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DNA, a Possible Site of Action of Aluminum in *Rhizobium* spp.

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Al was found to penetrate the cell envelopes of both sensitive and tolerant *Rhizobium* strains and bind to DNA in vivo. Despite causing a reduction in viability, Al stimulated DNA synthesis in the sensitive strain, which suggested that an excision repair mechanism was operating. The Al-stimulated DNA synthesis was reduced by the simultaneous addition of chloramphenicol. In contrast to the sensitive strain, DNA synthesis was unaffected by Al binding to DNA in the tolerant strain. It is proposed that Al enters the cell and binds to the DNA helix, increasing stabilization and preventing successful replication. Different repair mechanisms appear to operate in response to Al in tolerant and sensitive strains.

Aluminum, the most abundant metal in the Earth's crust, becomes more soluble as acidity increases and is often the major toxic element in acidic soils and water (2, 15). Many hypotheses have been proposed for the mechanism of Al toxicity to animals and plants (5, 6); however, Al-microorganism interactions have received little attention.

Al was shown to bind to *Staphylococcus aureus* cell walls at pH 4.3 (1), and consequently the cell wall was suggested to be a possible site of toxicity. Al has been observed to bind to the cell wall and intracellular polyphosphate granules of *Anabaena cylindrica* (12). Previous work on rhizobia has investigated direct and indirect mechanisms of Al toxicity (19). It has been shown that *Rhizobium* cells are most sensitive to Al at the moment of cell division (10) and during rapid cell division (exponential growth phase) (20).

A link between Al toxicity and DNA has been reported for peas (9) and wheat (S. J. Henning, Ph.D. thesis, Oregon State University, Corvallis, 1975). Al has also been shown to bind to mammalian DNA in vitro (7). Many toxic metals, in addition to binding to proteins and coenzymes, act by disrupting DNA; Mg^{2+}, Co^{2+}, and Ni^{2+} stabilize the helix, whereas Cd^{2+}, Cu^{2+}, and Hg^{2+} destabilize the DNA duplex (4).

The aims of this study with *Rhizobium* strains were to investigate the following questions. (i) Does Al bind to DNA? (ii) Does Al affect DNA synthesis? (iii) Are there differences in these respects between tolerant and sensitive strains?

MATERIALS AND METHODS

Bacteria and growth conditions. The strains used in this study were representative of their species (18). Al-sensitive *Rhizobium leguminosarum* bv. *trifolii* strains RDG 2015 and RDG 2002 (isolated in our laboratory, with similar responses to Al) and Al-tolerant *R. loti* NZP 2037 (obtained from the Department for Scientific and Industrial Research, Palmerston North, New Zealand) were maintained at 5°C on yeast extract-mannitol (YEM) agar containing 3 g of $CaCO_3$ $liter^{-1}$ (16). The number of viable cells was measured by using serial dilutions and YEM agar. Strains were exposed to Al in a defined arabinose-galactose-glutamate medium (20).

In this paper Al tolerance refers to the ability to multiply in liquid defined medium in the presence of 50 μM Al and Al sensitivity refers to the loss of viability (as indicated by a lack of growth on YEM agar) under the same conditions.

Cell harvesting. When using the following DNA extraction procedure, it is important to use containers made from plastic such as polypropylene whenever possible to avoid leaching of Al into the solution, as can happen with glassware.

Following exposure to Al, the cultures were centrifuged at 6,000 × *g* for 15 min. The supernatant fluid was discarded and replaced by 100 ml of filter-sterilized distilled-deionized water and centrifuged as above. The washing procedure was repeated with fresh distilled-deionized water. The pellets were resuspended in 4.5 ml of TE7 (10 mM Tris hydrochloride, 0.5 mM EDTA [pH 7.0]), and the replicate pellets were pooled. The cells were lysed by addition of 1 ml of 10% sodium dodecyl sulfate (final concentration, 1%) and incubated overnight at 47°C in a water bath.

