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SACCHARIDE COMPOSITION OF EXTRACELLULAR POLYMERS PRODUCED BY SOIL MICROORGANISMS

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Summary—Microorganisms vary widely in their ability to produce extracellular polymers (ECPs). The saccharide composition of ECPs (water-soluble and water-insoluble fractions) obtained from three soil *Pseudomonas* strains was compared to the saccharide composition of ECPs of *Arthrobacter viscosus*, *Azotobacter indicus*, *Bacillus subtilus*, *Chromobacterium violaceum*, *Cryptococcus laurentii*, *Hansenula holstii* and *Mucor rouxii*. The saccharide composition of the ECPs ranged from two homopolysaccharides (*B. subtilus*; *H. holstii*) composed of 770 and 930 mg saccharides g^{-1} ECP organic C, respectively, to heteropolysaccharides (*Pseudomonas* strains) consisting of <30 mg saccharides g^{-1} ECP organic C. The saccharide composition of the water-insoluble ECPs obtained from the *Pseudomonas* strains was independent of the saccharides added to the growth medium as a C and energy source. Glycoproteins were the major constituents of the water-insoluble *Pseudomonas* ECPs ranging from 315 to 510 mg protein g^{-1} ECP. These ECPs contained active fractions of acid and alkaline phosphatase, invertase, urease and β-glucosidase. Electron photomicrographs revealed massive excretion of ECPs possibly functioning in colonization and protection of exoenzymes.

INTRODUCTION

Production of extracellular polymers (ECPs) is a common property of most soil microorganisms (Allison, 1968). Such polymers often occur as gelatinous sheaths or capsules that contain homo- or heteropolysaccharides. A number of functions have been suggested for ECPs, which include adhesion, protection against desiccation, ion exchange, tolerance to metals, and recognition and immunological protection against predation (Dudman, 1977).

A great deal of evidence has been collected showing that soil polysaccharides are involved in the soil aggregation process (Cheshire, 1979; Tisdall and Oades, 1982). Research with ^{14}C-labeled substrates has shown that soil saccharides represent a mixture of saccharides of plant and microbial origin (Oades and Wagner, 1970; Cheshire *et al.*, 1971, 1973). Using [^{14}C]glucose and [^{14}C]xylose, Cheshire *et al.* (1971, 1973) found that both substrates were rapidly utilized by the soil microflora with very little transformation into other saccharides. Oades and Wagner (1971) reported that the labeled saccharides transformed by soil organisms persisted in soil and were assumed to be present in the resting cells of the soil microbiota. Such studies have shown that while soil microorganisms metabolize most of the added organic materials, they also produce ECPs. ECPs secreted by *Chromobacterium violeceum* (Martin *et al.*, 1965; Martin and Richards, 1963), *Azotobacter indicus* (Martin *et al.*, 1965) and *Bacillus subtilus* (Martin, 1946) were reported to be effective agents increasing aggregation and tilth when applied to soil. Addition of microbial gums isolated from soil have also been reported to increase soil aggregation when reapplied to soil (Rennie *et al.*, 1954). Little is known on the stability of saccharides released as ECPs in soil and even less is known about the saccharide composition of ECPs produced by soil organisms which are capable of stabilizing soil aggregates.

Our aim was to determine the composition of ECPs of diverse soil microbiota and to characterize the composition of ECPs produced by *Pseudomonas* spp isolated from organic-amended soil exposed to different growth substrates. The *Pseudomonas* ECP saccharide composition was compared with the saccharide composition of selected microbial polymers reported to improve soil tilth.

MATERIALS AND METHODS

Microorganism isolation and ECP extraction

The *Pseudomonas* strains were isolated on soil extract agar (10 g glucose l^{-1}) from an Arlington coarse-loamy, mixed Haplic Durixeralf (pH 7.9; 670 g sand and 250 g clay kg^{-1} soil; C/N 10.9) that had been amended with two applications of 25 metric tonnes barley straw (*Hordeum vulgare*) ha^{-1} soil over 9 months. The isolates were Gram-negative, catalase, oxidase, citrate, urease and arginine dehydrogenase positive, non-spore forming motile rods. One isolate was identified as *Pseudomonas aeruginosa* based upon pyocyanin and fluorescein pigment production on King's A and King's B agar, respectively. The other two *Pseudomonas* strains (I and II) produced fluorescein pigment on King's B agar but not pyocyanin on King's A agar, and could not be typed further based on assays outlined by Bergan (1981). The ECPs were isolated from inoculated nutrient broth (Difco, Detroit, Mich.) (10 g saccharide l^{-1}) after 2 weeks at 25°C. The broth was decanted and the water-insoluble polymer was separated from the cellular material by gentle vortex and centrifugation (1500 rev min^{-1} for 10 min). The pellet was rinsed twice with water and

