

Effects of Rhizobial Extracellular Polysaccharide on pH and Aluminum Activity¹

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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-precipitable 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 $P < 0.001$. 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. Differences in acid tolerance might result from an internal metabolic change or structural difference in cell membranes with increased EPS production either a by-product of this difference or only coincidentally correlated. However, there are several mechanisms by which EPS production could enhance

acid tolerance. Extracellular polysaccharide produced by "fast-growing" *Rhizobia* are not composed exclusively of sugar residues, but contain other constituents such as uronides and pyruvate. The composition of the acidic fraction of the EPS in *R. phaseoli* varies in the number of residues per repeating unit (ranging from 8-11), the number of acid groups within each repeating unit (23) and the structure and configuration of the side chains (4). The arrangement of the EPS components is such that free carboxyl groups could participate in the chelation or binding of a cation, including Al^{3+} or Mn^{2+} as found in acid soil conditions. Chelation with EPS might reduce the activities of these ions. Alginate, a commercially produced EPS from *Azotobacter*, is chemically similar to rhizobial EPS and has been shown to chelate numerous cations including aluminum (1). The variation in the number and arrangement of constituent carboxyl groups of the EPS of *R. phaseoli* could produce differences with respect to Al^{3+} chelation that would benefit the *Rhizobium*.

An EPS capable of chelating only moderate quantities of Al^{3+} and Mn^{2+} might, due to its nature and distribution, still substantially reduce the levels of these toxic ions at the cell surface. Studies on other organisms have shown that EPS can also act as a barrier to the diffusion of heavy metal ions (2). Rhizobial EPS is only moderately soluble, originates at the cell surface, and slowly diffuses into the solution. A gradient of EPS might induce a reciprocal gradient of free Al, and/or Mn" and thus increase the tolerance of a strain to acid conditions.

An exudate composed of EPS with many carboxyl groups, in addition to chelating Al^{3+} and Mn^{2+} , might also act as a diffusion barrier for H_3O^+ . Rhizobium that produce base as either a metabolic by-product or as a specific response to acidic conditions would benefit from a highly buffered viscous coating of this sort.

The overall objective of this experiment was to determine if the increased EPS production of acid-tolerant strains could modify the levels of Al^{3+} and H_3O^+ in the rhizobial microenvironment. The experiment had two specific purposes: to determine the extent to which EPS produced by *R. phaseoli* could modify the acidity or Al^{3+} activity of its microenvironment, and to attempt to improve the correlation coefficient between acid tolerance of a strain of *R. phaseoli* and its EPS production at pH 7.00. The linear regression correlation coefficients of the bulk EPS production vs. acid tolerance is 0.575 (3). Multiplying the total EPS produced by its relative Al^{3+} chelation or buffering ability gives an index of the ability of the rhizobial strain to modify its environment in that respect. If the r^2 value of the linear regression of these indices vs. acid tolerance of the strain were to show an increase over 0.331, then it could be assumed that ability of the EPS to chelate Al^{3+} or act as a buffer is an important mechanism by which the additional EPS production increases acid tolerance.

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MATERIALS AND METHODS

Twenty *Rhizobium phaseoli* strains were selected for this experiment. Their relative acid tolerance and their extracellular polysaccharide production were measured by methods previously described (3).

Buffering capacities were measured under the following conditions: A 0.100M solution of KCl was prepared using distilled deionized water, depleted of CO₂ by boiling for 20 min, then bubbling with N₂ gas for 2 h. The pH was lowered to pH 3.20 with HCl, and 30.0 mg of EPS was added to 40.00 mL of the KCl solution and stirred rapidly for 30 min. The quantity of EPS added exceeded its solubility. The resultant solution was highly viscous and contained suspended EPS. The pH of the solution was monitored while additions of standard base were made. Nitrogen gas was bubbled into the solution through a pasteur pipette. Except for a hole to allow the gas to escape and a micropipette for base additions the beaker was sealed with parafilm. The precautions against CO₂ contamination were necessary to restrict the effect of the HCO₃ equilibria on the titration curves.

