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Bacterial Exopolysaccharides and Productivity of Salt Affected Soils: I. Diversity of Exopolysaccharide-producing Bacteria Isolated from the Rhizosphere of Wheat (*Triticum aestivum* L.) Grown in Normal and Saline Pakistani Soils

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Abstract

This study was conducted to isolate and identify the EPS-producing bacteria associated with the roots of three wheat lines grown in saline and non-saline soils. Results indicated the presence of various EPS-producing bacterial genera in unplanted saline and non-saline soil, rhizosphere and rhizoplane of the three wheat fines. *Bacillus* sp. were more variable in unplanted saline than non-saline soil. In contrast to uniform distribution of *Bacillus* sp. in unplanted soils, other EPS-producing bacterial genera isolated from rhizosphere and rhizoplane were more numerous and diversified. Frequent occurrence of *Microbacterium* sp. in the soil and rhizosphere of the plants invoke the desire for more research work to explore the role of this bacterium in the saline environment.

Introduction

Natural salinity and forced salinization (through canal irrigation) of soils is a serious problem limiting crop productivity under arid and semi-arid conditions. Besides nigh salt content, degraded soil structure (Barzegar *et al*., 1996) is an important factor responsible for the failure of most plant types to grow and survive under saline soil conditions (Wiegand *et al*., 1996). A plausible reason for poor soil structure is the low organic matter content which is insufficient to sustain reasonable level of microbial activity. Besides several kinds of microbial activities responsible for sustained soil productivity and favourable soil structure, bacterial production of exopolysaccharides (EPS) is of particular significance under saline conditions.

Exopolysaccharides constitute a minor fraction (0.1 to 1.5%) of the total soil organic matter (Cheshire, 1979), but because of their unique water-holding and cementing properties, they play a vital role in the formation and stabilization of soil aggregates and regulation of nutrient and water flow across the plant roots (Tisdall, 1994). These EPS are added to the soil as either sloughed off plant parts and root exudates of intact plants or capsular and slime materials secreted by soil microbes (Tisdall and Oades, 1982). Among soil micro-organisms, EPS-producing bacteria inhabiting the vicinity of the plant roots are considered to the more important (Webley *et al*., 1965; Gouzou *et al*., 1993, 1995; Amellal *et al*., 1998). EPS produced by these acteria form the water stable micro-aggregates responsible for maintaining physico-chemical properties of the soil suitable for plant growth (Tisdall, 1994; He and Horikawa, 1997). However, limited information is available on the uantification and characterization of EPS-producing acteria from salt-affected soils. The objective of the present study was to isolate and identify the EPS-producing acteria from the rhizosphere of wheat grown in saline and normal Pakistani soils.

Materials and Methods

Soils: The soils were collected from experimental fields of the Nuclear institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. Physico-chemical characteristics of the air-dried and sieved (< 2 mm) soil are presented in Table 1.

Plants: Two salt tolerant lines (W-1073 and W-41) and one commercial variety (INQ-91) of wheat (*Triticum aestivum* L.) were obtained from the Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan, and Ayub Agriculture Research Institute, Faisalabad, Pakistan, respectively. The salt tolerant lines were developed through crossing wheat with Aegilops cylindrica (Farooq *et al*., 1995) and have rooting characteristics fairly different from those of commercial variety. Seeds were surface-sterilized and allowed to germinate on moist filter paper before transferring to potted soil (250 g soil pot^{-1}). The plants were grown for 15 days in a growth chamber under controlled conditions (day and night temperatures of 15 and 21°C, respectively and day light of 400 μmoles photons m^{-2} sec^{-1} for a period of 8 h day^{-1}). Initially, the plants were irrigated with H_20 at 60 percent water holding capacity (WHC) and subsequently the water content was maintained by weight-loss method.

