EFFECT OF SALINE AND ALKALINE WATER ON GROWTH AND SURVIVAL OF *RHIZOBIUM MELILOTI*

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ABSTRACT

The effect of salts commonly found in irrigation water on survival and growth of Rhizobium meliloti were determined. Sodium chloride at concentrations up to 250 mM did not influence viability of the bacterium suspended in water or in soil. A completely defined growth medium of low osmolarity was developed and the effects of added Na^{+}, K^{+}, Ca^{+2}, Mg^{+2}, PO_4^{-3}, HCO_3^{-1}, SO_4^{-2}, Cl^{-}, and CH_3COO^{-} (acetate) on the growth rate of various strains of R. meliloti was determined. Of these ions, only Mg^{+2}, PO_4^{-3}, and CH_3COO^{-} inhibited growth of concentrations of 200 mM or less. Sensitivity to some of the salts was affected by molybdate ion and by the presence of proline or glutamate. Strains of R. meliloti isolated from alfalfa fields in various parts of New Mexico were found to be comparable to laboratory strains with respect to salt tolerance.

These experiments suggest that salinity in concentrations normally encountered in irrigation water in arid regions does not influence survival or growth of R. meliloti growing independent of the host plant alfalfa. This work has been expanded to determine the effects of salinity on establishment of the symbiotic relationship between R. meliloti and alfalfa that results in biological nitrogen fixation.

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INTRODUCTION

The objectives of the proposed research were to determine the effects of salts commonly found in water in arid regions on the survival and growth of Rhizobium meliloti. The bacterium is agriculturally important because, when living in symbiotic relationship with alfalfa (Medicago sativa), the bacterium fixes nitrogen. The bacterium converts atmospheric nitrogen (N_2) into ammonia (NH_3), the form of nitrogen useful to plants. The bacterium fixes much more nitrogen than it requires for its own growth. This is excreted into the plant root tissue. The plant in return provides the bacterium with the energy required for the bacterium's growth and nitrogen fixing activity.

Before R. meliloti can establish this productive relationship with the host plant, it must proliferate in the soil independent of the plant. Furthermore, the bacterium must survive from one season to the next and in the absence of a host plant. A search of the literature showed that there were no reports of the effects of salts commonly found in arid regions on growth of R. meliloti. Several lines of evidence suggested, albeit indirectly, that Rhizobia in general and R. meliloti in particular are more sensitive to salts than more familiar bacteria.

The proposed research included a survey of many strains of R. meliloti isolated from local fields for salt tolerance to determine if strains indigenous to arid regions are any more resistant to salinity than other strains.

The long range goal of the proposed research, if it proved that R. meliloti is inhibited by the salinity encountered in arid regions, was to isolate mutant strains resistant to saline conditions. This trait could then be introduced into other strains of R. meliloti selected for other desirable traits. In the past, strains of Rhizobia selected for more efficient nitrogen fixation or some other trait of value to the symbiotic relationship but of no value to

the bacterium when it lives independently, have been found to be unable to compete with the indigenous Rhizobia (Jones and Bromfield, 1978; Ham, 1980). Salt tolerance could provide a selective advantage to the bacterium while living free prior to establishment of the symbiosis. We have found, however, that R. meliloti is quite resistant to the effects of salinity at the levels found in situations where alfalfa is grown. The bacterium is capable of withstanding much greater salinity than the host plant, at least when growing independent of the plant. Furthermore, strains of R. meliloti isolated from the field in New Mexico are no more salt tolerant than are laboratory strains. There seems to be no selection for salt tolerance in the natural situation. We are currently initiating experiments to determine if salinity affects the establishment of an effective root nodule, the next stage in biological nitrogen fixation by R. meliloti.

METHODS AND MATERIALS

Bacterial Strains: The strains of Rhizobium meliloti used and their sources are listed in Table 1. Field strains were isolated from soil samples taken from alfalfa fields. The soil samples were diluted and the suspension used to inoculate alfalfa plants growing gnotobiotically in test tubes (Vincent, 1970). Bacteria were isolated from nodules on plants receiving the highest dilution of the soil suspension (Vincent, 1970). Isolates were cloned several times and were again passaged through plants a second time and reisolated. This procedure provided isolates of bacteria capable of forming root nodules in alfalfa with 80% success.