The ability of the EPS to chelate aluminum was measured by first replacing previously chelated ions with Na⁺, then equilibrating the EPS against a slightly-soluble solid-phase aluminum compound.

Forty milligrams of EPS from each of the 20 *R. phaseoli* strains was weighed into 100 mL beakers. Forty milliliters of distilled H₂O was added and the solution stirred for 10 min. The EPS suspension was then transferred into a 25-cm length of standard cellulose dialysis tubing, with a dry flat width of 45 mm and a molecular weight cutoff of 12 000 to 14 000. Blanks were included as well as two replicates of each different EPS. The dialysis bags were immersed in a vat of flowing distilled H₂O for 24 h to reduce residual salt levels. The EPS was then Na⁺ saturated by equilibration for 24 h against Na⁺ saturated Dowex 50 W X-8 exchange resin. The pH of the solution was maintained at 4.50 ± 0.05 with additions of NaOH and HCl. The Na⁺ solution concentration in the bags was then decreased by equilibration with flowing distilled deionized H₂O for 3 h. The dialysis bags were then suspended for 24 h in a vat containing three dialysis bags filled with solid phase AlPO₄. This was prepared by addition of a saturated Al₂(SO₄)₃ solution to a saturated solution of Na₂HPO₄. The precipitate was filtered with a buchner funnel and washed 3 times. The pH of a suspension of the solid and water was adjusted to 4.5 with additions of HCl and NaOH. The solid was then refiltered and placed in cellulose dialysis bags. Before use, the bags were equilibrated against 5 separate 5-L volumes of distilled H₂O at pH 4.5.

After 24 h equilibration, the Al-saturated EPS in the dialysis bags was digested and analyzed. The bags were first

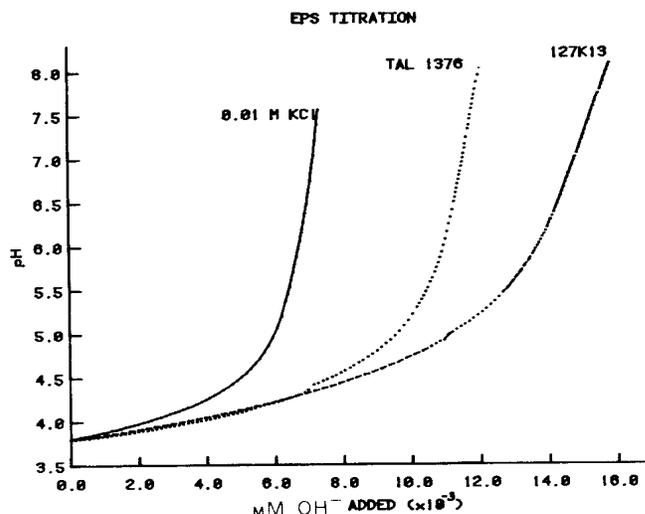


Fig. 1—Titration curves of 30.0 mg EPS of two *R. phaseoli* strains.

Acid tolerance limit*	Code no. and strain
4.2	1-CIAT 899, 2-CIAT 2545
4.3	3-CIAT 73
4.5	4-127K110, 5-CIAT 905, 6-127K11, 7-CIAT 166, 8-CIAT 2546, 9-CIAT 255
4.6	10-127K15, 11-127K26, 12-127K44, 13-TAL 1376, 14-CIAT 632, 15-127K16
4.8	16-127K46, 17-TAL 1383, 18-US2668
5.1	19-127K13, 20-US2667

*"Acid tolerance limit" is the pH of the most acidic agar plate on which the rhizobial strain successfully colonized (see ref. 3).

removed from the solution and weighed. The liquid was evaporated and the sample and cellulose bag placed in digestion tubes. The samples were digested with nitric and perchloric acid (5). Aluminum measurements were taken using a nitrous oxide-acetylene flame on a Perkin Elmer 560 atomic adsorption spectrophotometer. Phosphate readings were also taken on the digested samples to check for possible Al-phosphate complex formation or Donnan/double-layer phenomena that would affect the assumption that the Al found in the bag was in a chelated form. Measured phosphate levels did not differ significantly from vat solution levels.

