. Finally, the sequences were compared to the GenBank sequences using the BLAST search [1]. In addition, the 16S rDNA of some strains was analysed by using the ARB program package [38]. The alignment was based on secondary structure, and performed using the automatic aligner function (Version 2.0) of the ARB aligner (ARB_EDIT4) including manual evaluation. The physiological properties were performed using API identification strips ID 32 GN and ID 32 E according to the manufacturer's instructions (Biomérieux SA, Marcy-l'Etoile, France).

Production of polysaccharides

The isolates were cultivated for 5 days at 30°C in shake flasks in liquid medium with the same composition as the agar medium used for isolation containing $(NH_4)_2SO_4$ (0.6 g/l) (medium I), Bacto tryptone (Difco) (5 g/l) (medium II) or Bacto tryptone (Difco) (1 g/l) (medium III) as nitrogen source. Culture filtrate viscosities were measured using a Brookfield DV II viscometer at 20°C.

Isolation of polysaccharides

A 0.9% NaCl was added to the culture medium, the medium was lightly homogenised for 30–45 s and centrifuged at +4°C, 14,700×*g* for 45 min to separate the cells. Three volumes of ice-cold 100% ethanol was added to the supernatant. If the precipitate did not form at once, the solution was let to precipitate at 4°C overnight. The precipitate was either collected with a spoon and a strainer or it was centrifuged at +4°C, 4,000×*g* for 15 min. The precipitate was solubilised in a small volume of distilled water, dialysed using Medicell Visking (MWCO 12-14000 dalton) dialysis tube and freeze dried.

Analysis

Protein

Protein contents of the freeze-dried polysaccharides were estimated by Lowry method [23].

Sugar composition

The EPS sugar composition was determined using methanolysis as described by De Ruiter et al. [9]. The EPS was treated with 2N HCl in dry methanol for 16 h at 80°C, followed by 1 h of 2N CF_3CO_2H (TFA) at 121°C. The released sugars were analysed using High Performance Anion Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection (PAD) as described by Verhoef et al. [43].

MALDI-TOF MS

For Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight +Mass Spectrometry (MALDI-TOF MS) a Ultraflex workstation (Bruker Daltronics GmbH, Germany) was used. The mass spectrometer was calibrated with a mixture of malto-dextrins (mass range 365–2,309). The samples were mixed with a matrix solution (1 µl of sample in 9 µl of matrix). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid in a 1 ml mixture of acetonitrile : water (300 µl:700 µl). The prepared sample and matrix solutions 2 µl were put on a gold plate and dried with warm air.

Sugar linkage analyses

The EPS sample was methylated according to Hakomori [17] and subsequently dialysed against water and evaporated in a stream of dry air. The methylated samples were hydrolysed using 2 M TFA (2 h, 121°C). After evaporation in a stream of air ($T < 20$°C), the partially methylated sample was converted to alditol acetates and analysed by GC-FID [11]. Identification of the

compound was performed using GC-MS as described by van Casteren et al. [42].

Determination of the pyruvate and O-acetyl content

The amount of pyruvate was determined according to Troyano et al. [41]. The EPSs (1 mg) were dissolved in 1 ml of 1 N TFA and hydrolysed for 6 h at 120°C. The hydrolysate was centrifuged and analysed at 40°C using a Thermo Quest HPLC system equipped with both a Shodex RI71 refractive index detector and a Thermo Separation Products Spectra System UV2000 UV detector at 220 nm. HPLC separation was performed using a Bio-Rad Aminex HPX-87H column using 0.01 N H_2SO_4 (0.6 ml/min) as a mobile phase. The amount of pyruvate is calculated using a calibration curve of 0.2–2 mM sodium pyruvate. The acetyl content was determined after saponification with 0.4 M NaOH in 50%(v/v) isopropanol/water according to Voragen et al. [45] using the same chromatographic system.

Partial hydrolysis

The EPS (3–4 mg) was dissolved in 1 ml 0.05 N TFA and subjected to partial hydrolysis for 1 h at 100°C. After hydrolysis the samples were cooled, dried in a stream of dry air and redissolved in water before MALDI-TOF MS analysis.