Nucleic acid extraction. The lysate mixture was decanted into a 50-ml polypropylene Medical Scientific Equipment (MSE) centrifuge tube and mixed with an equal volume of Tris-saturated phenol. The phenol-lysate mixture was centrifuged at 1,460 × *g* for 15 min. The upper, aqueous layer was transferred with a Pasteur pipette to a glass 50-ml centrifuge tube and mixed with an equal volume of chloroform, isoamyl alcohol, and phenol (24:1:25). The mixture was centrifuged at 1,460 × *g* for 15 min. The upper, aqueous layer was transferred with a Pasteur pipette to another 50-ml glass centrifuge tube. The aqueous solution was then mixed with 5 M $NaClO_4$ (final concentration, 1 M) and allowed to stand for 20 min. An equal volume of chloroform and isoamyl alcohol (24:1) was then added, and the solution was mixed and centrifuged at 1,460 × *g* for 15 min. The upper, aqueous layer was then transferred with a Pasteur pipette to a 50-ml polypropylene MSE centrifuge tube. Sodium acetate was added to a final concentration of 0.3 M to the aqueous solution, and then 2 volumes of cold absolute ethanol was added. The extraction mixture was then frozen overnight at −20°C to precipitate the nucleic acids (NA). The precipitate was pelleted by centrifugation at 12,000 × *g* for 15 min. The supernatant was then discarded, and the pellet was resuspended in 2 ml of TE7.

NA fractionation and analysis. The crude NA sample was then fractionated on a gel filtration column. A Bio-Rad column was sterilized by autoclaving at 121°C for 15 min and packed aseptically with Sephadex G-50 beads in a TE7 buffer, which had been sterilized by autoclaving at 121°C for 15 min. The column was packed with 3.5 to 4 cm^3 of

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Sephadex G-50 and eluted with 4 volumes of TE7. The column was then loaded with 200 to 300 μl of the crude NA sample and eluted with TE7 buffer. Fractions (1 ml) were collected and analyzed for A_{260} (NA) in UV-visible plastic cuvettes by using a Cecil Instruments CE 272 UV spectrophotometer. The fractions were analyzed for Al in a Perkin-Elmer 3030 atomic absorption spectrophotometer with a graphite furnace.

The size of the extracted NA collected after fractionation was estimated by agarose gel electrophoresis (8). A 0.7% (wt/vol) gel was prepared in Tris-borate-EDTA buffer containing ethidium bromide. Each slot in the gel was loaded with 30 μl of the sample mixed with 5 μl of loading buffer. A lambda marker containing DNA fragments of known length was added to run alongside the samples. The gel was run at 100 V for 2 h before examination. Bands of NA stained with ethidium bromide were observed under a UV lamp.

Following fractionation of an NA sample from strain RDG 2015 which had been exposed to Al, the fraction showing the highest A_{260} was incubated with either 20 μg of RNase A (Sigma Chemical Company) per ml at 37°C for 24 h or 20 μg of DNase (Sigma Chemical Company) per ml at 37°C for 4 h. The samples were then fractionated by using gel filtration and analyzed for NA and Al as described above.

Cells exposed to Al in defined medium. Strains RDG 2015 and NZP 2037 were inoculated separately into 300-ml polypropylene MSE centrifuge tubes containing 200 ml of a defined arabinose-galactose-glutamate medium (pH 5.5) to give an initial cell density of 5×10^3 CFU ml^{-1}. There were two replicate tubes per strain, and they were incubated at 26°C on a rotary shaker at 80 rpm. Al was added to a final concentration of 50 μM after 60 h of incubation, at which point the cells were at the mid- or late exponential (log) growth phase. Incubation was continued for a further 36 h. At the end of the experiment the cultures were harvested, washed, and lysed, and NA was extracted and analyzed as described above.

Effect of Al on [^{3}H]thymidine incorporation. Strains RDG 2015 and NZP 2037 were inoculated separately into 300-ml propylene MSE centrifuge tubes containing 200 ml of defined medium at pH 5.5 (Al to be added at mid-exponential phase). The initial cell density was 5×10^3 to 5×10^4 CFU ml^{-1}. Incubation was carried out at 26°C on a rotary shaker at 80 rpm. Al and 10 μCi of [^{3}H]thymidine (Amersham; ^{3}H on the methyl group with a final concentration of 0.05 μCi ml^{-1} in 200-ml cultures) were added after 55 h of incubation. Incubation was continued for a further 20 h. At the end of the experiment the cultures were harvested, washed, and lysed, and NA was extracted as described above, with the size fractionation omitted.