Isolation of rhizosphere EPS-producing bacteria: After a specified growth period (between 16 to 26 days after transplantation), plants with attached roots were recovered from the soil and excessive soil from roots was removed by a mechanical stirring technique (Gouzou *et al*., 1993). The soil that was retained on the roots following mechanical stirring was termed "root adhering soil" or RAS. The RAS and the rhizoplane (RP) fractions of plant roots were

obtained by sequential washings of the roots (5 and 50 ml sterilized water) and manual maceration of the washed roots, respectively. Isolation of EPS-producing bacteria was then made from these two fractions as well as from unplanted soil. Both the RAS and plant roots were dried at 105°C for 24 h.

Table 1: Physico-chemical characteristics of the two soils.

	Soil	
Property	Normal	Saline
Sand %	27.7	56.3
Silt, %	57.1	29.2
Clay, %	14.4	12.0
Electrical conductivity, dS m^{-1}	2.7	8.3
pH (KCl extract)	7.6	8.0
Na^{+1}	3.6	21.0
K^{+1}	5.0	2.6
SO_4^{-2}	5.9	33.8
Cl^{-1}	3.2	8.1
Organic C, %	0.82	0.07
Total N, %	0.06	0.03
Mineral-N (μg g^{-1} soil) ($NH_4^+ + NO_2^- + NO_3^-$)	53.4	18.6

Serial dilution and pour plate method were used for the isolation of EPS-producing bacteria on RCV mineral medium (Heulin *et al*., 1987) enriched with mannitol, sucrose or glucose (40 g L^{-1}) and with or without 15 g L^{-1} of NaCl. Total viable counts of the three fractions were taken on the respective sugar media and in 10 times diluted tryptic soy agar medium. Bacterial colonies showing EPS-production on sugar media were randomly selected and purified by streak plate method using tryptic soy agar (TSA) medium. Purified strains were then grown overnight in liquid broth (LB) at 30°C under constant agitation and mixed with sterile glycerol (40%) for preservation at -80°C.

Identification of isolated bacteria by BIOLOG and API systems: Isolated and purified bacterial strains were classified into G (Gram-negative) and G (Gram-positive) by aminopeptidase test and identified by BIOLOG and API5OCHB (bioMerieux, France) systems of bacterial identification. For BIOLOG identification, purified strains cultured overnight in 5-10 ml of LB broth were centrifuged at 12000 rpm for 10 min. The bacterial pellets were washed twice in 0.85 percent KCl solution and then suspended in 2-3 ml of KCl solution arid used as inoculurn or adjusting the transmittance of the bacterial suspension (35 to 42% for G^- and 53 to 59% for G^+ bacteria) in BIOLOG tubes. The bacterial suspension was dispensed into the wells (150 μl per well) of BIOLOG microtiter plates and the plates were incubated for 24 hrs. Positive response was indicated by a colour change, which was measured at 590 nm using a spectrophotometer plate reader (MR 5000 Dynatech). For spore forming bacteria, the washed bacterial pellets were suspended in API5OCH (bioMerieux, France instead of BIOLOG solution. API strips representing 49 substrates for bacterial acidification were then inoculated with bacterial suspension arid observed for a positive change after recommended incubation period.

Data obtained from both the methods were submitted to the specific data base i.e. Microlog and Apilab (bioMerieux, France) and the bacteria were identified by numerical taxonomy giving a range of percentage identification. Strains isolated from saline soil were assigned the code MAS, while that from normal soil as MA. Isolated bacterial strains were again tested for polysaccharide production by a rapid spot plate screening method using glucose and sucrose media with and without NaCl at 25°C and 30°C.