Table 1 Identification of EPS-producing bacteria isolated from paper machine slimes

VTT code (origin)	Ribogroup, closest similarity to	Partial 16S rDNA sequence, closest similarity (%) to	API results, % id ID 32 GN/ID 32 E	Final identification
E-011926 (Fin B)	1248-S-1 0.67 *Citrobacter freundii*	96.1 *Enterobacter* sp. 95.3 *Salmonella* spp.	Low discrimination/ 95.2 *Enterobacter cloacae*	*Enterobacter* sp.
E-011927 (Fin B)	1301-S-6 0.90 K*lebsiella pneumoniae*	99.8 *K. pneumoniae*^{T}	93.8 *K. pneumoniae*/ 94.7 *K. pneumoniae*	*Klebsiella pneumoniae*
E-011929 (Fin A)	1303-S-5 0.61 *Methylobacterium* sp.	95.4 *Methylobacterium organophilum*^{T} 94.6 *Methylobacterium suomiense*^{T}	Not done	*Methylobacterium* sp.
E-011930 (Fin A)	502-S-8 0.91 *Enterobacteriaceae*, closest to *Enterobacter*	99.6 (97.3^T) *K. pneumoniae* 98.1 *Morganella morganii* 97.5 *Enterobacter cancerogenus*	Low discrimination/ 91.9 *Pantoea* spp. (doubtful profile)	*Klebsiella*/ *Enterobacter* sp.
E-011935 (Spa)	1253-S-2 0.94 *Enterobacter* sp.	99.4 *Enterobacter amnigenus*^{T} 99.0 *Buttiauxella izardii*^{T}	96.8 *Enterobacter amnigenus*/ 83.1 *Enterobacter amnigenus*	*Enterobacter amnigenus*
E-011937 (Spa)	1253-S-4 0.78 *Serratia marcescens*	99.6 *Rahnella aquatilis*	96.1 *Rahnella aquatilis*/ 94.5 *Rahnella aquatilis*	*Rahnella aquatilis*
E-011939 (Spa)	1253-S-6 0.79 *Raoultella terrigena*	99.2 *Raoultella terrigena*^{T}	96.1 *Klebsiella oxytoca*/ 99.9 *Raoultella terrigena*	*Raoultella terrigena*
E-011940 (Fin B)	502-S-8 0.92 *Enterobacteriaceae*, closest to *Enterobacter*	99.6 (97.3^T) *K. pneumoniae* 98.1 *Morganella morganii*97.5 *Enterobacter cancerogenus*	Low discrimination/ 88.1 *Pantoea* spp. (doubtful profile)	*Klebsiella*/ *Enterobacter* sp.
E-011941 (Fin B)	1254-S-2 0.48 *Citrobacter koseri*	99.6 *Citrobacter koseri*^{T}	Low discrimination/83.5 *Pantoea* spp. (doubtful profile)	*Citrobacter* sp.
E-011942 (Fin B)	1254-S-3 0.78 *Enterobacteriaceae*, closest to *Enterobacter*	99.6 (97.3^T) *K. pneumoniae* 98.1 *Morganella morganii* 97.5 *Enterobacter cancerogenus*	Low discrimination/ 83.3 *Pantoea* spp. (doubtful profile)	*Klebsiella*/ *Enterobacter* sp.
E-011943 (Fin B)	502-S-8 0.94 *Enterobacteriaceae*, closest to *Enterobacter*	99.6 (97.3^T) *K. pneumoniae* 98.1 *Morganella morganii* 97.5 *Enterobacter cancerogenus*	Low discrimination/ 90.6 *Pantoea* spp. (doubtful profile)	*Klebsiella*/ *Enterobacter* sp.
E-011944 (Fin B)	1254-S-5 0.73 *Enterobacter hormaechei*	99.4 *Citrobacter koseri*	74.5 *Enterobacter cloacae* (not valid)/low discrimination	*Citrobacter* sp.
E-022114 pa)	1386-S-3 0.69 *Enterobacter cloacae*	99.2 *Enterobacter cloacae*/*asburiae*	Low discrimination/ 97.4 *Enterobacter cloacae*	*Enterobacter cloacae*
E-022115 (Spa)	1386-S-4 0.84 *Bacillus simplex*	99.8 *Bacillus simplex*	Not done	*Bacillus simplex*
E-022116 (Spa)	1386-S-5 0.79 *Raoultella planticola*	100 *Raoultella planticola*^{T}/ *ornithinolytica* 100 *Serratia fonticola*	Low discrimination/ 99.3 *Raoultella planticola*	*Raoultella planticola*
E-022117 (Spa)	1386-S-6 0.79 *Serratia rubidaea*	98.4 *Yersinia enterocolitica* 98.1 *Serratia entomophila*^{T}	99.5 *Serratia rubidaea*/ 99.9 *Serratia rubidaea*	*Serratia* sp.
E-022118 (Spa)	1386-S-7 0.62 *Enterobacter cloacae*/ *aerogenes*	100 *Enterobacter asburiae*^{T}/*cloacae*/ *aerogenes*/*kobei*	Not valid/75.9 *Enterobacter asburiae* (doubtful profile)	*Enterobacter* sp.
E-022119 (Spa)	1386-S-8 0.82 *Enterobacter cloacae*	100 *Enterobacter asburiae*/ *cloacae*/*kobei*	Low discrimination/ 97.4 *Enterobacter cloacae*	*Enterobacter cloacae*

