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Characterization of exopolysaccharides produced by rhizobacteria

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Summary. Bacteria isolated from the rhizosphere, the rhizobacteria, of sorghum, pearl millet, wheat, alfalfa and rice were screened for the production of exopolysaccharide (EPS). Nearly a quarter of the strains produced exopolysaccharides, either capsular or hydrosoluble slime. A majority of the isolates produced slime. Physico-chemical analyses have indicated the ability of certain diazotrophic *Pseudomonas paucimobilis* isolates from millets and sorghum to produce unique types of EPS, which are highly viscous and thermostable.

Introduction

Bacterial exopolysaccharides are extensively used as thickening and gelling agents in a wide range of industrial products and processes (Kang and Cottrell 1979), but the strains used so far for industrial production of polysaccharides belong to a surprisingly small number of taxa such as *Agrobacterium radiobacter, Rhizobium* spp., *Xanthomonas campestris, Bacillus* spp., *Azotobacter vinelandii, Alcaligenes faecalis* and *Klebsiella pneumoniae* (Whitefield 1988). Several of these are able to synthesize more than one type of polysaccharide (Sutherland 1985).

It is worth noting that several of these taxa are soil-inhabiting diazotrophs and often plant-associated. It has been suspected that their ability to fix nitrogen in vitro or in association with the plants is related to their ability to produce copious amounts of polysaccharides. This relation was demonstrated in the case of two nitrogen-fixing soil bacteria *Beijerinckia* spp. and *Derxia gummosa* (Hill and Postgate 1969; Becking 1974), wherein smooth mucoid colonies fixed nitrogen more actively than the rough non-mucoid ones. The explanation was that as the enzyme nitrogenase is oxygen-sensitive, polysaccharides act as a barrier against oxygen diffusion, thus providing an optimal low pO_2 for N_2-fixation.

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In the course of a recent survey of bacteria associated with plant roots, it was observed that in an N-deficient C-rich medium a large number of these produced copious amounts of exopolysaccharide (EPS). It was therefore decided to evaluate root-associated microflora for the production of polysaccharide with a potential for industrial application.

Materials and methods

Bacterial strains. A total of 175 strains of rhizobacteria were screened for their ability to produce EPS, of which 50 strains were obtained from various research centres in India, Bangladesh and Egypt. Most of the strains associated with cereal roots were N_2-fixing, and were isolated using the spermosphere enrichment method (Thomas-Bauzon et al. 1982). The host plants were wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), pearl millet (*Pennisetum americanum* L.), sorghum (*Sorghum bicolor* L.) and lucerne (*Medicago sativa* L.).

Media. Purified bacterial strains were maintained on slopes of nutrient agar or N-deficient media: RCV (Weaver et al. 1975), malate medium (Döbereiner and Day 1976), or Watanabe's synthetic medium (Watanabe and Barraquio 1979); the latter was modified by the addition of 100 mg/l of yeast extract and the replacement of glucose by a mixture of carbohydrates (5 g glucose/l, 5 g sucrose/l, 3 g mannitol/l and 3 g malate/l). The pH was adjusted to 6.8 and the media autoclaved at 120° C for 20 min.

Identification of bacterial strains. Following preliminary Gram and oxidase reaction tests, API (Appareils et Procédés d'Identification, bioMérieux, La Balme les Grottes, F-38390, Montalieu Vercieu, France) microtube systems API 20NE and API 20B were used for bacterial identification.

Extraction of polysaccharides. To evaluate the production of EPS, bacteria were grown in one of the three N-deficient media. Initial inoculum was obtained from a 5 ml nutrient broth culture grown overnight at 28° C and centrifuged at 15000 *g* for 15 min. The pellet obtained was suspended in 5 ml of 0.85% KCl solution and inoculated into a 250-ml serum bottle containing 100 ml culture medium. The 100-ml batch cultures were incubated at 28°C for 3-5 days with constant agitation in an orbital shaker (150 rpm).

