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The Correlation Between Extracellular Polysaccharide Production and Acid Tolerance in *Rhizobium*[1]

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ABSTRACT

Rhizobial isolates from six host genera (*Cicer, Phaseolus, Leucaena, Lens, Melilotus* and *Trifolium*) were screened for ability to colonize successively more acidic agar plates. Sufficient isolates from *Cicer* and *Phaseolus* were screened to show that relative tolerance to acidic conditions was normally distributed. Extracellular polysaccharide (EPS) was isolated by ethanol precipitation from 10-d-old solution cultures of the most and least acid tolerant of these strains. Acid-tolerant isolates from *Cicer, Phaseolus, Leucaena* and *Melilotus* produced significantly more ($P < 0.001$) EPS than did those which were acid sensitive. The differences between isolates from *Trifolium* were significant at the $P < 0.05$ level. No significant difference in EPS production was found among the 8 isolates from *Lens*. The correlation between EPS production and acid tolerance was tested further with EPS isolated from 20 strains of *Rhizobium phaseoli*. The quantity of EPS produced varied from 18 to 75 mg 100 mL culture, was normally distributed, and was positively correlated with acid tolerance ($P < 0.01$), albeit with an r^2 value of only 0.331. It is postulated that EPS could modify the microenvironment of the rhizobia and so decrease the stresses induced by an acid soil.

Additional Index Words: rhizobial survival, nitrogen fixation, legumes, acid soils.

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MANY genera of bacteria excrete polysaccharides into their surroundings. The amount and chemical composition of these extracellular polysaccharides (EPS) varies both with genus and species, and in some cases may depend on environmental conditions (7).

The existence of extracellular polysaccharides excreted by rhizobia have been known for nearly a century (11). Rhizobial EPS has been shown to bind with lectins from the homologous host plant (2,3,5). This is perhaps a recognition mechanism partly responsible for the specificity of infection (6). It is doubtful, however, that this will prove to be the only function of rhizobial EPS as the production of these materials is widespread throughout the microbial world, even among genera not known to form symbiotic associations.

Commercial polysaccharide producers often can obtain an increase in the output of bacterial water-soluble polymers by growing the bacteria under nutrient-limiting conditions. *Azotobacter*, in steady-state continuous culture, produced EPS under all limitations tested (7). Limitations of molybdenum, phosphate, iron, carbon, nitrogen, calcium, and potassium all increase the amount of EPS produced per cell. This trait is not unique to *Azotobacter* as other bacteria have shown similar responses to nutrient limitation (10, 14).

The production of EPS represents a considerable energy investment by an organism, both in the sugars used as structural components and the energy required for synthesis and excretion. As such, one would expect that its production must confer some benefit on its producer. The EPS produced by some genera have been shown to chelate ions, ward off predator and viral attack, concentrate enzymes, and act as a food reservoir in leaner times (4). It is doubtful that the EPS of *Rhizobium trifolii* acts as the latter as it is produced in substantial quantities under both carbon-(mannitol) sufficient and limited conditions (8).

As the production of EPS is a response by numerous species of bacteria to a broad range of limiting factors, EPS may have an integral role in permitting an organism to survive under a variety of nonideal conditions.

One nonideal condition under which rhizobia exhibit differing degrees of tolerance is low pH. Acid soils occur worldwide, and commonly they are correlated with high activities of Al^{3+} and Mn^{2+} as well as deficiencies of phosphate, molybdenum, and less often calcium.

Ayanaba et al. (1), in a screening of *Rhizobium* for acid tolerance, have reported that strains which produced dry, pinpoint colonies on YEM agar were extremely sensitive to the acid-Al conditions, whereas strains with large "gummy or wet" colonies exhibited a greater tolerance.

This experiment was undertaken to determine if there was a correlation between acid tolerance in rhizobia and the genetic propensity to produce extracellular polysaccharide.

MATERIALS AND METHODS

The agar plate screening method of Ayanaba et al. (1) was used for screening *Rhizobium* for tolerance to low pH. The yeast-mannitol agar medium for the plates was modified from that of Keyser and Munns (13). In addition to the 1.0-g yeast extract and 5.0 g mannitol L^{-1}, the solution contained 2.0 mmol KNO_3 and (in μmol) 300 $MgSO_4$, 50.0 FeEDTA, 300 $CaCl_2$, 100 K_2HPO_4, 150 KH_2PO_4, 2.0 $MnSO_4$, 1.0 $ZnSO_4$, 0.50 $CuSO_4$, 0.10 Na_2MoO_4, and 0.020 $CoCl_2$. The pH of the medium was adjusted, using sterilized acid and base, after the agar had been added (19 g L^{-1}) and the solution autoclaved. After an initial screening to determine representative strain response, eight pH levels were used (6.4, 5.3, 5.1, 4.8, 4.6, 4.5, 4.3, and 4.2). The highest pH level was included to check the viability of the bacterial culture.