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centrifuged after each rinse. The water-insoluble ECP was then dried at 50°C and stored at −20°C. The decanted broth was decreased in volume by gentle heating (50°C) and a stream of N_2 gas. The water-soluble ECPs were precipitated by addition of 3× methanol volume. The solution was covered and the ECPs allowed to precipitate overnight at 4°C and the solution decanted. The precipitate was then solubilized in water and the process repeated three times. The water-soluble ECPs were then dried at 50°C and stored at −20°C. The water-soluble and water-insoluble ECPs were light brown in color.

Extracellular polymers were also extracted from the following soil organisms: *Arthrobacter viscosus* (ATCC 19584, Cadmus *et al.*, 1963), *Azotobacter indicus* (ATCC 9037, Martin *et al.*, 1965), *B. subtilus* (ATCC 15192, Martin, 1946), *Chromobacterium violaceum* (ATCC 9544, Martin and Richards, 1963), *Cryptococcus laurentii* (ATCC 10668, Cadmus *et al.*, 1962), *Hansenula holstii* (ATCC 2448, Anderson *et al.*, 1960), *Mucor rouxii* (ATCC 24905, Bartnicki-Garcia and Reyes, 1968).

Saccharide and protein analyses

The saccharide composition of the isolated ECPs was determined as described by Martens and Frankenberger (1990). This involved treatment of 1 mg microbial polymer C (dry) with 0.4 ml 6 M H_2SO_4 for 2 h and heated under a reflux apparatus for 16 h with 0.5 M H_2SO_4. Samples were then treated with 0.1 M EDTA and titrated to pH 4.0 with 5 M KOH. The hydrolyzed monosaccharides were separated by high performance anion chromatography and determined with pulsed amperometric detection.

Total protein content was determined with a micro-amino acid analyzer Model 420-A-03 (Applied Biosystems, Foster City, Calif.) using norleucine as an internal standard.

Enzyme isolation

Enzyme activities in the water-insoluble *Pseudomonas* ECPs were extracted as follows: all broth was decanted from the ECP and the ECP was placed into a polypropylene centrifuge tube, shaken with water and separated from the cell debris by centrifugation at 2000 rev min^{-1} for 10 min. The cellular material in the supernatant was decanted, the ECP pellet gently vortexed with water and the centrifuge process repeated three time. The ECP pellet was then mixed with 50 ml 10 mM phosphate buffer, vigorously vortexed and centrifuged at 10,000 rev min^{-1} for 10 min. The clear supernatant was decanted, diluted to 50 ml with the appropriate buffer for acid and alkaline phosphatase (Tabatabai, 1982), invertase (Frankenberger and Johanson, 1983), urease (Tabatabai, 1982) and β-glucosidase (Bastic *et al.*, 1980) and assayed for each of the enzyme activities. Organic C content was determined by a modified Mebius procedure (Nelson and Sommers, 1982).

Transmission electron microscopy was conducted on a Hitachi H-600 electron microscope (Hitachi Sci. Instr., Mountain View, Calif.) at a 60-kV accelerating voltage utilizing a ruthenium red treatment (Balkwill and Casida, 1979). Tests indicated that the *Pseudomonas* strains could be separated from the ECP by centrifuging. Thus the isolation, fixation and dehydration procedures were conducted after allowing the

Table 1. Total carbon and saccharide content of selected microbial polymers and reference compounds