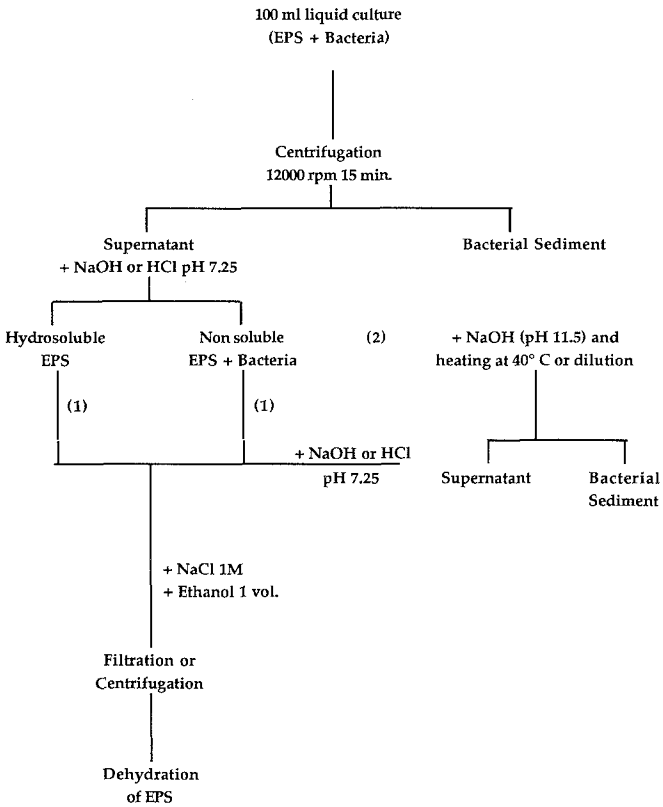

Fig. 1. Flow chart for polysaccharide extraction

The general procedure used for exopolysaccharide extraction is described in Fig. 1. The first step was the centrifugation of the culture solution at 18000 *g*; the pH of the supernatant was then adjusted to 7.25. Polysaccharides were precipitated by the addition of 6 g of NaCl (1 M final concentration) followed by addition of one volume (100 ml) of 95% ethanol. The precipitate was recovered either by swirling a spatula or a glass rod or by centrifugation at 3000 *g* for 15 min. Polysaccharides were then dehydrated in an alcohol series (60, 70, 80, 95% ethanol) and vacuum-dried at 35° C.

The above procedure was also used for extracting hydrosoluble slime. However if a capsular EPS was present, dilution of the culture solution followed by pH adjustment to 11.5 and subsequent heating (40° C) was necessary to separate the bacterial cells from the polysaccharide (pathway 2).

Chemical analysis of EPS. Hexoses and acid substituents such as succinate, pyruvate and uronic acids were determined by HPLC. Purified EPS (20 mg) was hydrolysed with 1 M H_2SO_4 (2 ml) in sealed tubes at 100° C for 8–16 h. The hydrolysate was neutralised using $BaCO_3$, filtered and separated into two fractions. Quantitative determination of neutral carbohydrates was done with the first fraction by HPLC at 85° C on an Aminex HPX 87P column (Bio-Rad), after elimination of the acid components using Amberlite MB3 resin. The column was eluted with deionized water at a flow rate of 0.5 $ml \cdot min^{-1}$. Uronic and organic acids were identified with the second fraction by HPLC on a Radial Pak C_{18} Cartridge (Waters), eluted with 0.05% dodecyl trimethylammonium chloride in a 0.05 N sodium nitrate solution. Liquid chromatography was performed using Waters equipment: solvent delivery system (model M 6000A), universal injector (model U6K) and R 401 refractometric detector (Courtois et al. 1986).

^{1}H-Nuclear magnetic resonance (NMR). A better characterization of organic substituents was obtained by using proton magnetic resonance. Spectra were recorded at 100 MHz in a D_2O solution (5 g/l) on a Brucker WP 100 spectrometer at 85° C. Peak areas assigned to the methyl protons of the pyruvate, *O*-acetate and *O*-succinate were compared to the peak area of free acetate as an internal standard (sodium acetate $5 \cdot 10^{-3}$ M) (Heyraud et al. 1986).