A random selection of rhizobial strains was screened, as well as CIAT 899 and its antibiotic resistant mutant (CIAT 2545), both strains known to be infective on *Phaseolus* and to do well under acid soil conditions in unrelated soil trials. Screening included strains infective with *Cicer, Phaseolus, Leucaena, Melilotus/Medicago, Trifolium*, and *Lens*.

Subsamples from each culture were gram-negative stained using the procedure outlined by Vincent (15, p. 17–18).

Rhizobium stock cultures were provided by the Nitragin

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Co., CIAT, USDA Univ. of Hawaii, and NifTAL (see Table 1).

Bacteria from each stock culture were transferred to nutrient solution at pH 7.0 and grown from 4 to 6 d. The culture was then diluted so that a single drop applied to the agar plates contained roughly 100 bacteria, as variation in inoculation level influenced colonization ability.

The four replicate plates from each strain plate were kept at 25°C and monitored daily for up to 10 d as some rhizobial strains had a considerable lag period before establishing visible colonies at the lower pH levels. The pH of all plates on which rhizobia multiplied was noted.

For the isolation of the EPS, the culture medium was slightly altered, with yeast extract, replaced by 1 mg L^{-1} Ca pantothenate, 1 mg L^{-1} thiamine and 0.1 mg L^{-1} biotin; the KNO_3 by 1.1 g L^{-1} Na^+ glutamate; and the potassium phosphate was adjusted to 500 μmol to replace that previously supplied in the yeast extract.

One hundred milliliters of this solution adjusted to pH 7.0 was sterilized in a 250-mL flask stoppered with dacron wool, then the medium inoculated with *Rhizobium* to give an initial population of 1000 bacteria mL^{-1}. Three replicates of each strain were then incubated at 24 ±2°C for 10 d. This time period was chosen after initial experiments to monitor the population numbers and the quantity of EPS produced. The cultures were swirled on a rotary shaker for 15 min every 2 h. On the 10th day the cultures were counted using the dilution plate count method and the pH of the solution taken prior to isolating the EPS. The pH of the culture solution ranged from 6.5 to 7.5 and was not correlated with total EPS produced or the relative acid tolerance of the strains. The log of the population per milliliter varied between 9.22 and 10.1.

The extracellular polysaccharide was isolated by centrifugation and ethanol precipitation, using a modification of the procedure of Humphrey and Vincent (12). The cultures were vortex mixed for 2 min then centrifuged for 30 min at 9200 × *g* and 15°C. A microscopic examination of the rhizobia after centrifugation showed no major cells lysis or distortion. The viscous supernatant material was removed by suction into 350 mL ethanol leaving some liquid in the centrifuge tubes to avoid disturbing the pelleted bacteria. The culture flask was rinsed with 50.0 mL of a solution 0.3 mmol in $CaCl_2$ and 0.25 mmol K_2SO_4. The pellet was then resuspended in this solution, vortex mixed an additional 2 min, and recentrifuged. This supernatant was also suctioned into the ethanol. In cases where the original culture media was extremely viscous, half of the 50.0-mL wash solution was added before the first vortex mixing. This decreased the viscosity and aided the formation of a pellet during the first centrifugation. Four drops of 2*M* HCl were added to the ethanol and supernatant solution. This acidification of the solution hastened the precipitation of the EPS from the ethanol. The flask was then stored, for a minimum of 12 h, at −15°C. After removal from the freezer the solution was centrifuged for 30 min at 28 000 *g* at 15°C. The supernatant was decanted and the pellet resuspended in 50 mL distilled water. This resuspended pellet was then added to 125 mL of ethanol and 6 drops of 2*M* HCl. Chilling, centrifuging, decanting, and resuspending in 50 mL distilled water followed. The sample was then freeze-dried and weighed.

Transmission electron micrographs of *Rhizobium trifolii* strain 162X99 before EPS isolation showed a distinct EPS sphere around the bacteria. Transmission electron micrograph photographs after EPS isolation showed little to no EPS present.