Code	Microbe	Organic C content (mg g^{-1} ECP)	Total saccharide content (mg g^{-1} ECP-C)
		Bacteria	
Ai	*Azotobacter indicus*	377	810
Cv	*Chromobacterium violaceum*	362	506
Bs	*Bacillus subtillus*	453	867
Av	*Arthrobacter viscosus*	392	303
	P. aeruginosa		
Paws	water-soluble fraction	605	26
Pawi	water-insoluble fraction	635	24
	Pseudomonas sp I		
PIws	water-soluble fraction	514	74
PIwi	water-insoluble fraction	575	186
	Pseudomonas sp II		
PIIws	water-soluble fraction	522	61
PIIwi	water-insoluble fraction	628	26
		Deuteromycetes	
Cl	*Cryptococcus laurentii*	407	636
Hh	*Hansenula holstii*	347	996
Mr	*Mucor rouxii*	53	43
		Reference*	
	Hydroxyethyl guar	483	698
	Glucose	380	956

*Total C content based on 1 mg sample; total saccharide content based on 1 mg C sample.

Table 2. Saccharide composition of microbial ECPs and hydroxyethyl guar

Microbe code	Inositol	Mannitol	Fucose	Arabinose	Rhamnose	Galactose	Glucose	Xylose	Mannose	Fructose	Ribose
						$\mu g\ g^{-1}$ C					
Ai	34	60	ND*	ND	56	ND	188	ND	511	ND	ND
Cv	27	30	ND	60	ND	282	68	49	ND	ND	18
Bs	27	4	67	ND	ND	ND	20	ND	ND	775	ND
Av	26	8	ND	ND	ND	203	91	ND	ND	ND	ND
Paws	25	2	6	ND	ND	ND	6	ND	11	ND	ND
Pawi	28	3	8	ND	ND	ND	16	ND	ND	ND	ND
PIws	26	8	3	ND	6	12	24	ND	11	ND	9
PIwi	36	31	21	ND	14	113	ND	ND	ND	ND	8
PIIws	33	7	22	ND	ND	ND	24	ND	8	ND	ND
PIIwi	35	1	14	ND	ND	ND	10	ND	ND	ND	ND
Cl	40	48	ND	ND	ND	25	35	243	284	ND	ND
Hh	46	8	ND	ND	48	ND	12	ND	927	ND	ND
Mr	63	17	ND	ND	ND	11	15	ND	ND	ND	ND
Hydroxyethyl guar	64	36	6	19	ND	141	25	7	359	ND	44

*ND, not detected.

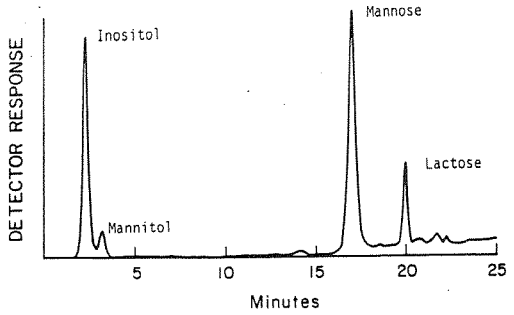

Fig. 1. HPAC-PAD chromatogram of an acidic extract of *Hansenula holstii*. Lactose was added as an internal standard.

Table 3. Metabolism of saccharides by *Pseudomonas* spp*

Saccharide	Organisms		
	Pseudomonas I	*Pseudomonas* II	*P. aeruginosa*
Arabinose	NM	NM	NM
Cellobiose	NM	NM	F/O
Fucose	NM	NM	NM
Galactose	F/O	F/O	F/O
Glucose	F/O	F/O	F/O
Lactose	NM	NM	F/O
Mannitol	NM	NM	F/O
Mannose	F/O	F/O	F/O
Rhamnose	NM	NM	NM
Xylose	F/O	F/O	F/O

*NM, not metabolized; O, oxidation of saccharide; F, fermentation of saccharide. The organisms were grown in Burk's medium with the saccharide indicated at 37°C for 7 days.

majority of organisms to settle, and not centrifuged, before proceeding to the next step.

RESULTS AND DISCUSSION

Pseudomonas strains are active microbiota in the mineralization of organic matter in soil (Dooren de Jong, 1926). Screening soil bacteria from the amended Arlington soil indicated that the majority of bacteria isolated were *Pseudomonas* strains. The three *Pseudomonas* strains used in this study were selected on their ability to produce large amounts of water-insoluble material when cultured in nutrient broth (10 g glucose l^{-1}).