RESULTS AND DISCUSSION

Graphs and figures presented here are coded in the following sequence:

Titration curves for the EPS with the highest and lowest buffering capacity as well as the blank salt solution are shown in Fig. 1. All other EPS titration curves fell between these two extremes. From these titrations, the number of millimoles of base needed to raise the pH of 1.0 mg of EPS from 4.0 to 5.5 was calculated. This pH range represents the approximate range where the buffering capacity of the EPS would be biologically significant as the range of tolerance to acid conditions by *R. phaseoli* was from 4.2 to 5.1. Values for this buffering ability ranged from 0.104 to 0.203 mmol charge/g EPS with an average of 0.147. Three replicates of 3 strains chosen at random gave a standard error of 0.003 and a LSD₀₅ of 0.014.

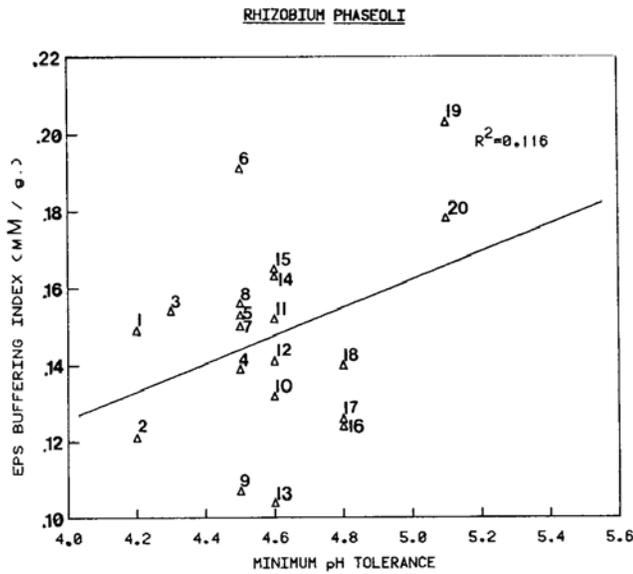


Fig. 2—Relative buffering abilities of the EPS of 20 strains of *R. phaseoli* vs. minimum pH tolerance.

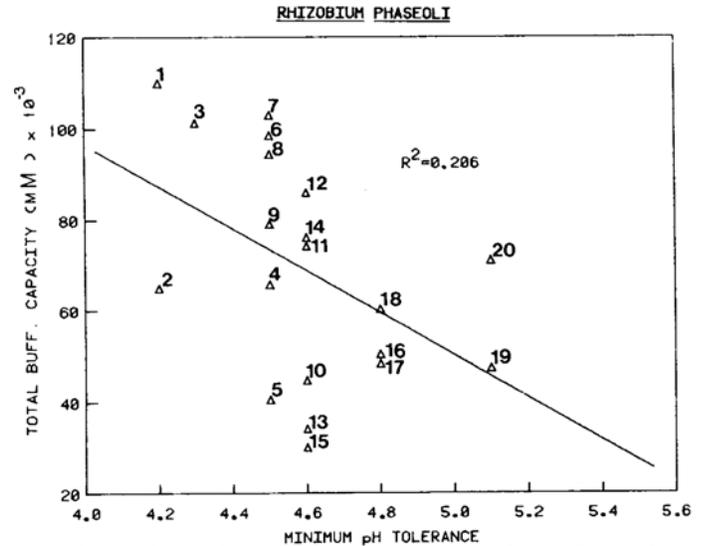


Fig. 3—Buffering ability of the total amount of EPS produced by a strain vs. acid tolerance of a strain.