^{T}type strain

Results

Characterisation and identification of polysaccharide-producing isolates

Slimy bacteria were isolated from slime deposits collected from paper machines. A total of 18 isolates, originating from three different mills, were selected for detailed study. The isolates were first characterised by ribotyping. Altogether 16 different ribogroups (ribotypes) were generated, indicating that only three isolates represented the same ribotype (E-011930, E-011940 and E-011943, isolated from two different Finnish paper mills) (Table 1). RiboPrint databases could reliably identify to the species level (similarity >0.85) only one isolate, E-011927, as *Klebsiella pneumoniae*. Almost all (16/18, 89%) of the isolates were atypical members of the family *Enterobacteriaceae* belonging to the genera *Citrobacter*, *Enterobacter*, *Klebsiella*, *Rahnella*, *Raoultella* (formerly *Klebsiella*) or *Serratia* (Table 1). One isolate was identified as *Methylobacterium* sp. and one as *Bacillus simplex*. Many of the isolates potentially represent new species. The clustering (relationship) among the generated RiboPrint patterns and those of relevant type strains is presented in Fig. 1.

Production and isolation of EPSs

Exopolysaccharides of the 18 isolates were produced for characterisation by cultivating the corresponding bacteria in liquid medium. During cultivation, EPS production was estimated by measuring the increase of culture medium viscosity. The viscosities of the culture filtrates of the 18 isolates varied from 1.0 to 44 cP (Table 2). The lowest viscosity was detected in *B. simplex* E-022115 culture filtrate and the highest in *Klebsiella/Enterobacter* sp. E-011930 culture filtrate. The amounts of EPSs precipitated from the cultures varied from 0.01 to 2.82 g/l. The highest amount of polysaccharide was produced by *Citrobacter* sp. E-011941 and *Klebsiella/Enterobacter* isolates E-011930 and E-011943 (2.82, 1.67, 1.19 g/l, respectively) whereas only 0.12 g/l of polysaccharide was produced by *Klebsiella/Enterobacter* sp. E-011940, belonging to the same ribogroup as E-011930 and E-011943 and isolated from the same mill as E-011943. Due to this difference in the quantity of EPS produced, the isolates E-011940 and E-011943 were both included in further studies together with the third representative of the same ribotype originating from a different mill (E-011930).