In a second experiment the Al-sensitive strain RDG 2002 was grown in defined medium to the late-exponential phase as in the previous [^{3}H]thymidine incorporation experiment. After 88 h, Al (50 μM), [^{3}H]thymidine (0.5 μCi ml^{-1} in 20-ml cultures), and chloramphenicol (50 μg ml^{-1}) were added and incubation was continued for a further 24 h. The treatments were as follows: (i) no Al, no chloramphenicol; (ii) chloramphenicol, no Al; (iii) Al, no chloramphenicol; and (iv) both Al and chloramphenicol.

The DNA extraction procedure was the same as that described above, with the final gel filtration stage omitted. The ethanol-precipitated pellet was resuspended in 1 ml of TE7 buffer. The total NA content was measured by A_{260}. The RNA content was assessed by the Orcinol method (3), with RNA standards. The DNA content of a sample was calculated as the total NA (A_{260}) minus the RNA content.

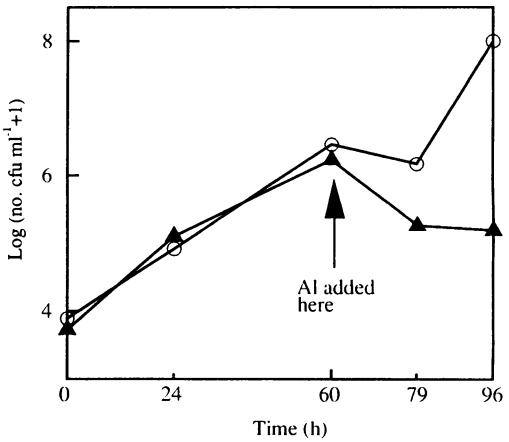

FIG. 1. Effect of 50 μM Al at pH 5.5 on the viability of *Rhizobium* strains RDG 2015 (▲) and NZP 2037 (○) in defined medium (mean of two observations).

The [^{3}H]thymidine content of an NA sample was measured by using a Beckman LS 1801 scintillation counter. A 0.3-ml portion of the NA sample was transferred to a scintillation vial and mixed with 3 ml of Ecoscint fluor (National Diagnostics). The samples were then left in the dark for 30 min before being counted. The samples were quench corrected; no appreciable differences between quench values were recorded. The results were expressed as comparative counts per minute.

RESULTS

Effect of Al on viability of cells and its binding to DNA. The effect of Al on cell viability is shown in Fig. 1. It inhibited the multiplication of RDG 2015 and caused a prolonged decrease in the viability of the population. It also initially inhibited the multiplication of NZP 2037, but after 19 h the cells were multiplying normally in the presence of Al.

Figure 2 shows the concentration of Al, A_{260}, and presence of high-molecular-weight DNA following gel filtration of nucleic acid from the Al-tolerant NZP 2037 strain after

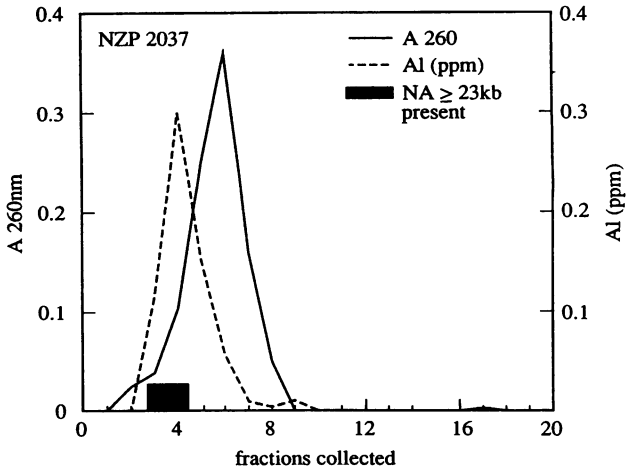

FIG. 2. Gel filtration (Sephadex G-50) of NA extracted from *R. loti* NZP 2037 (exposed to Al in vivo). Fractions that contained NA fragments larger than or equal to 23 kb are indicated.