The correlation between the buffering ability of the EPS and the acid tolerance of its producer are shown in Fig. 2. The positive slope with a low linear regression r^2 value suggests that there is a slight tendency for the EPS of the acid-tolerant strains to be less efficient at buffering solution pH changes when compared to EPS excreted by the acid-sensitive strains.

The ability of the total amount of EPS produced by a strain to buffer its surroundings with its relative acid tolerance is compared in Fig. 3. The r^2 of 0.206 for this regression was less than that of the regression of bulk EPS produced and acid tolerance (0.331). This indicates that although the EPS has some ability to function in this manner, it is not its major function in according acid tolerance on a strain.

Aluminum chelation values ranged from 0 to 0.62

mg Al^{3+} chelated per g EPS with an average of 0.22. The standard error was 0.03 with a $LSD_{0.5}$ of 0.12 between means. There were significant differences between strains at the 0.1% level.

The correlation between the ability of 1 mg of EPS to chelate Al^{3+} and the acid tolerance of its producer is shown in Fig. 4. Chelation capacity was not consistent with the acid tolerance of the strain. If anything, the acid-tolerant strains produced EPS less capable of chelating Al^{3+} than acid-sensitive ones.

Figure 5 indicates lack of correlation between acid tolerance and the amount of Al that could be chelated by the EPS excreted by a strain.

CONCLUSIONS

Significant differences in EPS buffering capacity exist among strains of *R. phaseoli*. This capacity is not,

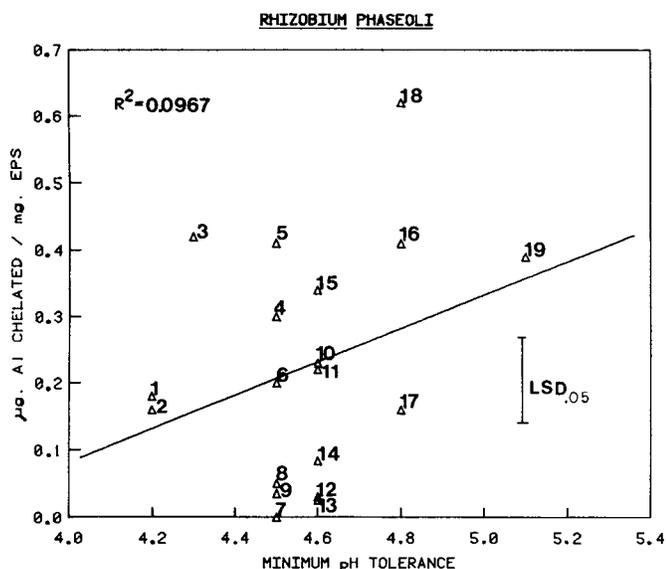


Fig. 4—Aluminum chelation ability of the EPS vs. acid tolerance of the strain.

however, significantly correlated with the acid tolerance of the strain. In addition, the linear regression correlation coefficient between EPS produced and acid tolerance limit is decreased when the EPS values are weighted by their buffering ability. This indicates that although the EPS has some capacity to act in this manner, it is not the root of the observed correlation between EPS produced and acid tolerance. The general trend, albeit with a low r^2 value, is that the EPS from the most acid-sensitive strains has the greater buffering ability.

The magnitude of the buffering ability of the EPS is not large, ranging between 0.1 and 0.2 mmol/g. Using average values of 0.147 mmol/g, 47.7 mg/culture flask, and $10^{9.5}$ bacteria/mL, the EPS of a single *Rhizobium* has a total buffering capacity of 2.21×10^{-15} equivalents. If the soil solution around a *Rhizobium* of length 1.4 μm and diameter 0.69 μm contains solid EPS to 1 μm (estimates obtained from TEM scans) then the affected volume is approximately 19 μm^3 , the EPS density is 0.794 g EPS/ cm^3 , and there is a 0.12 mmol of possible buffering capacity (between the pHs of 4 and 5.5) per cubic centimeter.