Organic carbon analyses indicated that the water-soluble and water-insoluble polymers collected from the *Pseudomonas* strains contained a higher amount of C than the non-*Pseudomonas* ECPs (Table 1). However, the *Pseudomonas* strains were lower in total saccharide content when compared with the other microbial polymers (Table 1).

Saccharide composition and metabolism

The saccharide composition of the 13 microbial polymers and a reference compound, hydroxyethyl guar, is shown in Table 2. Basically, two types of ECPs, homopolysaccharides and heteropolysaccharides, were present in the non-*Pseudomonas* ECPs. The bacterial ECP isolated from *B. subtilus* was composed mainly of fructose (775 mg g^{-1} ECP-C) and was the only non-*Pseudomonas* ECP to contain fucose (67 mg kg^{-1} ECP-C). The deuteromycete, *H. holstii*, was composed of repeating mannose units (927 mg g^{-1} ECP-C). Figure 1 is a chromatogram of an acidic extract of the *H. holstii* ECP separated by high performance anion chromatography and detected by pulsed amperometry. High resolution was obtained upon detection of inositol, mannitol and mannose with this ECP. The remaining non-*Pseudomonas* ECPs were heteropolysaccharides composed mainly of mannose–glucose units (*Azotobacter indicus*), galactose–glucose units (*Arthrobacter viscosus*, *Mucor rouxii*) and mannose–xylose units (*Cryptococcus laurentii*). Martin and Richards (1963) showed that the *Chromobacterium* ECP was among the most effective organic polymers tested for increasing soil aggregation and tilth. The *Chromobacterium* ECP was composed mainly of galactose (282 mg g^{-1} ECP-C), glucose (68 mg g^{-1} ECP-C), arabinose (60 mg g^{-1} ECP-C), and xylose (49 mg g^{-1} ECP-C). The appearance of arabinose and xylose in the ECPs of microbial saccharides was not expected since they are considered as being of plant origin (Murayama, 1981). However, Cheshire *et al.* (1973) reported that small amounts of [^{14}C]xylose and [^{14}C]arabinose were found in soils exposed to [^{14}C]glucose and persisted for at least 112 days.

Previous work has indicated that the production of homopolysaccharides and heteropolysaccharides by chemoheterotrophs is a function of the C source available for growth. The biosynthesis of homopolysaccharide-ECPs such as fructosan by *B. subtilus* occurs by a transglycosylation process (Anderson, 1963). A specific saccharide source usually is required (Wilkinson, 1958). However, Wilkinson (1958) reported that changes in substrates (saccharides) have little effect on the composition of heterosaccharide ECPs and appears to be a genetic characteristic (Austrian, 1952). In our work, the effects of modifying the growth substrates (saccharides) in nutrient broth had little to no effect on the saccharide composition of the water-insoluble *Pseudomonas* ECPs. The saccharide composition of the *Pseudomonas* ECPs listed

Table 4. Total protein and saccharide content and amino acid composition of selected microbial ECPs

Composition (mg g^{-1} ECP)	*Chromobacterium violaceum*	*H. holstii*	*P. aeruginosa*	*Pseudomonas* I	*Pseudomonas* II
Total saccharide content*	506	996	24	140	26
Total protein content	116	6	510	460	315
Aliphatic amino acids†	43	<1	225	207	145
Basic amino acids	18	<1	72	74	47
Acid amino acids	30	<1	123	110	76
Aromatic amino acids	4	<1	52	41	28
Methionine + proline	6	<1	37	28	19

*Saccharides as mg g^{-1} ECP-C.

†Aliphatic amino acids include glycine, alanine, valine, leucine, isoleucine, serine and threonine; basic amino acids include lycine, arginine and histidine; acidic amino acids include aspartate and glutamate; and aromatic amino acids include tyrosine and phenylalanine.

Table 5. Enzyme activity of microbial extracellular polymers

Microbe	Enzymes				
	Acid phosphatase	Alkaline phosphatase	Invertase	Urease	β-Glucosidase
			Units*		
P. aeruginosa	1.80	0.30	0.02	23.70	8.00
Pseudomonas I	4.20	0.10	0.08	9.50	6.80
Pseudomonas II	9.00	0.40	0.10	20.60	7.10

*Units of enzyme activity expressed as μM product formed l^{-1} min^{-1}.