Results and Discussion

Population density of bacteria was determined only for one of the salt tolerant wheat lines i.e., W-1073. Of the two soils (unplanted), saline soil showed a lower mean viable bacterial counts as compared to the normal soil (Table 2), an observation in line with other reports (Bilal and Maliik, 1987). Rhizospheric fraction (root adhering soil or RAS) contained higher population of bacteria compared to unplanted saline soil. In normal soil, there was a rhizospheric effect only for population growing on mannitol-containing medium. Rhizosphere soil is reported to maintain a higher microbial population through providing easily oxidizable rhizodeposits that include sloughed off root material and root exudates (Heulin *et al*., 1987; Liljeroth *et al*., 1990; Martens, 1990). A higher bacterial enrichment was found for RAS of saline as compared to that of normal soil, probably due to higher rhizodeposition under saline conditions. Several reports indeed suggest greater release of carbonaceous material from roots exposed to salinity (Giordano *et al*., 1994). Addition of NaCl (15 g L^{-1}) in the medium had, in general a depressing effect on bacterial population. These observations indicated a marked effect of plants on the development of microbial populations in the rhizosphere.

Table 3 and 4 list EPS producing bacteria isolated from different fractions of wheat roots in normal and saline soils, respectively. The results indicated uniform distribution of EPS-producing bacterial genera (*Bacillus* sp.) in unplanted soil. However, the two soils varied in the number of species of dominating bacterial genera. Saline soil contained *Bacillus* species more than normal soil. The variation in number of *Bacillus* species could be attributed to the harshness of the saline environment for survival of bacteria, which supported only the subsistence of spore tormers. A variation among different plant cultivars and between the plants grown in normal and saline soil was observed for rhizosphero are rhizoplane EPS-producing bacterial genera and it was more prominent on the rhizoplane of the three cultivars grown in normal soil. The effect of rhizoplane and rhizosphere on bacterial diversity and population density was also observed by others (Rouatt and Katznelson, 1961; Liljeroth *et al*., 1990; Mavingui *et al*., 1992).

Table 2: Total viable bacterial counts (CFU, Colony Forming Units) on different media in unplanted and rhizosphere soil (root adhering soil) of a wheat line W-1073 grown in normal and saline soils.

		CFU × 10^6 g^{-1} soil				
Soil fraction		TSA/10	Glucose	Mannitol	Sucrose	Mean
Non-saline soil						
Unplanted soil	Control	17	31	1	22	18
	NaCl, 15g/l	7	25	2	20	14
Rhizosphere soil	Control	9	11	20	21	15
	NaCl, 15 g/l	3	4	7	7	5
Saline soil						
Unplanted soil	Control	3	1	9	20	8
	NaCl, 15g/l	4	1	5	9	5
Rhizosphere soil	Control	41	ND	27	ND	34
	NaCl, 15 g/l	28	ND	13	17	15

Table 3: Bacterial strains isolated and identified from normal unplanted soil, rhizoplane (RP) and root-adhering soil (RAS) of the three wheat lines grown in normal soil.