Media: Vincent's mannitol medium (VM) was used as noted. Cells were grown in liquid medium consisting of, per liter, K_2HPO_4 (0.5g); NaCl (0.1g); $MgSO_4 \cdot 7H_2O$ (0.2g); yeast extract (0.4g); and mannitol (10g). Cells grew equally in this medium when sodium succinate was the carbon source (10 g/l). A minimal defined medium (MDM) was used for determining the effects of solutes on growth of R. meliloti. This medium consists of: 10 mM MOPS (Morpholinopropane sulfonic acid); 1 mM Tricene (N-tris(Hydroxymethyl)methyl-glycine); 10 µM ferric sulfate; 270 µM potassium sulfate; 600 µM magnesium chloride; 10 mM ammonium sulfate; Trace minerals including: 0.01 µM zinc sulfate, 0.04 µM boric acid, 0.007 µM copper sulfate, 0.008 µM cobalt chloride, 0.005 µM molybdic acid, 0.009 µM manganese sulfate; Vitamins including, per liter, 20 µg riboflavin, 20 µg p-amino benzoic acid, 20 µg nicotinic acid, 20 µg biotin, 20 µg thiamin, 20 µg pyridoxin HCl, 20 µg Ca^{+2} pantothenate, and 120 µg inositol. Mannitol (10 g/l) was the carbon source. The medium was adjusted to pH 7.5 and filter sterilized.

Growth Conditions: Cultures were maintained on VM agar slants. Cultures were recloned and transferred several times each year. Contamination of the

cultures by an unidentified spore forming bacterium happened on several occasions. This contaminant could be readily identified by its colony morphology. Experimental cultures were routinely plated to verify the purity of cultures. Inocula for growth experiments were grown in VM for 24 to 36 hours. Cells were washed three times in sterile twice distilled water and were resuspended in water. 0.2 to 0.5 ml cell suspension were used to inoculate the experimental cultures (10 ml of medium in a 50 ml Erlenmeyer flask). Cells were then grown overnight in the experimental medium (for example, MDM with 250 mM NaCl added). In the morning, the cultures were diluted into 10 ml of the same experimental medium in 50 ml Erlenmeyer flasks fitted with side arms permitting optical measurements. Cells were incubated in a rotary incubator shaker. All growth was at $30^{o}C$. The medium used for the inoculum was not critical. Similar results were obtained when cells were grown in MDM and washed prior to inoculation into the experimental medium.

Measurements of Growth: Growth was estimated by measurements of optical absorbance using the Klett-Summerson Colorimeter fitted with the green filter. Growth was linear to 150 Klett Units (KU). Experiments were usually terminated when growth reached 150 KU. If not, cultures were diluted before optical measurements. The growth rate μ (in dimensions of hr^{-1}) was calculated from a linear regression of the absorbancy versus time. Points not on the linear portion of the curve were rejected. The calculations were assisted by a computer program prepared by Gary M. Schlosser. In an experiment to determine the variation between cultures, six identical cultures were grown and the growth rates determined. The coefficient of variation ($\sigma x/\bar{x}$) was 13%. Duplicate control cultures typically resulted in growth rates within 10% of one another. Differences in growth rates of less than 10% were not considered to be significant. Nearly all the experiments reported here were performed many

times with several different strains. We have reported representative experiments. We have reported only those results that were consistently obtained with all strains unless noted otherwise.

RESULTS

The Medium: The research goals required development of a defined medium of low osmolarity so that the effects of added minerals could be determined. Use of an organic buffer rather than a mineral buffer in the medium was thought desirable to minimize contamination by unidentified trace minerals. The MOPS Defined Medium (MDM) developed (see methods and materials section) is similar to that developed by Neidhardt et al., (1974). The buffer concentration in this medium is quite low, 11 mM. Typically when cultures had grown to an absorbancy greater than 250 Klett Units, enough acidic end products had accumulated so that the pH fell below the initial pH of the medium. In most experiments growth was followed until the absorbancy of the culture was only 150 Klett Units. The buffering capacity of the medium was sufficient to maintain the original pH at this culture density. For some strains, the trace element mixture was not required. For some strains, the complete vitamin supplement was not necessary. However, with this medium, all strains grew. Most strains grew with generation times of 4 to 8 hours.