Characterisation of the polysaccharides

The 18 polysaccharides produced were analysed for their sugar and substituent composition as shown in Table 3. In general it is interesting to see that all of the EPSs analysed show the presence of charged groups in the form of uronic acid or pyruvate revealing the polyanionic nature of these polysaccharides. Furthermore most of the EPSs analysed are substituted with *O*-acetyl groups. With respect to the sugar compositions half of

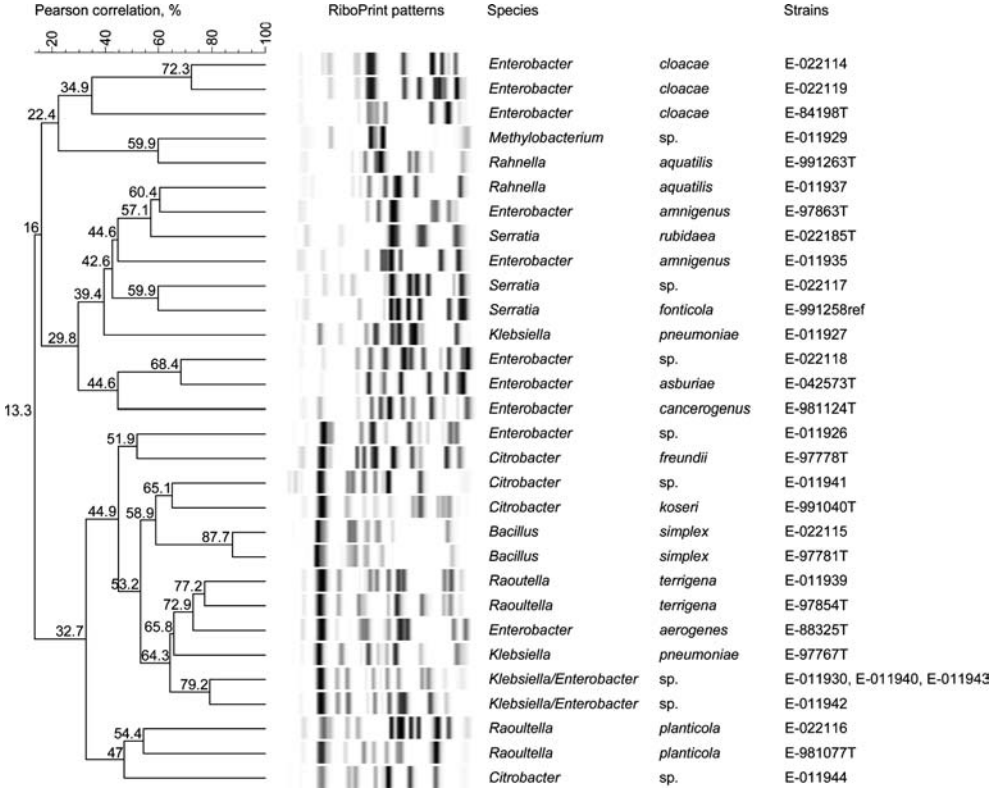

Fig. 1 Dendrogram of the RiboPrint patterns of isolated strains and 14 reference strains (T and ref)

Table 2 Polysaccharides obtained by ethanol precipitation from liquid cultures of the EPS-producing bacteria isolated from paper machine slimes

VTT code	Strain	Medium	Culture viscosity (cP)	Yield (total sugars, g/l)	Protein (g/l)
E-011926	*Enterobacter* sp.	III	2.1	0.13	0.04
E-011927	*K. pneumoniae*	III	16.3	0.53	0.02
E-011929	*Methylobacterium* sp.	II	16.1	0.46	0.04
E-011930	*Klebsiella*/*Enterobacter* sp.	III	44	1.67	0.10
E-011935	*Enterobacter amnigenus*	III	3.0	0.25	0.02
E-011937	*Rahnella aquatilis*	III	3.0	0.28	0.02
E-011939	*R. terrigena*	III	8.0	0.70	0.02
E-011940	*Klebsiella*/*Enterobacter* sp.	III	1.3	0.12	0.04
E-011941	*Citrobacter* sp.	I	28.9	2.82	0.43
E-011942	*Klebsiella*/*Enterobacter* sp.	III	2.9	0.21	0.03
E-011943	*Klebsiella*/*Enterobacter* sp.	III	15.8	1.19	0.03
E-011944	*Citrobacter* sp.	III	2.4	0.90	0.02
E-022114	*Enterobacter cloacae*	I	1.2	0.03	0.02
E-022115	*B. simplex*	I	1.0	0.02	0.02
E-022116	R. planticola	I	2.3	0.36	0.02
E-022117	*Serratia* sp.	I	1.1	0.10	0.03
E-022118	*Enterobacter* sp.	I	1.1	0.10	0.01
E-022119	*Enterobacter cloacae*	I	1.1	0.01	0.004