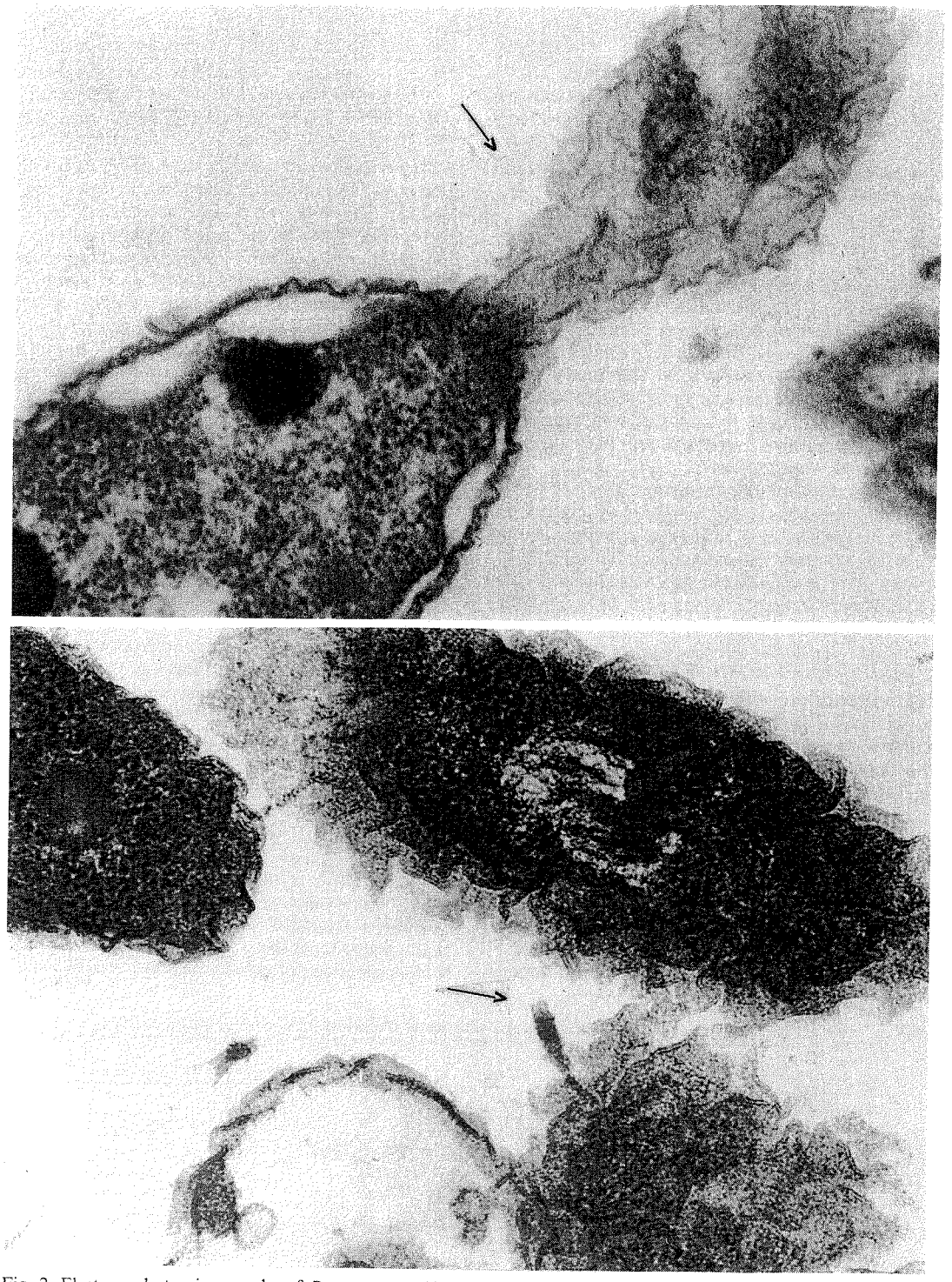

Fig. 2. Electron photomicrographs of *P. aeruginosa* (60,000×) showing excretion of ECPs. Arrows indicate ECPs.

in Table 2 were a result of adding glucose as a sole saccharide source to the growth medium. In another study, arabinose, fucose and rhamnose added to the growth medium were the only added saccharides found in the *Pseudomonas* ECPs at amounts comparable to those added. Vortexing the polymer with water removed nearly all traces of the three saccharides from their respective ECPs indicating that they are weakly associated but not incorporated into the *Pseudomonas* ECP. *Pseudomonas* strains incubated in nutrient broth without added saccharides always produced ECP of the same composition as the nutrient broth spiked with different saccharides.