Optimal pH for Growth: The optimum pH for growth of all strains of *R. meliloti* tested was in the range of pH 7.5 to pH 8.5. None of the strains grew well at 6.5 or less. The pH of most surface water in New Mexico is in the range of pH 7.5 to 8.5. The alkalinity of the water available to *R. meliloti* locally should not be detrimental to the bacteria.

Osmolarity of the Medium: To determine if *R. meliloti* is sensitive to conditions of high osmolarity *per se* rather than to some specific solute in the medium, several solutes were added to the MDM in high concentration. The resulting osmolarity of the medium was calculated. Representative results of one experiment appear in Table 2. In support of these experiments, it should be pointed out that at pH 7.5, the addition of 100 mM potassium phosphate

buffer increase the osmolarity of the medium to 187 meq. This concentration of potassium phosphate inhibits growth of most strains completely.

The growth of R. meliloti also appears to be unaffected by the ionic strength of the growth medium. The addition of either 200 mM NaCl or 400 mM glycerol results in the same increase in osmolarity. But the addition of NaCl increases the ionic strength of the medium from $\frac{\rho}{2}$ = 18.04 to 418.04 while the addition of 400 mM glycerol has essentially no effect on the ionic strength. Neither addition inhibits growth of R. meliloti (Table 3).

Effect of Sodium on Growth: A series of experiments were run to determine if sodium chloride inhibits growth of R. meliloti. Sodium chloride is the most common form of salinity in arid regions. Representative data appear in Table 4. As can be seen, under most conditions, the addition of NaCl at concentrations of 100 mM or less did not inhibit growth of R. meliloti strain NSI. The only exception was when cells grew with 1/10 the usual amount of phosphate (e.g. 0.1 mM rather than 1.0 mM). This lower concentration of phosphate did not appear to affect growth in the controls. However, cells did appear to be more sensitive to the addition of sodium chloride when phosphate was present at the low concentration. Other strains of R. meliloti were tested for inhibition of growth by NaCl and inhibition was observed only when the concentration exceeded 100 mM. Sodium chloride, sodium sulfate and sodium bicarbonate had comparable effects indicating that the anion did not influence growth. Potassium added as KCl, K_2SO_4, or $KHCO_3$ had no inhibitory effect in any of the strains tested at concentrations of 100 mM or less.

The addition of sodium or potassium stimulated growth under most conditions. Initially it was presumed that the addition of these minerals increased the concentration of a minor trace element present as a contaminant in the sodium or potassium salts. Increasing the concentration of the trace minerals

used in MDM up to 10 fold had no effect on growth. Increasing each trace mineral individually had no effect on growth. However, when molybdate was omitted from the medium, sodium chloride and potassium chloride had significantly greater inhibitory effects (Table 5). The basis for this apparent interaction between molybdate and sodium, a component of nitrate reductase and nitrogenase complex but to have no other known roles in bacteria (Weinberg, 1977), is not known.

When first isolated, two field strains appeared to have a requirement for sodium (Table 6). These strains were passed through alfalfa plants a third time and subcultured several times. After this treatment, they no longer required sodium, and potassium no longer inhibited their growth. Unfortunately, the original isolates are no longer viable and this has not been pursued further. The effects of sodium and potassium on three additional field isolates are presented in Table 7. These field strains appear to be no more salt tolerant than typical laboratory strains.

Inhibition of Growth by Magnesium: In a survey of metal ions inhibiting growth magnesium was found to reduce the growth rate of all strains tested. The results of one representative experiment are shown in Table 9.