the isolates produce EPSs with the same sugar composition, e.g. EPSs from isolates E-011926, E-011930, E-011935, E-011940, E-011941, E-011942, E-011943, E-011944 and E-022114 all contain fucose, galactose, glucose, glucuronic acid, pyruvate and *O*-acetyl as their main sugar moieties and substituents in a molar ratio of 2:2:1:1:1:1, respectively. Apart from some deviation due to traces of rhamnose, mannose and galacturonic acid all of these EPSs have the same composition. The sugar/substituent compositions of the nine EPSs described above are the same as the composition found for colanic acid [16, 40], commonly produced by several members of the family *Enterobacteriaceae* [16].

The EPS produced by *Methylobacterium* sp. E-011929 is a pure galactan with two pyruvate ketals substituted at two out of three galactoses per repeating unit (RU). The chemical fine structure of this EPS was determined by Verhoef et al. [44]. Detailed structural analysis of the EPS produced by *Raoultella terrigena* (basonym *Klebsiella terrigena*) E-011939 (unpublished results) showed that this EPS, containing high amounts of rhamnose together with glucose, galactose, glucuronic acid and pyruvate, has the same chemical fine structure as the one reported for *Klebsiella* serotype K70 [10]. *K. pneumoniae* E-011927 and *Raoultella planticola* E-022116 EPSs also have the same sugar and substituent compositions. These latter two EPSs show a high-mannose level together with glucose, galactose, glucuronic acid and pyruvate similar to the sugars and substituents found typical for *Klebsiella* sp. EPSs [26–28].

Based on the results shown above it is clear that colanic acid seems to be one of the major EPSs found to be produced by the slimy bacterial isolates from the paper mills studied. Based on these findings it was decided to look more into detail to the structure of one of the potential colanic acid EPSs (E-011941) and compare the nine potential colanic acid samples with each other using linkage analysis and partial hydrolysis followed by MALDI-TOF MS.

Linkage analysis of colanic acid

The polysaccharide of *Citrobacter* sp. E-011941 was chosen for linkage analysis by per methylation. The results obtained for both the native and carboxyl-reduced sample (Table 4) agree with the sugar composition found in Table 3. Linkage analysis of the carboxyl reduced sample revealed the presence of 12 mol% 4-linked fucose, 29 mol% 3,4-linked fucose, 21 mol% 3-linked glucose, 21 mol% 3-linked galactose and 5 mol% 4-linked glucuronic acid. Furthermore 4 mol% terminal and 8 mol% 4,6-linked galactose could be found. These results do not exactly fit the molar proportions of the RU. The rather high level of 3,4-linked fucose, when compared to the 4-linked fucose, could be explained by the possible incomplete removal of the *O*-acetyl group, known to be attached to position 2 or 3 of the 4-linked fucose during methylation, leading to an overestimation of 3,4-linked fucose. Furthermore the low amount of 4-linked glucuronic acid found is easily explained by incomplete carboxyl reduction of this moiety to its glucose analogue. Taking into account that bacterial polysaccharides always consist of RUs it was concluded that this EPS consists of 4-linked fucose, 3,4-linked fucose, 3-linked glucose, 3-linked galactose, 4,6-linked galactose and 4-linked glucuronic acid with a molar ratio of 1:1:1:1:1:1, respectively. The results found agree with previous linkage analysis [13, 14, 21] performed on colanic acid. The 4,6-linked galactose represents the terminal galactose substituted with a pyruvate ketal at position 4 and 6.