Pseudomonas strains can grow at the expense of a large variety of organic compounds (Palleroni, 1981). Metabolic studies showed that these *Pseudomonas* strains could not metabolize arabinose, fucose or rhamnose (Table 3) and may explain why large amounts of these three saccharides were found associated but not incorporated into the ECP. Only galactose, glucose, mannose and xylose were metabolized by all three *Pseudomonas* isolates. *P. aeruginosa* metabolized these saccharides present in Burk's medium within 2 days of addition and was an active denitrifier. *Pseudomonas* strains I and II required at least 10 days for positive production of acid and denitrified at a much slower rate.

Oades and Wagner (1970) and Cheshire *et al.* (1971, 1973) found that when [^{14}C]glucose or [^{14}C]xylose were added to soil, low concentrations of [^{14}C]soil saccharides persisted during incubation. Cheshire *et al.* (1971) reported that ^{14}C-labeled rhamnose and [^{14}C]-fucose detected upon incubation of [^{14}C]glucose in soil became the most strongly labeled saccharides persisting during the study. They concluded that certain saccharides persist in soil due to "selective decomposition". The inability of the isolated *Pseudomonas* strains to decompose rhamnose and fucose supports their "selective decomposition" theory.

Protein composition and function

The non-*Pseudomonas* ECPs had a relatively high saccharide content comprising the total C content (Table 1). The *Pseudomonas* ECPs had less saccharides but ranged from 514 to 635 mg C g^{-1} ECP indicating that amino acids may constitute a significant fraction of these ECPs since amino acids contain between 320–650 mg C g^{-1} amino acids. Protein analyses indicated that the water-insoluble *Pseudomonas* polymers ranged from 315 mg protein g^{-1} ECP (*Pseudomonas* II) to 510 mg protein g^{-1} ECP (*P. aeruginosa*) (Table 4). It is of interest that even though the *Pseudomonas* ECPs varied in their protein contents, the percentage of the different classes of amino acids were nearly identical (Table 4). In comparison, the *Chromobacterium* ECP was 116 mg protein g^{-1} ECP and the *Hansenula* ECP contained very little protein (6.0 mg g^{-1} ECP).

The high proportion of protein present in the *Pseudomonas* ECPs suggests that the polymers may have an enzymatic function. After separation of the polymers from the cellular material, enzyme assays indicated a wide range of enzymatic activity (Table 5). The high level of urease activity may be due to the use of nutrient broth consisting of beef broth and peptone as organic N sources. The ECPs of each of the three *Pseudomonas* strains contained active fractions of acid and alkaline phosphatase, invertase, urease and β-glucosidase.

Further research is needed on the fate of water-soluble and water-insoluble ECPs in the soil environment. The water-soluble ECPs are no doubt rapidly utilized by other soil organisms as a C and energy source. Electron microphotographs showed that the water-insoluble ECP secretion appears to be associated with the cell membrane of *P. aeruginosa* and may provide increased surface area for bacterial attachment (Fig. 2). Burns (1982) reviewed the ecology of soil enzymes and proposed a microbe-exoenzyme–substrate interaction. In theory, microorganisms may retain their extracellular enzymes within these ECPs and possibly protect the proteins from proteases by being associated with the polysaccharides in the ECP (Barker and Gray, 1983). The extensive production of ECPs may extend the colonization of the bacterial cell within the surrounding environment.

Our study shows that the saccharide composition of ECPs from isolated *Pseudomonas* strains were not affected by the saccharide composition of their nutrient growth medium and were of a proteinaceous nature. The persistence of certain saccharides in the soil environment is probably due to "selective decomposition" by microorganisms. The *Pseudomonas* ECPs in addition to providing a mechanism for adhesion and avoiding desiccation may also function to protect the bacterial exoenzymes and increase exploitation for nutrients in the soil environment.

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