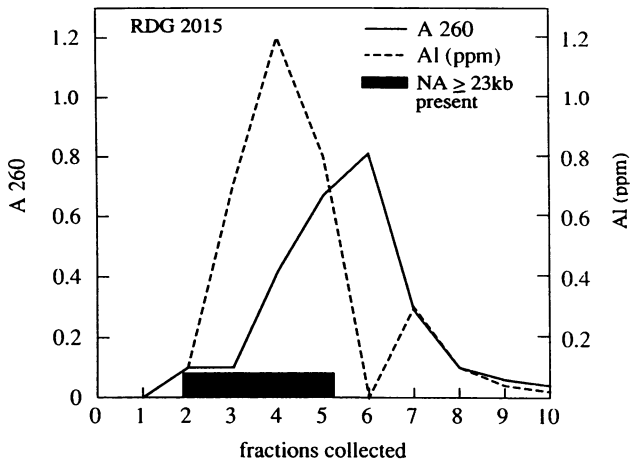

FIG. 3. Gel filtration (Sephadex G-50) of NA extracted from clover *Rhizobium* strain RDG 2015 (exposed to Al in vivo). Fractions that contained NA fragments larger than or equal to 23 kb are indicated.

exposure to Al. There was some overlap between the fractions showing positive A_{260} values and those containing Al. Double-stranded DNA corresponding to the 23-kb lambda marker was observed in fractions 3 and 4. No other stained NA was observed by agarose gel electrophoresis in these or any other fractions. The other material with positive A_{260} values may represent DNA or RNA free nucleotides or perhaps protein, although the A_{260} exceeded the A_{280} in each fraction (data not shown). The peak Al concentrations coincided with the fractions which contain high-molecular-weight DNA. Figure 3 shows data for Al-sensitive RDG 2015 strain. A higher concentration of NA was eluted through the Sephadex column, and high-molecular-weight DNA was observed in fractions 2 to 5, which coincided with the peak Al concentrations and not the peak A_{260} as before.

Figure 4 shows the effects of RNase or DNase on the Al and A_{260} profiles for NA extracted from RDG 2015 after exposure to Al. The Al-NA profile was unaffected by RNase, whereas after DNase treatment 45% of the Al recovered was displaced from the NA peak and eluted in later fractions.

Effect of Al on [^{3}H]thymidine incorporation. Both strains continued to multiply in the absence of Al (Table 1). The addition of Al after 55 h to strain RDG 2002 stopped the cells from dividing in the medium and caused a drop in the viability of the population (Table 1). Al halted division of NZP 2037 cells in the medium, yet did not noticeably affect the viability of the cells when they were transferred to Al-free YEM agar (Table 1).

Table 2 shows the radioactivity counts for the extracted DNA of the two strains. Al appeared to stimulate [^{3}H]thymidine incorporation in the Al-sensitive strain by a factor of three or four times that in the control. In contrast, for the Al-tolerant strain the [^{3}H]thymidine incorporation value for the Al treatment was similar to that in the control.

The sensitive strain RDG 2002 multiplied normally in the absence of chloramphenicol (an inhibitor of protein synthesis) and Al, but chloramphenicol reduced its viability (Table 3). Al alone and Al plus chloramphenicol both reduced cell viability, but the treatment with Al plus chloramphenicol was no more toxic than that with Al alone. Chloramphenicol alone did not affect [^{3}H]thymidine incorporation during the 24-h treatment (Table 4). This suggested that normal DNA