Optical rotation. Optical rotation was measured on 0.1% (w/v) polysaccharide solutions in 0.1 M NaCl with a Fica spectropolarimeter (Model Spectropol 1b), operating at 300 nm with a 5-cm thermostatted cell. Temperature was controlled by a Haake circulating water bath (from 10° C to 85° C ± 0.5).

Viscosity measurement. Viscosities at low shear rates were measured with a Contraves Low-Shear LS 30 coaxial cylinder viscometer.

Results

Screening of isolates for polysaccharide production

Among the strains screened, nearly a quarter (43 strains) were able to produce polysaccharides under the conditions used. Most of the strains produced either a capsular polysaccharide or slime (Table 1). An hydrosoluble slime was the only type of polysaccharide produced by a majority of the strains from wheat, pearl millet and lucerne, whereas several rice isolates produced also a capsular EPS. Only ten strains (5Aj, 29Aj, 5ATr, RMDP17a and b, RMDP19, RMDP10, 7jDa, 7jDb and Mucl) produced both capsular and slime EPS.

Strains B5, M43, M48, M38, M51 from the rhizosphere of rice, strains B6, BG12, BG43, BG44 from wheat, strains 7jC2, 7jC3, 7jC4, 7iC from lucerne, strains RMDP1, RMDP15, RMDP22a from pearl millet and strains B7C, L33, L28A, L40B from maize did not produce extractible polysaccharide under the growth conditions used (data not presented). Strains such as *Xanthomonas* sp. TW38, 14G, 7CSF, *Bacillus polymyxa* ATCC 842 and ATCC 21551, unidentified strains MFH, F7M, MFG2A and *Pseudomonas paucimobilis* 2395 did not produce any polysaccharide in a liquid medium, although they seemed to produce polysaccharide-like substances on agar medium (data not presented).

Bacterial identification

Most of the strains were identified after analysis of phenotypic characters (API 20NE and API 20B) with an APILAB software programme (Table 1). EPS-producing strains identified as *Agrobacterium radiobacter* were isolated frequently from wheat and pearl millet. Several bacteria isolated from rice, pearl millet and lucerne were identified as *Pseudomonas* spp.

Chemical analysis

The chemical composition of EPS produced by 39 different strains is given in Table 1. A majority of the rhi-

Table 1. Nature and composition of exopolysaccharides (EPS) produced by rhizobacteria