Effect of Phosphate on Growth of R. meliloti: Potassium phosphate was found to inhibit the growth of most strains of R. meliloti at concentrations typically used in minimal defined medium for enteric coliforms. The results of one representative experiment are shown in Table 8. The growth of strains RM2011 and RM1826 obtained from Ethan Signer (Massachusetts Institute of Technology) was not inhibited by concentrations of phosphate of 100 mM or less. However, these strains are unusually tolerant of sodium (M. Osburne, personal communication) and are presumably tolerant of other ions as well.

At pH 7.5, 100 mM potassium phosphate has an osmolarity of 284.2 meq. This osmolarity, per se, is not inhibitory for growth of R. meliloti. However, phosphate buffers particularly at or above pH 7.5 are known to complex a variety of inorganic and organic materials making them unavailable to the cell. Phosphate could inhibit growth of R. meliloti by making the calcium in the medium unavailable. Calcium is required for growth of R. trifolii and presumably for growth of R. meliloti and other "fast growing" Rhizobia (Vincent, 1962). However, increasing the calcium concentration 10 fold did not affect sensitivity to phosphate.

Inhibition of Growth by Acetate: In an initial experiment, various carbon sources were tested for their ability to support growth of R. meliloti. Acetate was found to inhibit the growth of the bacterium regardless of the carbon source available to the cell. Sodium propionate did not inhibit growth of R. meliloti. The effect appears to be specific for acetate (Table 10).

Effect of Anions on Growth of R. meliloti: Of the anions tested only phosphate and acetate appeared to affect growth of R. meliloti. Sodium chloride, sodium sulfate, and sodium bicarbonate all had no effect on growth at comparable concentrations. Acetate and phosphate inhibited growth dramatically as already discussed.

Effect of Osmotica: The addition of glutamate increases the tolerance of several bacteria to sodium chloride (Measures, 1975). Apparently the cell concentrates glutamate to increase the osmolarity of the interior of the cell and thus prevents loss of intracellular water to the medium. Mutants of Salmonella typhimurium more resistant to sodium chloride have been isolated. These mutants accumulate high concentrations of proline which presumably act like glutamate as an osmoticum (L. Csonka quoted in an article appearing in Science 206:1168). The effect of added glutamate and proline on the ability

of R. meliloti to tolerate inhibitory concentrations of potassium phosphate, magnesium chloride, and sodium acetate was tested (Table 11). These data show that the addition of proline and glutamate have the same stimulatory effect on growth in the absence of an inhibitory solute. Neither putative osmoticum has much effect on inhibition of growth by 100 mM phosphate. Both osmotica are partially effective in relief of inhibition by magnesium. Both completely reverse the effect of added acetate and stimulate growth as well. These data suggest that since the osmotica affect the cell's response to the three solutes differently, the inhibition of growth by the three solutes must have three different modes of action. Since simply increasing the osmolarity of the growth medium does not inhibit growth (Table 2), presumably these three inhibitory solutes do not inhibit growth simply as a consequence of making the medium hypertonic. And conversely, glutamate and proline do not reverse the inhibition simply by increasing the intracellular osmolarity. The physiological basis for the effects of proline and glutamate is currently under investigation.

Survival of R. meliloti: The effect of salinity on the survival of R. meliloti was determined in two experiments. In the first, cells of strain NSI were washed and resuspended in twice distilled water and in solutions of 50, 100 and 250 mM NaCl. Cells were held at 4° and 30°C. Cell numbers were determined at weekly intervals by plate counts. After 20 weeks the cell numbers had decreased less than an order of magnitude. Neither the salinity nor the holding temperature had any effect on the final cell numbers.

In a second experiment, the survival of R. meliloti strains NSI and MV_1 in soil saturated with water of increasing salinity was determined. The rhizobium strains were genetically marked by isolating spontaneous mutants resistant to 100 µg/ml streptomycin. 10^{10} cells were used to inoculate pots

of soil (approximately 400 cm^3) that had been saturated with twice distilled water with 50 mM, 100 mM or 250 mM NaCl added. The pots were held in a greenhouse. Soil cores were taken at weekly intervals. The soil moisture was determined from the weight loss. Total cell numbers were determined from plate counts using VM. Numbers of surviving Rhizobium meliloti were determined from plate counts using VM + 100 µg/ml streptomycin. Cell numbers decreased rapidly as the soil dried. However, once the soil lost no more moisture, total cell numbers and numbers of rhizobia (streptomycin resistant cells) become constant to a value of 10^5 - 10^6 cells/gm soil. The salinity of the soil had no effect on the total cell numbers or on the numbers of surviving rhizobia. This experiment was continued for 9 weeks. From these two experiments, we can conclude that NaCl in the water does not affect the ability of R. meliloti to survive independently in the soil.