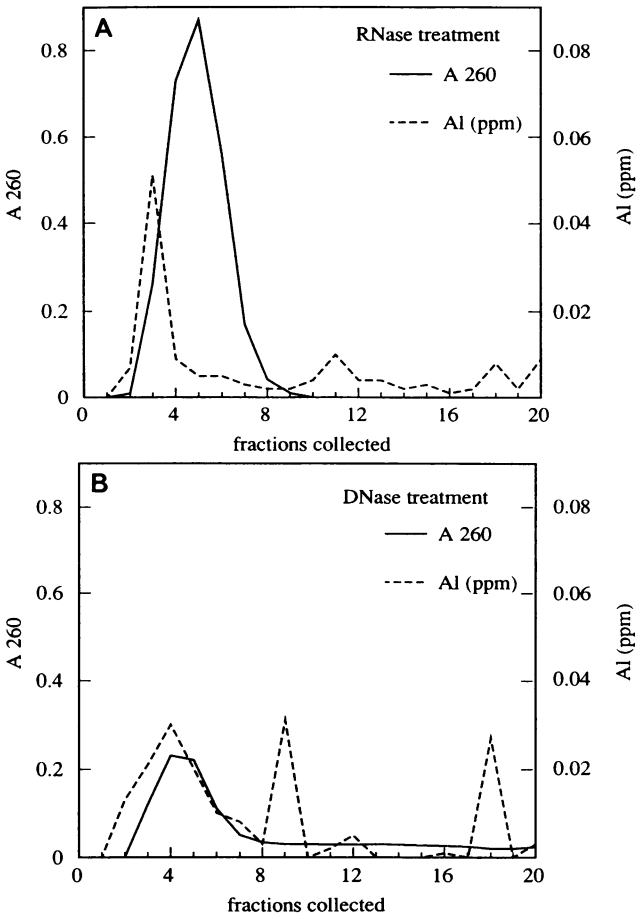

FIG. 4. Gel filtration (Sephadex G-50) of prefractionated NA extracted from clover *Rhizobium* strain RDG 2015 (exposed to Al in vivo) following RNase (A) or DNase (B) treatment.

replication was unaffected by chloramphenicol. Al increased the [^{3}H]thymidine incorporation to almost four times that of the control, but this figure was almost halved by the simultaneous addition of chloramphenicol (Table 4). This experiment could not be repeated with the Al-tolerant strain as chloramphenicol interfered with DNA synthesis in the untreated control cells.

DISCUSSION

Does Al bind to DNA in rhizobia? The data presented here indicate that Al penetrates the *Rhizobium* cell and binds to the DNA of both Al-sensitive and Al-tolerant strains. Al

TABLE 1. Effect of 0 or 50 μM Al (pH 5.5) on the viability of two *Rhizobium* strains in defined medium

Time (h)[a]	Viability (log CFU/ml) of[b]:			
	RDG 2002		NZP 2037	
	− Al	+ Al	− Al	+ Al
0	4.41	4.50	5.96	5.87
55	7.41	7.41	7.84	7.79
75	7.72	6.66	8.11	7.72

[a] The Al was added after 55 h of incubation.
[b] Results are means of two observations (standard error of the mean = 0.21).

TABLE 2. Effect of 0 or 50 μM Al (pH 5.5) on [^{3}H]thymidine (0.05 μCi ml^{-1}) incorporation for two *Rhizobium* strains in defined medium

Replicates	[^{3}H]thymidine incorporation (cpm/μg of DNA) in:			
	RDG 2002		NZP 2037	
	−Al	+Al	−Al	+Al
1	145	467	99	86
2	90	319	122	79
Mean[a]	118	393	111	83

[a] Standard error of the mean = 58.

entering the cell from an acidic medium would polymerize when it encountered the near-neutral internal cell environment (11). The fractionation profiles in Fig. 2 and 3 show some association between A_{260} and Al concentration, but the peak Al concentrations coincide with the fractions containing high-molecular-weight DNA.

To ensure that Al did not coincidentally elute together with NA at fractions 3 to 6, we passed an Al solution through a gel filtration column. Al analysis (data not shown) revealed that the Al concentrated in fractions 2, 5, 9, 13, 17, 19, and 21, each peak perhaps representing a different chemical form of Al (14). Following elution of the NA extracts, the Al was concentrated in fractions 2 to 8, suggesting that it was present in only a small number of chemical forms and therefore that it was associated with the NA. Strong evidence in support of this is also provided by the experiment in which DNase was used; following this treatment, which degraded the DNA, 45% of the Al recovered had dissociated from the NA peak (Fig. 4). That the majority of the Al is attached to DNA and not RNA is shown by the contrasting effects of RNase and DNase (Fig. 4).