Plant origin and strain code	Bacterial identification	Media[a]	EPS production: Capsular	EPS production: Slime	EPS yield g/l/5 days growth	Composition: Neutral sugars: Glu	Gal	Man	Uronic acids	Substituents: Acet	Pyr	Succ	Remarks
Rice													
SY1	*Pseudomonas paucimobilis*	RCV + sucrose	+ + +	–	1.0	+	+	+	+	–	–	–	
5Aj	*P. paucimobilis*	RCV + sucrose	+ + +	+ +	1.3		nd		nd		nd		Slight
29Aj	*P. paucimobilis*	RCV + sucrose	+ +	+	1.3		nd		nd		nd		solubility
5ATR	*P. paucimobilis*	RCV + sucrose	+ +	+	1.3		nd		nd		nd		
40P	*Pseudomonas* sp.	NB + sucrose	+ +	–	0.3	+	+	–	+	–	–	–	Fucose in EPS
MSR	Unidentified	WAT + glucose[b]	–	+ + + +	4.5	+	+	+	+	+	–	–	
S55ASP	*Azospirillum* sp.	WAT + glucose[b]	–	+ + +	1.6	+	+	–	–	–	+	+	Succinoglycan
445BP	*Azospirillum* sp.	WAT + 4 sugars	–	+ +	1.0	+	+	–	–	–	+	+	
Wheat													
Aa	*Agrobacterium radiobacter*	RCV + mannitol[b]	–	+	1.7	+	+	–	–	+	+	+	
Ab	*A. radiobacter*	WAT + glucose[b]	–	+ +	1.0	+	+	–	–	+	+	+	
BG1, BG2, BG5	*A. radiobacter*	RCV + mannitol	–	+ + +	1.3–1.5	+	+	–	–	–	+	+	Succinoglycan
BG9	*A. radiobacter*	RCV + 4 sugars	–	+ + +	1.9	+	+	–	–	–	+	+	
BG11, BG41,	*A. radiobacter*	RCV + glu + suc[b]	–	+ + +	0.6–1.2	+	+	–	–	–	+	+	
BS28, BS28a,													
BS28b, BS21, BS8b													
CF41	*Bacillus polymyxa*	RCV + sucrose[b]	+ + +	–	4.0	+	+	+	+	+	+	+	Proteins in EPS
M1	Unidentified	RCV + suc + mann	–	+ + + +	2.6	+	+	–	–	+	+	+	
M2	Unidentified	RCV + mannitol	–	+	0.4	+	+	–	–	+	+	+	Succinoglcyan
N	Unidentified	RCV + mannitol	–	+	0.8	+	+	–	–	+	+	+	
Pearl millet													
RMDP 17a	*P. paucimobilis*	RCV + 4 sug	+ +	+ +	1.8	+	+	–	–	+	–	–	Highly viscous
RMDP 17b	*P. paucimobilis*	WAT + glu + suc	+ +	+ +	1.6	+	+	–	–	–	–	–	polysaccharides
													containing rhamnose
RMDP19	*Beijerinckia* sp.	RCV + sucrose	–	+ + + +	4.0		nd		nd		nd		
		RCV + glucose	+ +	+	1.2								
RMDP10	*Beijerinckia* sp.	RCV + 4 sugars	+ + +	+ + +	2.0	+	+	–	–	+	+	+	
RMDP16	*A. radiobacter*	RCV + 4 sugars	–	+ + + +	4.1–4.5	+	+	–	–	+	+	+	
RMDP23, GM85,	*A. radiobacter*	RCV + sucrose	–	+ + + +	2.5–3.8	+	+	–	–	+	+	+	Succinoglycan
GM570, GM1848													
GM290a, GM290b,	*A. radiobacter*	RCV + mannitol	–	+ + + +	2.5	–	+	+	–	–	+	+	
GM117	Unidentified	RCV + glu + suc	–	+ +	1.0	+	+	–	+	+	+	–	Rhamnose in EPS
Lucerne													
7jB1	*Pseudomonas* sp.	RCV + mannitol[b]	–	+ + +	4.4	+	+	–	(+)	+	–	–	
7iB, 7jC1	*Pseudomonas* sp.	RCV + mannitol[b]	–	+ + +	0.5–0.8	+	+	–	–	+	–	–	
7jDa, 7jDb	*Pseudomonas* sp.	RCV + mannitol[b]	+	+ + +	1.0–1.2	+	+	–	–	–	+	+	
Sorghum													
Muc1	*P. paucimobilis*	WAT + sucrose	+ + +	+	2.5	+	+	+	+	–	+	–	Proteins in EPS
													Highly viscous

nd = not determined; + to + + + + = positive to highly positive; – = negative; (+) = traces; Glu = glucose; Suc = sucrose; Gal = galactose; Man = mannose; Acet = Acetate; Pyr = Pyruvate; Succ = Succinate; WAT = Watanabe; RCV = after Weaver et al. (1975); NB = nutrient broth; DOB = Döbereiner; 4 sugars (sug/l) = 5 g glucose, 5 g sucrose, 3 g mannitol (mann), 3 g malate