DISCUSSION

The results of these investigations show that growth and survival of R. meliloti, growing independently as a typical vegetative bacterium, are not affected by salinity. Of the minerals examined, only high concentrations of magnesium and phosphate inhibited growth. The concentrations required are much higher than are encountered in irrigation waters presently used in New Mexico (New Mexico Water Resources Assessment for Planning Purposes, 1974). R. meliloti can tolerate much higher concentrations of sodium chloride than can its host alfalfa (Bula and Massengale, 1972). Furthermore, salt tolerance of all strains of R. meliloti were similar. The field isolates from irrigated alfalfa fields in New Mexico were no more tolerant of salt than laboratory strains.

While salinity does not affect growth and survival of R. meliloti growing independent of a host plant, salinity could affect the ability of the bacterium to establish a useful symbiotic relationship with the plant. Salinity could affect the ability of the bacterium to locate the appropriate host (chemotaxis), the ability of the bacterium to move towards the host (motility), the ability of the bacterium to adsorb to the surface of the root and then to dissolve the outer layer of root tissue to gain entrance. Currently we are investigating these possibilities.

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Table 1

Strains:

All are strains of Rhizobium meliloti. All could nodulate alfalfa (Medicago sativa) under laboratory conditions (Vincent, 1970).

Strain	Source	Note
NSI	Harold Evans	Laboratory Strains
I02FSI	Winston Brill	"
F-28	Joseph Cowels	"
Rm 2011	Ethan Signer	"
Rm 1811	"	"
I0312	American Type Culture Collection	"
MV_1	Mesilla Valley, N.M.	Field Isolate
MV_2	"	"
MV	"	"
CHA	Chavez County, N.M.	"
POR	Portales, N.M.	"
BES	Chavez County, N.M.	"
LC	Mesilla Valley, N.M.	"
A108	San Juan County, N.M.	"
A110	"	"
A111	"	"
H550	"	"

Table 2

Effect of increasing osmolarity on growth of *R. meliloti* I02FSI.

Addition	Osmolarity[a]	Rel μ[b]
None	73.06 meq.	1.00
MOPS 50 mM	142.56 "	1.26
" 100 "	212.06 "	1.27
" 250 "	347.00 "	0.92
" 500 "	768.06 "	0.38

[a]Osmolarity calculated as the sum of dissociated ions and undissociated molecules in the medium. Reported as milliequivalents (meq.).

[b]Growth rate relative to the control = 1.00.

Table 3

Effect of increasing osmolarity on growth of R. meliloti I02FSI.

Addition	Osmolarity[a]	Rel μ[b]
None	73.06 meq.	1.00
NaCl	473.06 "	1.16
Glycerol 200 mM	473.06 "	1.17
Sodium Acetate	473.06 "	0.13

[a]Osmolarity calculated as the sum of dissociated ions and undissociated molecules in the medium. Reported as milliequivalents (meq.).

[b]Growth rate relative to the control = 1.00.

Table 4

Effect of sodium on growth of *R. meliloti* NSI.