Code	Bacteria identified from diffferent samples	Gram	*Suc30	*Suc30 Na	*Suc25	*Suc25Na
	Unplanted soil					
MA1	*Bacillus insolitus* (62%)**	+	+	+ + +	+ + +	+ + +
MA2	*B. insolitus* (39%)	+	+	+ +	-	-
MA3	*B. insolitus* (39%)	+	+	+ + +	+ +	+ + + +
MA10	*B. licheniformis* (81%)	+	±	+ + +	±	+ + +
	Soil planted with W-1073					
	a) RAS					
MA114	*B. coagulans* (70.6)	+	+	+ + +		+ +
MA101	*B. insolitus* (73%)	+	+	+ + +	+ +	+ + +
MA102	*B. insolitus* (40%)	+	+	+ + + +	+ + +	+ + + +
MAI28	*B. insolitus* (88%)	+	+ + +	+ + + +	+ + +	+ + + +
MA104	*B. licheniformis* (58%)	+	+	+ +	+ +	+ + +
MA119	*B. licheniformis* (61%)	+	+	+ + + +	+ + +	+ + +
	b) RP	+	+	+ + +	+	+ +
MA206	*B. coagulans* (12%)	+	+ + + +	+ + +	+ + +	+ + + +
MA205	*B. insolitus* (89%)	+	+ + + +	+ + + +	+ + + +	+ + +
MA207	*B. insolitus* (81%)	+	+	+ + +	+	+ +
MA218	*B. insolitus* (88%)	+	-	+ + +	+ +	+ + + +
MA228	*B. insolitus* (88%)	+	+	+ + +	+ +	+ + +
MA230	*B. insolitus* (76%)	+	+ + +	+ +	+ + +	+ + + +
MA261	*B. insolitus* (84%)	+				
MA268	*B. insolitus* (94%)	+	n.d	n.d	n.d	n.d
MA286	*B. insolitus* (85%)	+	n.d	n.d	n.d	n.d
MA283	*Pseudomonas fluorescens* type A (58%)	+	+ + +	+ + +	+ + +	+ + +
MA214	*Microbacterium* sp.	+	+	+	+ + +	+ + + +
MA227	*Microbacterium* sp.	+	+	+ +	+ +	+ + +
MA248	*Microbacterium* sp.	+	n.d	n.d	n.d	n.d
MA271	*Microbacterium* sp.	-	+ +	+ + +	+	+ + +
MA217	*Delya marina* (55%)	-	n.d	n.d	n.d	n.d
MA285	*D. marina* (32%)	-	-	+ + +	+ +	+ + +
MA202	*Agrobacterium rhizogenes* A (87%)	-	n.d	n.d	n.d	n.d
MA287	*Klebsiella planticola* (18%)	-	+	+ + +	+	+ + +
MA201	*Salmonella* sub spp. 1G (17%)					
	Soil planted with W-41					
	a) RAS					
MA305a	*A. rhizogenes* A	-				
MA305b	*A. rhizogenes* A (82%)	-	+ +	-	+ + + glc	+ /-glc
MA306	*Salmonella* sub sp. 1G (30%)	-	+ +	+	+ glc	-glc

	b) RP					
MA404a	*B. amyloliquefaciens* (31%)	+	±	+ + +	-glc	-glc
MA407a	*B. coagulans* (67%)	+	±	+ + +	-glc	-glc
MA411	*Microbacterium* sp.	+	+ + +	+ + +	-	-
MA405a	*A. rhizogenes* A (88%)	-	+	-	+ glc	± glc
MA408b	*Serratia marcescens*	-	+ + + +	+ +	+ + + glc	+ glc
	Soil planted with Inq-91					
	a) RAS					
MA103	*Variovorax paradoxis* (77%)	-	n.d	n.d	n.d	n.d
MA302a	*Agrobacterium rhizogenes* A	-	+ + +	+ +	+ + glc	+ glc
	b) RP					
MA401	*B. circulans* (52%)	+	+ + + +	-	-glc	-glc
MA402b1	*Microbacterium* sp.	+	+ + +	+ + +	+ + glc	± glc
MA402b2	*Microbacterium* sp.	+	n.d	n.d	n.d	n.d
MA403a	*Serratia odorifera* (51%)	-	+ + + +	+ + + +	+ glc	-gic
MA402a	*Salmonella* sub sp. 1G (60%)	-	+ + + +	+ + + +	+ + glc	+ gic

*Polysaccharide production (-,poor; +, + +, + + +, + + + +, moderate to very high) observed by spot plate method on RCV-sucrose medium incubated at 25°C (Suc25) and 30°C (Suc30)., Na, medium supplemented with Naa (15 g L^{-1}); Gram staining (aminopeptidase test); gic, glucose medium.; n.d, not determined **Percentages given by identification systems with Biology or Apilab data bases.

Table 4: Bacterial strains isolated and identified from unplanted saline soil, rhizoplane (RP) and root-adhering soil (RAS) of the three wheat lines drown in saline soil.