Partial hydrolysis of colanic acid

To prove whether the different glycosyl moieties found are linked together in the same sequence as found for colanic acid all the nine potential colanic acid EPSs were subjected to weak acid treatment to release oligomeric

Table 3 Sugar and substituent composition (in mol%) of the EPSs produced by the isolated strains

VTT code	Strain	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Xylose	GalA	GlcA	O-Acetyl	Pyruvate	total sugar
E-011926	*Enterobacter* sp.	19	–[a]	–	23	15	16	–	–	13	8	5	63
E-011927	*Klebsiella pneumoniae*	–	–	–	13	15	42	–	–	19	–	10	80
E-011929	*Methylobacterium* sp.	–	–	–	53	–	2	–	–	–	–	45	46
E-011930	*Klebsiella*/*Enterobacter* sp.	31	–	–	23	15	3	–	1	15	8	4	66
E-011935	*Enterobacter amnigenus*	25	–	–	21	14	2	–	5	12	11	9	65
E-011937	*Rahnella aquatilis*	–	27	–	41	2	1	–	2	27	–	–	71
E-011939	*R. terrigena*	–	41	-	19	17	1	–	–	18	1	4	69
E-011940	*Klebsiella*/*Enterobacter* sp.	16	4	–	23	18	9	–	3	11	8	6	46
E-011941	*Citrobacter* sp.	31	–	–	26	13	2	–	1	16	7	5	55
E-011942	*Klebsiella*/*Enterobacter* sp.	20	4	–	22	11	7	–	2	11	13	10	64
E-011943	*Klebsiella*/*Enterobacter* sp.	27	1	–	24	15	2	–	2	14	10	6	56
E-011944	*Citrobacter* sp.	23	1	–	23	19	4	–	2	14	9	5	59
E-022114	*Enterobacter cloacae*	26	2	–	21	14	-	–	–	12	13	7	25
E-022115	*B. simplex*	–	18	–	14	19	30	–	3	15	2	–	13
E-022116	*R. planticola*	–	–	–	18	12	47	–	1	10	1	10	61
E-022117	*Serratia* sp.	–	37	2	–	3	–	–	30	–	11	17	38
E-022118	*Enterobacter* sp.	12	–	–	3	6	67	2	9	–	–	–	52
E-022119	*Enterobacter cloacae*	–	22	–	13	16	–	–	–	–	1	47	22

[a]not detected

fragments. These oligomers were analysed by MALDI-TOF MS and the results were compared with the known structure of colanic acid. Figure 2 shows an example of the obtained MALDI TOF MS spectra after partial hydrolysis. The proposed structures of the oligomers released upon weak acid treatment are summarised in Table 5.

The first oligomers with *m*/*z* 495.5 and 537.4 represent a trimer of two fucose and one hexose with or without *O*-acetyl followed by the oligomers at *m*/*z* 511.4 and 553.6 representing a trimer of two hexose and one fucose with or without *O*-acetyl. Judging from acid stability of the different glycosidic linkages present within colanic acid the first oligomer most probably is a trimer representing the core of the RU composed of two fucoses and one glucose with fucose at both the terminal and reducing end of the oligomer. The oligomer at *m*/*z* 511/553 was more striking, since its release involves the hydrolysis of the aldobiuronic acid linkage between the glucuronic acid and galactose present in the side chain of the RU. This oligomer represents a trimer with fucose at the reducing end, glucose at the non-reducing end and a terminal galactose attached to the fucose. The oligomer found at *m*/*z* 611.6 was a trimer composed of galactose, glucuronic acid and pyruvated galactose. This trimer represents the full side chain of colanic acid and has a galactose at the reducing end and a pyruvated galactose at the terminus of the oligomer. No acetylated form of this oligomer was found. The hexamers found with mass *m*/*z* 1,192, 1,149, 1,107, 1,165, 1,037 and 995 all represent different forms of the RU of colanic acid: RU with three, two, one or no *O*-acetyl group and RU without pyruvate with one or 0 *O*-acetyl groups, respectively (Fig. 2). The results indicate that colanic acid has more *O*-acetyl substituents then previously assumed [13, 14] and that these *O*-acetyl groups are distributed at given locations over the RUs forming the polysaccharide.