It is difficult to assess the relative impact of the buffering capacity of the EPS on the maintenance of a pH gradient across it. The buffering ability of the EPS is only one of many factors involved. Other factors would be the presence of dissociable acid groups, the physical impediment of a thick viscous layer of EPS, the amount of cross-linking exhibited by the EPS, its relative solubility, and actual density. Other factors that also must be considered are variations in solution pH, minimum rhizobial survivable pH at the cell surface, and the cell's ability to excrete base. Although the pH buffering capacities of the EPS produced by the strains is not positively correlated with acid tolerance, the hypothesis that the EPS may have a role in the creation of a buffering zone is not refuted.

While the amount of Al^{3+} chelated per mg EPS also varied significantly between strains, Al^{3+} chelation was not positively correlated to the acid tolerance of the

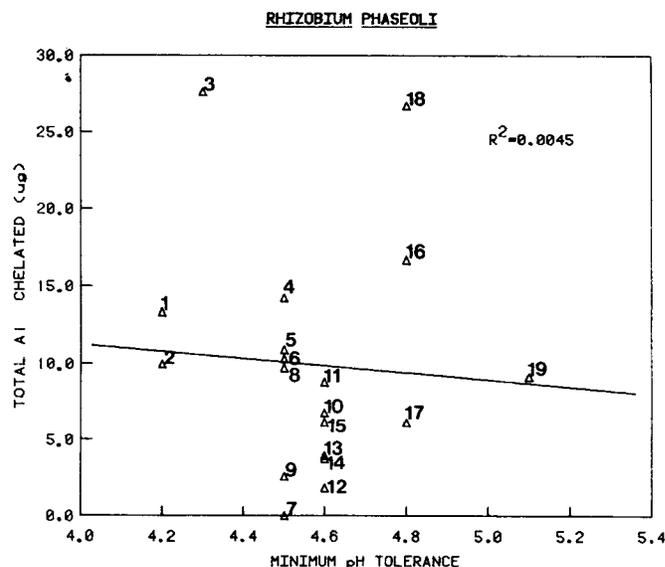


Fig. 5—Aluminum chelation ability of the total amount of EPS produced by a strain vs. acid tolerance of that strain.

Rhizobial strain that produced it. One might predict that the Al^{3+} tolerance of a rhizobial strain varies independently of its acid tolerance. Paralleling the results of the pH buffering work, the slope of the linear regression line and the comparison between most-and least-acid-tolerant strains would suggest that the least acid-tolerant rhizobia produced the EPS most capable of chelating Al^{3+} .

An additional point involved in chelation of cations is that with an increase in highly charged and strongly bound cations there will be a decrease in the negative charge density of the EPS surrounding the bacteria, thus making it easier for anions to diffuse to the cell surface. Difference in chelation ability might have a profound influence on bacterial nutrition, especially considering those anions, such as phosphate, that are not normally abundant in acid soils.

Increased EPS production is positively correlated with the tolerance of a strain to acid conditions (3). If the main effect of EPS on the rhizobial microenvironment were to provide a buffering envelope or to chelate toxic levels of Al^{3+} , then the EPS produced by the acid-tolerant bacteria would have a greater capacity to act in this regard. They do not. This suggests that the main function of EPS in helping to overcome acid stress is not in buffering or Al^{3+} chelation. It also suggests that acid-sensitive *Rhizobium* might have evolved EPS more suited to buffering or Al^{3+} chelation because of their acid sensitivity. A positive correlation between acid sensitivity and both buffering and Al^{3+} chelation as well as a decrease in r^2 values of the weighted EPS vs. acid tolerance tends to support this hypothesis. Other mechanisms which might account for the correlation between a strain's ability to withstand acid conditions and its EPS should be examined. In addition to high Al^{3+} and H_3O^+ , other common acid soil constraints include low levels of soluble phosphate, a greater abundance of positively charged clay surfaces, and a generally lower level of available cations. Rhizobial EPS may interact with any of the solution, inorganic, or host plant phases to pro

duce the correlation between acid tolerance and EPS production.

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