Code	Bacteria identified from different samples	Gram	*Suc30	*Suc30Na	*Suc25	*5ur25Na
	Unplanted soil					
MAS4	*Bacillus* amyloliquefaciens (41%)**	+	+	+ + +	+ + +	+ + +
MAS10	*B. insolitus* (90%)	+	+	+ +	-	-
MAS17	*B. insolitus* (99%)	+	+	+ + +	+ +	+ + + +
MAS26	*B. insolitus* 199%)	+	+	+ + +	+ +	+ + +
MAS31	*B. insolitus* (72%)	+	+	+ + + +	+ + +	+ + + +
MAS25	*B. licheniformis* 163%)	+	+/-	+ + +	+/-	+ + +
MAS35	*B. pumilus* (60%)	+	+	+ +	+ +	+ + +
	Soil planted with W-1073					
	a) RAS					
MAS114	*B. amyloliquefaciens* (50%)	+	+	+ + + +	+ + +	+ + +
MAS157	*B. coagulans* (40%)	+	+ +	+ + +	+	+ + +
MAS162	*B. hemoglucosidasus* (17%)	+	+	+ + +	+	+ +
MAS113	*B. insolitus* (72%)	+	+	+ + +	-	+ + +
MAS124	*B. insolitus* (82%)	+	+	+ + +	+	+ +
MAS128	*B. insolitus* (77%)	+	+	+ + +	+ +	+ + +
MAS125	*Pseudomonas mendocina* (40%)	-	-	+ + +	+ +	+ + +
MAS129	*Ps. syringae* (18%)	-	+ + + +	+ + +	+ + +	+ + + +
MAS133	*Microbacterium* sp. (68%)	+	+ + + +	+ + + +	+ + + +	+ +
MAS143	*Deleya marina* (26%)	-	+ + +	+ + +	+ + +	+ + +
MAS122	N.D	+ + +	+ + + +	+ + +	+ + + +	
	b) RP					
MAS205	*B. azotoformans* (57%)	+	+	+	+ + +	+ + + +
MAS238	*B. coagulans* (79%)	+	+	+ + +	+	+ +
MAS225	*B. insolitus* (16%)	+	+	+ + +	+	+ + +
MAS227	*B. pumilus* (53%)	+	+	+	+	+ + +
MAS207	*P. mendocina* (67%)	-	-	+ + +	+ +	+ + + +
MAS236	*Microbacterium* sp. (32%)	+	+ + +	+ +	+ + +	+ + + +
	Soil planted with W-41					
	RAS					
MAS305	*B. amyloliquefaciens* (35%)	+	+	+ + + +	-gic	-glc
MAS316	*B. coagulans*	+	+ +	+ + +	-glc	-glc
MAS301	*B. megaterium* (60%)	+	+ +	+ + +	+/-glc	-gic
MAS317	*Paenibacillus macerans* (70%)	+	+ + + +	+ + +	+ + glc	+/-glc

MAS302a	*Pantoea agglomerans* biogroup 2b (68%)	-	+ + +	+ +	+ + gic	+ glc
MAS302b	*Klebsiella oxytoca* (41%)	-	+ + +	+ +	+ + gic	+ glc
M AS318	*Enterobacter gergoviae*	-	+	+ + + +	-gic	-glc
	Soil planted with INQ-91					
	a) RAS					
MAS308	*Burkholderia cepacia*	-	-	-	-gic	-glc
MAS309	*Aeromonas trots* DNA gp 13	-	-	-	-gic	-glc
MAS310	*E. cloacae* A	-	+	+ + + +	-glc	-gic
MAS322	*E. cloacae* A (86%)	-	+	+	+ + glc	+ + gic
MAS323	*E. cloacae* (99%)	-	+	+	+ /-glc	+ gic
	b) RP					
MAS412	*B. thermoglucosidasrus*	+	-	-	-glc	-gic
MAS423	*Salmonella subspecies* 1G	-	+	-	+ glc	-gic
MAS424	*Salmonella. subspecies* 1G	-	+ + +	+	+ + glc	-gic

*Polysaccharide production (-,poor; +, + +,+ + +, + + + +, moderate to very high) observed by spot plate method on RCV-sucrose medium incubated at 25°C (Suc25) and 30°C (Suc30) respectively; Na, medium supplemented with or without NaCl (15 g L^{-1}); Gram staining (aminopeptidase test); glc, glucose medium.
**Percentages given by identification system with Biolog or Apiiab data bases.