Since pyruvate is rather acid labile the RUs without pyruvate could be explained by the removal of this substituent by acid. A mass difference of -146 between the RUs with one or zero O-acetyl groups and without pyruvate showed the release of oligomers representing the latter without fucose at *m*/*z* 961, 919 and 849, respectively. Furthermore the oligomeric fragment of the RU without the terminal pyruvated galactose (-232) at *m*/*z* 833 and the oligomeric fragment with *m*/*z* 875 representing the same oligomer with one additional *O*-acetyl group could be found. These structures agree with that of colanic acid. To confirm this for the other eight EPSs they were treated with the same weak acid conditions to see whether the same oligomers were released.

Analysis of the oligomeric fragments released after weak acid treatment of the other eight EPSs revealed that all of the samples except E-022114 are 75% or more similar to the reference sample. Although the sample of E-022114 only contains 38% of these oligomeric fragments, still these do indicate the presence of colanic acid, especially the ones representing the RU with one or two *O*-acetyl moieties at *m*/*z* 1,107.8 and 1,149.7. Again the

Table 4 Glycosidic linkage composition in mol% of the partially methylated alditol acetates of the native and reduced EPS of the isolate E-011941

	Native (mol%)	Reduced (mol%)	
2,3-di-O-Me-Fucp	13	12	→ 4)Fucp(1 →
2-O-Me-Fucp	32	29	→ 3,4)Fucp(1 →
2,4,6-tri-O-Me-Glcp	22	21	→ 3)Glcp(1 →
2,3,6-tri-O-Me-Glcp	–	5	→ 4)Glcp(1 → (GlcA)
2,4,6-tri-O-Me-Galp	20	21	→ 3)Galp(1 →
2,3-di-O-Me-Galp	9	8	→ 4,6)Galp(1 →
2,3,4,6-tetra-O-Me-Galp	3	4	Galp(1 →

Fig. 2 MALDI-TOF ms spectrum of the partially hydrolysed (0.05 N TFA) EPS produced by the isolate E-011930

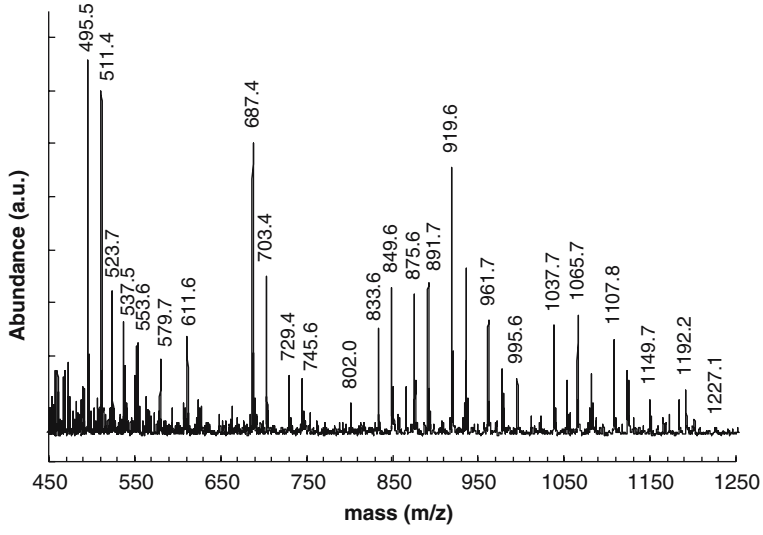

Table 5 Fragments released upon mild acid treatment (0.05 M TFA 100°C 1 h) of the EPS produced by the isolate E-011930 with their corresponding *m*/*z* values

m/z	O- acetyl	pyruvate	oligomer
495.5	-[a]	-	[fuc]-[glc]-[fuc]-OH or [fuc]-[fuc]-OH[b]
537.4	+	-	└[gal]
511.4	-	-	[glc]-[fuc]-OH
553.6	+	-	└[gal]
611.6	-	+	[gal]-[glcA]-[gal]-OH
687.4	-	-	[glc]-[fuc]-OH
729.4	+	-	└[gal]-[glcA]
833.6	-	-	[fuc]-[glc]-[fuc]-OH
875.6	+	-	└[gal]-[glcA]
849.6	-	-	[glc]-[fuc]-OH
919.6	-	+	└[gal]-[glcA]-[gal]
961.7	+	+	
995.6	-	-	
1037.7	+	-	
1065.7	-	+	[fuc]-[glc]-[fuc]-OH
1107.8	+	+	└[gal]-[glcA]-[gal]
1149.7	++	+	
1192.2	+++	+	