Table 1—Extracellular polysaccharide production of acid tolerant and sensitive strains of *Rhizobium*.

Host plant	Number of strains screened	Acid tolerance†	Strain/s‡	mg EPS 100 mL§
Cicer	19	Tolerant (4.3)	TAL 619	104.8
			TAL 620	93.1
		Sensitive (6.4)	TAL 263	59.6
			TAL 621	49.1
Phaseolus	20	Tolerant (4.2)	CIAT 899	74.0
			CIAT 2545	53.6
		Sensitive (5.3)	US2667	39.9
			127K13	23.4
Leucaena	9	Tolerant (4.2)	TAL 1145	43.7
		Sensitive (53.)	TAL 82	15.6
Lens	8	Tolerant (4.3)	TAL 638	30.4
		Sensitive (4.6)	TAL 634	27.4
Melilotus	6	Tolerant (5.1)	TAL 388	43.0
		Sensitive (6.4)	TAL 1372	1.3
Trifolium	5	Tolerant (4.5)	162X95	22.7
		Sensitive (4.6)	162X68	13.4

† The results of the agar plate screening are presented in this column. The number in brackets following the "tolerant" designation represents the pH of the most acidic plate colonized by isolates from this host plant (e.g., 2 cicer isolates survived at pH 4.3 but none at 4.2). The "sensitive" designation refers to the most acid-sensitive strains tested (e.g) of the 19 cicer isolates tested, only two survived at pH 6.4 but could not colonize agar plates at (the next most acidic pH 5.3).

‡ The column headed "strain/s" represents those strain or strains from the total number screened which exhibited the most or least acid tolerance.

§ mg of water-soluble ethanol-insoluble extracellular polysaccharide produced per 100 mL culture.

RESULTS AND DISCUSSION

In all cases acid-tolerant strains produced more EPS than sensitive strains. Except for rhizobia from *Lens* and *Trifolium* these differences were significant at the 0.001 level. Differences between *Trifolium* bacteria were significant at the 0.05 level but there was no significant difference between the EPS produced by the acid-tolerant and acid-sensitive *Lens* isolates. Table 1 summarizes these differences.

The least significant difference (at the 1% level) between EPS produced by two different strains is 10.0 mg with a standard error of 2.5 mg. In addition two groups were screened extensively enough so that a frequency distribution histogram could be constructed (Fig. 1a and b).

The EPS produced represented a significant proportion of the initial substrate supplied. From 500 mg of mannitol and 86 mg glutamate present upon inoculation, a population of ~ 10^9 bacteria mL^{-1} plus a quantity of polysaccharide ranging from 1.3 to 125.6 mg was produced. Randomly selected EPS was tested from both nitrogen and phosphate to check for contamination of the samples by nonpolysaccharide components or rhizobial cell material. Kjeldahl nitrogen ranged from 5 to 32 mg kg^{-1}, while phosphate determined after nitric–perchloric acid digestion (16) was 15 to 45 mg kg^{-1}. From this we conclude that the EPS was relatively free of contamination.

EPS production was tested in culture medium at pH 7.0 and could be expected to vary with environmental conditions. Thus only the genetic propensity to produce EPS under farily ideal conditions was tested and correlated with the acid-tolerance of the rhizobia. In preliminary studies, the quantities of EPS produced varied with the pH of the medium as did the absolute differences between the EPS produced among strains.

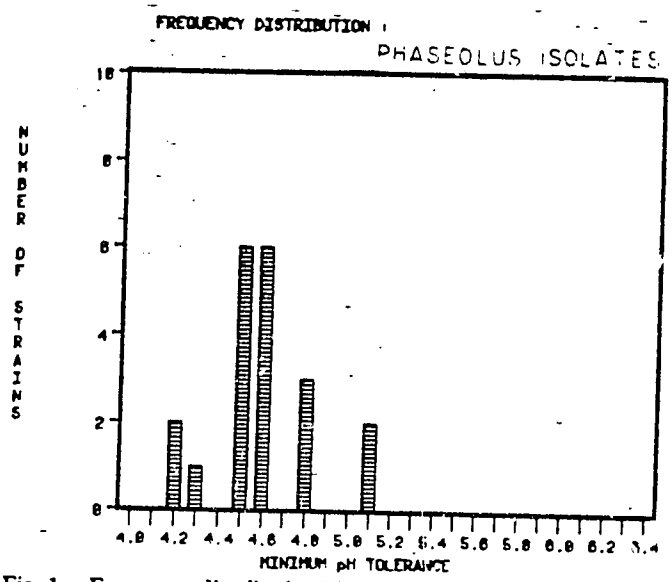

Fig. 1a—Frequency distribution histogram of acid tolerance in *R. phaseoli*.