Once inside the cell the Al, would form predominantly positively charged polymeric complexes (14), which could bind to, among other things, the negatively charged phosphate groups of DNA. This may stabilize the helix and prevent melting in a manner similar to that for the metals Mg^{2+}, Co^{2+}, and Ni^{2+} (4), thus stopping replication. It has been suggested that in plants large Al polymers bind across from the phosphate group of one strand of DNA to the phosphate group of the other strand (9). However, despite Al binding to DNA, the Al-tolerant cells continued to multiply in the presence of Al.

Does Al affect DNA synthesis? DNA synthesis in NZP 2037 (as indicated by [^{3}H]thymidine incorporation) was unaffected by Al, in contrast to DNA synthesis in the Al-sensitive strain RDG 2002 (Table 2). The increase in DNA synthesis by the Al-sensitive cells in response to Al did not prevent a decrease in viability. If Al inhibited cell division by preventing septum formation, the [^{3}H]thymidine incorporation by the control and Al treatments should have been similar. If Al stopped replication by blocking the active site of polymerase enzymes, little or no [^{3}H]thymidine incorporation would have occurred.

TABLE 3. Effect of 50 μM Al and 50 μg of chloramphenicol ml^{-1} (pH 5.5) on the viability of Al-sensitive strain RDG 2002 in defined medium

Time (h)[a]	Viability (log CFU/ml) of RDG 2002[b]			
	−Al, −chlor	−Al, +chlor	+Al, −chlor	+Al, +chlor
0	3.88	3.80	3.91	3.93
88	7.61	7.61	7.62	7.65
112	7.77	7.05	6.67	7.01

[a] The Al and/or chloramphenicol (chlor) was added after 88 h of incubation.
[b] Results are means of two observations (standard error of the mean = 0.33).

TABLE 4. Effect of 50 μM Al and/or 50 μg of chloramphenicol ml^{-1} (pH 5.5) on [^{3}H]thymidine (0.5 μCi ml^{-1}) incorporation for the Al-sensitive strain RDG 2002 in defined medium

Replicates	[^{3}H]thymidine incorporation (cpm/μg of DNA) in RDG 2002 with:			
	−Al, −chlor	−Al, +chlor	+Al, −chlor	+Al, +chlor
1	602	769	2,181	1,401
2	482	422	1,673	965
Mean[a]	542	596	1,927	1,183

[a] Standard error of the mean = 269.

[^{3}H]thymidine cannot normally be incorporated into RNA, but there may be a danger that some contaminating RNA or protein caused errors in the calculation of cpm of DNA per microgram. However, experiments involving Al-sensitive strains exposed to [^{3}H]thymidine with and without Al were repeated on four separate occasions. A significant difference in [^{3}H]thymidine incorporation of 50 to 75% was always observed. The simultaneous addition of chloramphenicol (an inhibitor of protein synthesis) with Al almost halved the amount of [^{3}H]thymidine incorporation from that when the cells were exposed to Al alone (Table 4). This indicates that new proteins are involved in the DNA synthesis following DNA damage. That chloramphenicol did not entirely stop [^{3}H]thymidine incorporation in the presence of Al may be because constitutive DNA repair enzymes were also involved in the attempt to repair Al damage.

If the repair of regions of DNA containing Al involved excision, new DNA would be synthesized to maintain chromosomal integrity. Such a response has been reported for *Escherichia coli* (13, 17).

Comparison of tolerant and sensitive strains. We found no evidence that the tolerant strain NZP 2037 prevented Al binding to DNA (Fig. 2), although the data do not allow quantitative comparisons between tolerant and sensitive strains. Al caused a large increase in [^{3}H]thymidine incorporation for the Al-sensitive strain (Table 2), but this did not occur with the Al-tolerant strain. The dramatic increase in DNA synthesis in the Al-sensitive cells may have represented a repair response (involving new proteins), which was, however, unsuccessful in preventing a reduction in cell viability. This response did not occur with the Al-tolerant cells which also had Al bound to DNA (Fig. 2). This suggests that the repair mechanism used to overcome damage by Al to DNA in tolerant cells is quite different from that in sensitive cells.

In conclusion, the data presented here indicate that DNA is a possible site of action of Al in the common soil bacterium *Rhizobium*. This could have important genetic and ecological consequences.

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