[a] + one *O*-acetyl or pyruvate moiety present; – not present
[b] OH represents the reducing end based upon preferred hydrolyses according to the most labile glycosidic linkage

between sample variance points to an inhomogeneous distribution of *O*-acetyl groups over the RU of colanic acid, since 89% of the samples shows the release of RUs with two *O*-acetyl groups, instead of only one. Future enzymatic studies will be used to determine the positions and distribution of these different *O*-acetyl groups.

Discussion

In this study, polysaccharide-producing bacteria were isolated from slimes collected from two Finnish and one Spanish paper mill and the EPSs produced by the isolates were characterised. The selection of isolates was based on slimy colony morphology and the isolates do not necessarily represent the most numerous bacteria in the deposits. Almost all (16/18) of the isolates were members of the family *Enterobacteriaceae* with *Enterobacter* and *Klebsiella* (*Raoultella*) as the most frequently represented genera. *Enterobacter*, *Klebsiella* and *Citrobacter* species are common in soil, healthy and decaying wood and in plants and also appear to be normal inhabitants in many paper machines [2, 24, 25, 33, 47]. According to a Canadian study, these bacteria grew continuously in many of the mills studied with the most likely growth areas being paper machines, biofilms on machinery and piping and the primary clarifier [15]. Polysaccharide-producing enterobacteria were found especially in the Finnish mill B and in the Spanish mill, slimy bacteria belonging to, e.g. genera *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Brevundimonas*, *Deinococcus*, *Methylobacterium*, *Paenibacillus* and *Pseudomonas* have previously been identified from various other mills [24, 34, 47].

The results of the carbohydrate analysis showed that the EPS produced by nine of the enterobacterial isolates is colanic acid consisting of 4-linked fucose, 3,4-linked fucose, 3-linked glucose, 3-linked galactose, 4,6-linked galactose and 4-linked glucuronic acid with a molar ratio of 1:1:1:1:1:1, respectively. The colanic acid—producing isolates belonged to the genera *Enterobacter* or *Citrobacter* or were atypical members of the *Enterobacteriaceae* most closely resembling *Klebsiella* and *Enterobacter*. Colanic acid is common to the EPS of many genera in the *Enterobacteriaceae* [16]. Small amounts of colanic acid are normally produced constitutively, but production can be increased by osmotic shock [37], β-lactam antibiotics [36] or during growth on solid surfaces [12]. Increase in colanic synthesis has been found to occur in *E. coli* cells growing in biofilms [32]. *E. coli* mutants deficient in colanic acid production are able to attach to abiotic surfaces and form bacterial films of one to two cell layers thick but are unable to form the three-dimensional, complex biofilm structures typical of the colanic acid producing parent strain [6, 31].

Colanic acid proved to be the major EPS produced by 9 of the 18 slimy bacterial strains isolated from the three paper mills selected for this study. Future studies will focus on the occurrence of colanic acid in slimes collected from the mills and on the potential of enzymatic slime control using colanic acid degrading enzymes on mills where enterobacteria commonly exist.

Acknowledgements The work described has been carried out with financial support from the Commission of European Communities, specific RTD programme "Competitive and Sustainable Growth", G1RD-CT2000-00387, "Eco-efficient novel enzymatic concepts for slime control in pulp and paper processing (Slimezymes)". It does not necessarily reflect the Commission's views and in no way anticipates the Commission's future policy in this area. We thank the technical staff of VTT Biotechnology for skilful technical assistance and the staff of Identification Service of DSMZ for the sequences.

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