[a] Culture medium most favourable for EPS production

[b] NH_4Cl used at concentrations from 10 to 500 mg/l

Table 2. Characteristics of EPS produced by rhizobacteria

Plant origin and strain code	Bacterial identification	EPS produced		Solubility in water	Filterability (0.8–0.45 μm)	η_{rel} at $\gamma = 0.015\ s^{-1}$ [C] = 1 g/l in 0.1 M NaCl	T_M in NaCl 0.1 M (°C)	$[\alpha]^{25}_{300}$ in 0.1 M NaCl	Remarks
		Capsular	Slime						
Rice									
SY1	*P. paucimobilis*	+	–	Fair	Fair	2.0	nd	– 58	
29Aj	*P. paucimobilis*	+	+	Poor	–	nd	nd	nd	Slight solubility
5ATr	*P. paucimobilis*	+	–	Poor	–	nd	nd	nd	
40P	*P. paucimobilis*	–	–	Good	Good	14.3	27.0	–140	Fucose replaces mannose
MSR	Unidentified	–	+	Good	Good	5.3	66.5	+ 64	
S55ASP	*Azospirillum* sp.	–	+	Good	Good	108.5	66.5	–132	Succinoglycan
445BP	*Azospirillum* sp.	–	+	Good	Good	55.0	67.0	–108	
Wheat									
Aa	*A. radiobacter*	–	+	Good	Good	175.0	67.0	–166	
Ab	*A. radiobacter*	–	+	Good	Good	305.0	67.0	–160	
BG1	*A. radiobacter*	–	+	Good	Good	581.0	70.0	–128	
BG2	*A. radiobacter*	–	+	Good	Good	172.0	60.0	–116	
BG5	*A. radiobacter*	–	+	Good	Good	96.0	74.0	–132	
BG9	*A. radiobacter*	–	+	Good	Good	230.0	68.0	–108	Succinoglycan
BG11	*A. radiobacter*	–	+	Good	Good	271.0	68.5	–136	
BG41	*A. radiobacter*	–	+	Good	Good	1269.0	68.0	–144	
BS28	*A. radiobacter*	–	+	Good	Good	363.0	68.0	–128	
BS21	*A. radiobacter*	–	+	Good	Good	743.0	68.5	–124	
BS8b	*A. radiobacter*	–	+	Good	Good	454.0	68.5	–142	
CF41	*B. polymyxa*	+	–	Fair	Fair	1.1	nd	+150	EPS with proteins
Pearl millet									
RMDP 17a	*P. paucimobilis*	+	+	Good	Good	6200 (2477.0)	70.0	–148	Highly viscous EPS
RMDB 17b	*P. paucimobilis*	+	+	Good	Good	1640.0	nd	nd	
RMDP19	*Beijerinckia* sp.	+	+	Poor	Poor	nd	nd	nd	
RMP10	*Beijerinckia* sp.	+	+	Fair	Fair	nd	nd	nd	
RMDP16	*A. radiobacter*	–	+	Good	Good	89.0	67.0	–128	
RMDP23	*A. radiobacter*	–	+	Good	Good	670.0	67.0	–118	
GM85	*A. radiobacter*	–	+	Good	Good	nd	nd	nd	
GM570	*A. radiobacter*	–	+	Good	Good	100.0	67.0	nd	Succinoglycan
GM1848	*A. radiobacter*	–	+	Good	Good	599.0	66.0	–124	
GM290a	*A. radiobacter*	–	+	Good	Good	1050.0	66.0	–110	
GM290b	*A. radiobacter*		+	Good	Good	74.0	66.0	–118	
GM117	Unidentified	–	+	Good	Good	28.0	nd	+108	
Lucerne									
7jB1	*Pseudomonas* sp.	–	+	Good	Good	Slight viscosity	66.5	– 30	
7iB	*Pseudomonas* sp.	–	+	Good	Good	3.8	nd	+300	
7jC1	*Pseudomonas* sp.	–	+	Good	Good	14.7	nd	+380	
7jDa	*Pseudomonas* sp.	+	+	Good	Poor	4254	70	–108	Succinoglycan
7jDb	*Pseudomonas* sp.	+	+	Good	Good	2466 (620)	80	–136	
Sorghum									
Muc1	*P. paucimobilis*	+	(+)	Fair	Poor	2160	nd	+290	EPS with proteins, gel forming

η_{rel} = Relative viscosity; T_M = temperature for conformational transition; $[\alpha]^{25}_{300}$ = specific rotation measured at 300 nm at 25° C; ( ) viscosity only partially lost after heating zosphere bacteria produced EPS containing glucose and galactose with a molar ratio of 7:1 and different proportions of pyruvyl, acetyl and succinyl substituents. These are typical succinoglycans. Chemical analyses have revealed some unique and highly complex polysaccharides from unidentified strains MSR, *Pseudomonas* sp. 40P and *P. paucimobilis* strains SY1, RMDP 17a, RMDP 17b and Muc1. EPS from *P. paucimobilis* strains are constituted not only of glucose (1 to 4) and galactose (1 to 5) subunits along with acetate or pyruvate residues, but also, at times, of mannose (1 to 3) and uronic acids. However, in strains RMDP 17a and 17b mannose and uronic acids are absent. Fucose is present in the EPS of strain 40 and rhamnose in the EPS of strains RMDP 17a and 17b and unidentified strain GM117.