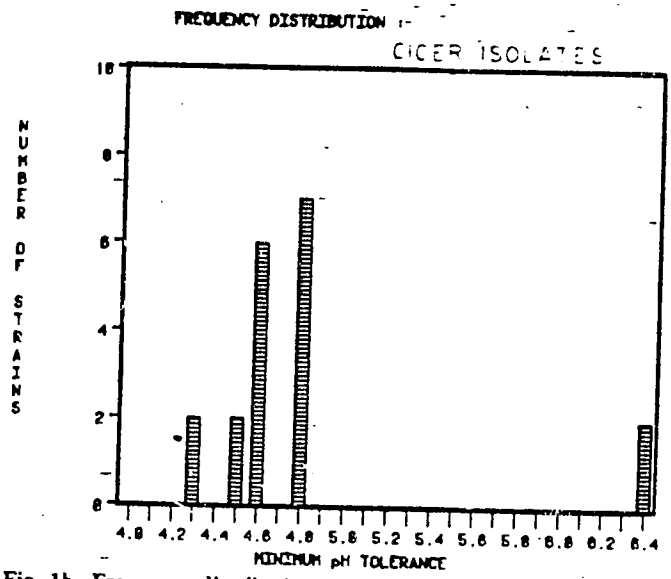

Fig. 1b—Frequency distribution histogram of the acid tolerance of *Rhizobium* strains from Cicer.

A pH of 7.0 was chosen to maximize the number of strains that could be tested for both acid sensitivity and EPS production so that the two phenomena could be examined for correlation. In addition, in trials run at an initial pH of 5.5, the pH of the culture solution varied considerably with population increases. This did not occur when the medium was pH 7.0.

To test the closeness of correlation of EPS with acid tolerance, one must consider not only the extremes of tolerance but the intermediate levels as well. The *Phaseolus* cross-inoculation group was chosen for this as 20 strains had been screened. The production of EPS by *R. phaseoli* is normally distributed (Fig. 2) and ranged from 18.1 mg to 74.0 with a mean of 47.7 mg. A Bartlett's test for homogeneous variances is significant at the 0.0007 level with an $LSD_{.05}$ of 7.8 mg. The correlation coefficient of the linear regression between EPS produced and acid tolerance of the 20 strains of *R. phaseoli* tested was 0.575 (Fig. 3). This is significant at the 1% level.

CONCLUSIONS

The quantities of EPS produced as well as the acid tolerance of the strains were normally distributed. High precision was achieved with measurement of EPS production (standard error 2.5 mg) and acid tolerance (98% of all strains had no variance among the 4 replicate plates).

The differences in quantity of EPS produced by the most and least acid-tolerant strains in 4 out of the 6 groups tested were significant at the 0.1% level and in another group at the 5% level. The acid-tolerant strain in each case produced more EPS than the acid-sensitive strains infective on the same host.

As the error in measurement of EPS production and acid tolerance is low, the low r^2 value of the linear regression is not a reflection of high variance in the data, but rather a weakness in the correlation between the measured EPS production and the acid tolerance

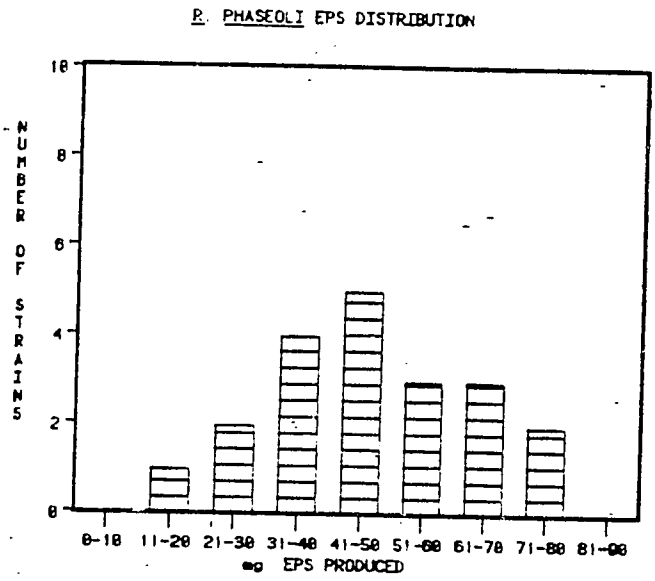

Fig. 2—Frequency distribution EPS produced per 100 mL of culture from 20 strains of *R. phaseoli*.