Experiment I

Addition	Rel μ[a]
None	100
NaCl 100 mM	138
NaCl 250 mM	59

Experiment II, Testing for interaction between Na^+ and phosphate

	Phosphate[b]		
Addition	0.1 mM	1.0 mM	10.0 mM
None	100	104	116
NaCl 50 mM	65	105	113
Na_2SO_4 33.3 mM	78	105	103

Experiment III, Testing for interaction between Na^+ and MOPS buffer

	MOPS		
Addition	10.0 mM	25.0 mM	50.0 mM
None	100	92	112
NaCl 50 μM	101	120	126
NaCl 100 μM	112	132	117

Experiment IV, Testing for interaction between Na^+ and pH

pH	Rel μ[d]
6.5	100
7.0	149
7.5	118
8.0	115

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[a] Relative growth rate, control = 100

[b] Relative growth rate, no sodium, 0.1 mM phosphate = 100

[c] Relative growth rate, no sodium, 10 mM MOPS = 100

[d] Relative growth rate, growth rate of cells grown with 100 mM NaCl/growth rate of cells grown without additional NaCl x 100. The growth rate varied with the pH from 0.025 hr^{-1} (pH 6.5) to 0.180 hr^{-1} (pH 8.0). When the pH of the medium was raised above pH 8.2, a precipitate formed.

Table 5

Interaction between molybdenum and sodium or potassium in R. meliloti I02FSI.

$MO_4^=$	Relative μ[a] NaCl Concentration 0	50 mM	100 mM	250 mM
0	104	98	71	25
5 nM	100	122	88	43
25 nM	100	104	82	42
50 nM	106	105	109	58

$MO_4^=$	Relative μ KCl Concentration 0	50 mM	100 mM	250 mM
0	98	89	83	37
5 nM	100	90	69	56
10 nM	100	81	88	50
50 nM	127	94	93	no growth

[a]Growth rate relative to the control having the usual concentration of molybdate (5 nM) and no additional sodium or potassium chloride.

Table 6

Salt sensitivity as affected by subculturing.

Experiment 1 (November, 1979)

Addition	Strain[a]		
	MV_1	MV_2	MV_3
None	0.05	0.07	0.06
NaCl 100 mM	0.15	0.09	0.11
KCl 100 mM	no growth	no growth	0.05

Experiment 2 (April, 1980)

Addition	Strain		
	MV_1	MV_2	MV_3
None	0.15	0.15	0.12
NaCl 100 mM	0.14	0.16	0.11
KCl 100 mM	0.17	0.18	0.19

[a]Results are expressed as the μ (hr^{-1}) rather than the growth relative to a single control.

Table 7

Effect of sodium ion on growth of field strains of *R. meliloti*[a].

Strain	NaCl 100 mM	KCl 100 mM	Na_2SO_4 100 mM
CHA	88	80	66
LC	99	109	128
BES	133	112	83

[a]Results expressed as relative growth rate with the control culture without additional sodium ion = 100.

Table 8

Effect of phosphate concentration.

Phosphate Concentration[a]	Rel μ[b]
1 mM	100
10 "	111
50 "	110
100 "	no growth
250 "	"
500 "	"

[a]Phosphate added as potassium phosphate buffer, pH 7.5. The buffer was made by combining KH_2PO_4 and K_2HPO_4 to a final pH of 7.5.

[b]Growth rate relative to the control = 100. R. meliloti strain I02FSI was used in this experiment.

Table 9

Inhibition of growth by magnesium.

Addition	Rel μ[a]
None	100
0.66 mM	92
6.66 "	52
16.66 "	32
33.33 "	no growth

[a]Relative growth rate. Control having only the Mg^{+2} present in MDM = 100. These results are for R. meliloti strain I02FSI.

Table 10

Inhibition of growth by acetate.

Addition	Rel μ[a]
None	100
Sodium Acetate 10 mM	92
" 25 "	69
" 50 "	57
" 100 "	44

[a]Growth relative to control = 100. *R. meliloti* strain I02FSI used in this experiment.

Table 11

Effect of glutamate and proline on inhibition of growth by phosphate, magnesium and acetate.

	Rel μ		
	Osmoticum[a]		
Addition	None	Proline	Glutamate
None	100	108	118
K_2HPO_4 100 mM	45	55	51
$MgCl_2$ 30 mM	7	35	21
Acetate 100 mM[b]	36	126	126

[a]Osmotica: 10 mM proline or 10 mM potassium glutamate. Results are expressed as relative to the control. Results with R. meliloti strain NSI are presented here.

[b]Acetate added as sodium acetate.