Physical and rheological properties

Polysaccharides exhibiting good solubility (in H_2O) and good filterability (pore size 0.8 μm to 0.45 μm) were further analysed for their rheological properties. Characteristics such as specific rotation $[\alpha]^{25}_{300}$, temperature of conformational transition (T_M) and relative viscosity (η_{rel}) are presented in Table 2.

As already mentioned, a majority of the EPS-producing rhizobacteria screened produced highly viscous succinoglycans, with various proportions of succinate and pyruvate, and whose relative viscosities (η_{rel}) of 1 g EPS/l in 0.1 M NaCl at γ $10^{-2}\,s^{-1}$ varied from 55 to 1269. Succinoglycans produced by the *Pseudomonas* sp. strains 7jDa and 7jDb exhibited higher viscosity (2466 and 4254, respectively) than those produced by other strains. EPS produced by pearl millet *P. paucimobilis* strains, RMDP 17a and 17b, and the sorghum strain Muc1 were also highly viscous (η_{rel} 6200, 1640, and 2160 respectively), but not the rice strain SY1. EPS varied in their specific rotation ($[\alpha]^{25}_{300}$) from −58 to −166. Exceptions were unidentified strains MSR (+64), GM117 (+108), *B. polymyxa* CF41 (+150), *P. paucimobilis* Muc1 (+290), and *Pseudomonas* spp. 7iB (+300) and 7jC1 (+380).

The most important effect of temperature on EPS, except for degradation in some exceptional cases, was an order-disorder conformational change, easily followed by optical rotation measurements. The average transition temperature (T_M) for the succinoglycans was $68 \pm 4°$ C and the relative viscosity decreased with increasing temperatures. This phenomenon was only partially reversible. In the case of *P. paucimobilis* strains RMDP 17a and 17b, no conformational change of EPS was observed between 10 and 90° C, and heating the polysaccharide for 10 min at 90° C caused only a 14% decrease in relative viscosity. In the presence of 0.1 M NaCl, heating increased this relative viscosity.

Discussion

A majority of the EPS-producing rhizobacteria screened in this study produced a highly viscous succinoglycan, with various proportions of succinate and pyruvate. Such succinoglycans are already known to be the most common EPS produced by soil bacteria and especially by N_2-fixing bacteria (Berthellet et al. 1984), and their pyruvyl, acetyl and succinyl contents are largely dependent upon the growth conditions (Jansson et al. 1977). Beside these, two strains (RMDP 17a and 17b) identified as *P. paucimobilis* produce an unusual highly viscous and thermostable polysaccharide, meeting the objective of the study, which was screening of rhizobacteria for selecting those producing copious amounts of EPS with high viscosity and thermostability, two important characters for industrial application.

Anson et al. (1987), have also reported a *P. paucimobilis* strain (NCIB 11942) producing a highly viscous polysaccharide. However, the reported chemical analysis of this polysaccharide (1 glucose, 0.66 rhamnose, 16% uronic acid, 10.4% acetate, and 2.5% pyruvate) shows that this EPS is quite different from the polysaccharide produced by strains RMDP 17a and 17b, as it contains a high proportion of uronic acids. The less viscous EPS produced by *P. paucimobilis* strain SY1 is also different from the Anson's type of EPS containing glucose, galactose, mannose and uronic acid.

The EPS produced by *P. paucimobilis* strains RMDP 17a and 17b were not only unique in their chemical composition but also exceptionally attractive for industrial application considering their viscosity, thermostability and absence of uronic acid. A European patent no. 0353145 has been registered.

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