Table 5: Comparison of various soil and root fractions of three wheat cultivars for the presence/absence of different EPS producing bacterial genera.

	Saline	Normal
Soil	B. amyloliguefaciens	*Bacillus insolitus*
ba.	*B. insolitus*	*B. licheniformis*
	B. licheniformis	*B. coagulans*
	B. pumilus	*B. insolitus*
Rhizosphere		
	Aeromonas trota	*B. licheniformis*
	B. amyloliquefaciens	*Salmonella* sub sp. IG
	B. coagulans	*Varivorax paradoxes*
	B. hemoglucosidus	*A. rhizogenes* A
	B. insolitus	
	B. megaterium	
	Panibacillus macerans	
	Burkholdena cepacia	
	Deleya marina	
	Enterobacter cloaceae	
	E. gergoviae	
	Klebsiella oxytoca	
	Pantoea agglomerans	
	Pseudornonas mendocina	
	P. syringae	
	Microbacterium sp.	
Rhizoplane		
	B. coagulans	*B. arnyloliquefaciens*
	B. insolitus	*B. azotoformans*
	B. pumilus	*B. circulans*
	B. thermoglucosidasius	*B. coagulans*
	Deleya marina	*B. insolitus*
	Salmonella sub sp. IG	*Deleya marina*
	Microbacterium sp.	*K. planticola*
		P. fluorescens
		Salmonella sub. sp. IG
		Serratia marcescens
		S. odonfera
		Microbacterium sp.

A comparison of bacteria grown at 25 or 30°C with and without the addition of NaCl in the medium, showed a variable EPS production by different strains (Table 3 and 4). In some strains the presence of salt in the medium stimulated an increase in polysaccharide production (MAS17), while in others it showed inhibitory effect (MA401) or no effect (MAS133). Strains belonging to *Microbacterium* sp. were those which produced *in vitro*, the most abundant exopolysaccharide, whatever the conditions we have tested especially the strains MAS133 and MAS236 isolated from saline soil. However, for the strains producing more EPS in the presence of salt it was difficult to inter that the higher production and spreading of polysaccharide on the medium was due to an increase in production or elasticity of the polysaccharide. Although the response of bacterial population of the two soil fractions (rhizoplane and rhizosphere) was variable, the rhizoplane population was more sensitive to salt addition.

Although the biochemical tests are considered to be a good method of bacterial identification, they have the disadvantage of retying on expression of bacterial metabolism depending on the growth conditions. Moreover, they arc less sensitive and frequently give false results (Wachsmuth, 1986; Versalovic *et al*., 1992; Lebaron *et al*., 1998). A variation in identification percentages (Table 3 and 4), therefore, indicated that these systems are unable to completely identify the strains and still there is a need of more reliable and confirmatory techniques for identification and classification of bacteria. The strain MAS133 was also identified with molecular techniques which confirmed that this strain was belonging to the genus *Microbacterium*.

A diversified EPS-producing bacterial population in the RP and RAS (Table 5) provided the evidence of root involvement in determining specific population dynamics.

The variability would be due to carbon richness and availability of the essential vitamins in the root zone (Patriquin *et al*., 1983). Others have reported a pronounced effect of plant roots on the size and composition of bacteria (Miller *et al*., 1989; Rovira and Davey, 1974). Among various identified EPS-producing bacterial genera, the *Bacillus* species were more abundant in different root fractions and in the two soils which indicated its dominance over other bacteria for survival under salt stress conditions.

The important finding of this study is that EPS-producing strains belonging to the Gram positive *Microbacterium* sp. were isolated for the first time from soil and rhizosphere. The role of this species in this ecosystem is not yet understood and would be very important especially in saline soils.

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