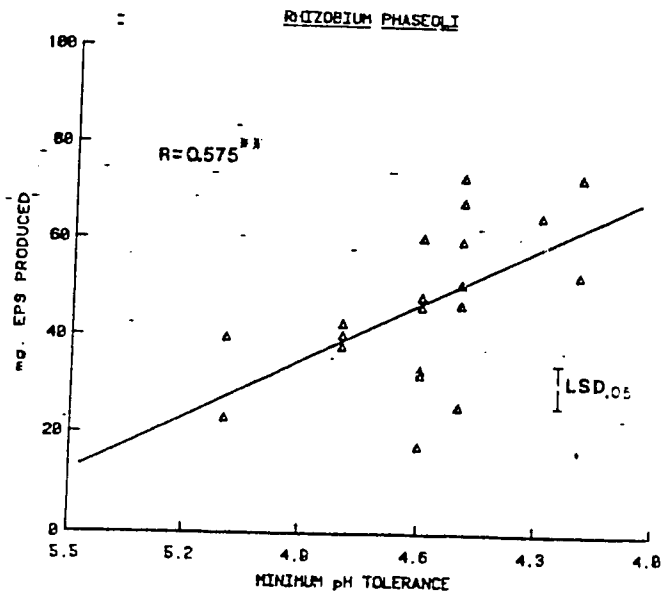

Fig. 3— EPS production vs. acid tolerance of 20 strains of *R. phaseoli*.

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of the strain. The r^2 value of only 0.331, although significant at the 0.01 level, suggests that there are other factors in addition to the quantity of EPS produced which affect the acid tolerance of a strain. An envelope of EPS is only one of many possible strategies for survival.

The correlation between acid tolerance and EPS produced might also be increased by considering variation in the composition of EPS. This study tested only the correlation between bulk EPS produced and acid tolerance. In studies on the acidic fraction of the EPS, it has been shown that the structure and composition varies between strains of *Rhizobium phaseoli* (9). If extracellular polysaccharide has a specific role in increasing the acid tolerance of its producer, such as chelating toxic Al^{3+} or Mn^{2+} ions, or buffering the extracellular environment, then chemical composition of the EPS as well as quantity should be considered.

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Effects of Rhizobial Extracellular Polysaccharide on pH and Aluminum Activity[1]

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ABSTRACT

A positive correlation has been demonstrated between the ability of *Rhizobium* strains to colonize acidic agar surfaces and their production of extracellular polysaccharide (EPS) in solution culture at pH 7.0. This set of experiments examined the effects of EPS on solution pH levels and Al activity, two common growth-limiting factors in acid soils. Samples of EPS from each of 20 previously acid-screened *Rhizobium phaseoli* isolates were titrated under controlled conditions and a buffering capacity was calculated. In addition, EPS from all strains were compared for ability to chelate aluminum by sodium saturation followed by equilibration against an aluminum-phosphate solid phase. Total aluminum of the digested samples was measured by atomic adsorption spectrophotometry. The buffering ability of the EPS varied considerably between strains but was not correlated with the acid tolerance of the strain from which each was isolated. Similarly, while there were significant differences in the ability of the EPS to chelate aluminum, these were not correlated with acid tolerance. Acid tolerance did not correlate with EPS production weighted for either buffering capacity of the EPS or its ability to chelate Al. Thus, while the amount of ethanol-precipitatable EPS from different rhizobial strains varied significantly in these two capacities, rhizobial EPS does not appear to confer acid tolerance on a strain by means of buffering pH or aluminum levels. Other possible causes of the observed relationship between acid tolerance and EPS production should be examined. These could include a role in increasing solution phosphate levels and/or the necessity of increased EPS for binding to plant lectins in an acidic environment.

Additional Index Words: **soil acidity, rhizobial survival, aluminum chelation.**

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ACID-tolerant strains of rhizobia produce more extracellular polysaccharide (EPS) than do acid-sensitive strains (3). Rhizobial isolates from 6 different host plants were studied and in 5 of the 6 cases these differences were significant at $P \leq 0.001$. Further testing of 20 strains of *Rhizobium phaseoli* with varying acid tolerance gave a linear regression correlation coefficient of 0.575** (**denotes significance at the 0.01 level), suggesting that bulk EPS production accounts for only part of a strain's ability to withstand acid soil conditions.

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