7. Deb, B. M. and Chattaraj, P. K., *Chem. Phys. Lett.*, 1988, **148**, 550–556.
8. Deb, B. M., Chattaraj, P. K. and Mishra, S., *Phys. Rev.*, 1991, **A43**, 1248–1257.
9. Chattaraj, P. K., *Int. J. Quant. Chem.*, 1992, **56**, 845–859.
10. Deb, B. M. and Dey, B. K., *Int. J. Quant. Chem.*, 1995, **56**.
11. Chattaraj, P. K. and Nath, S., *Int. J. Quant. Chem.*, 1994, **49**, 705–725.
12. Nath, S. and Chattaraj, P. K., *Pramana*, 1995, **45**, 65–73.
13. Chattaraj, P. K. and Nath, S., *Chem. Phys. Lett.*, 1994, **217**, 342–348.
14. Chattaraj, P. K. and Nath, S., *Proc. Indian Acad. Sci.*, 1994, **106**, 229–249.
15. Holland, P. R., *The Quantum Theory of Motion*, Cambridge Univ. Press, Cambridge, 1993.
16. de Broglie, L., *Nonlinear Wave Mechanics: A Causal Interpretation*, Elsevier, Amsterdam, 1960.
17. Bohm, D., *Phys. Rev.*, 1952, **85**, 166–179, 180–193.
18. Gutzwiller, M. C., *Chaos in Classical and Quantum Mechanics*, Springer, Berlin, 1990.
19. Eckhardt, B., *Phys. Rep.*, 1988, **163**, 205–297.
20. Jensen, R. V., *Nature*, 1992, **355**, 311–317, 1995, **373**, 16 and references therein.
21. Chattaraj, P. K. and Sengupta, S., *Phys. Lett.*, 1993, **A181**, 225–231; Sengupta, S. and Chattaraj, P. K., *Phys. Lett. A*, in press.
22. Chattaraj, P. K., *Indian J. Pure Appl. Phys.*, 1994, **32**, 101–105; Chattaraj. P. K. and Sengupta, S., *Indian J. Pure Appl. Phys.*, in press.
23. Dewdney, C. and Hiley, B. J., *Found. Phys.*, 1982, **12**, 27–48.
24. Takabayasi, T. *Prog. Theor. Phys.*, 1952, **8**, 143–182; 1953, **9**, 187–222; 1955, **14**, 283–302.
25. Kan, K. K. and Griffin, J. J., *Phys. Rev.*, 1977, **C15**, 1126–1157.
26. Weiner, J. H. and Partom, Y., *Phys. Rev.*, 1969, **187**, 1134–1147; Weiner, J. H. and Askar, A., *J. Chem. Phys.*, 1972, **54**, 3534–3541.
27. McCullough, E. A. and Wyatt, R. E., *J. Chem. Phys.*, 1971, **54**, 3578–3591.
28. Hirschfelder, J. O., Christoph, A. C. and Palke, W. E., *J. Chem. Phys.*, 1974, **61**, 5435–5455; Hirschfelder, J. O. and Tang, K. T., *J. Chem Phys.*, 1976, **64**, 760–785; 1976, **65**, 470–486.
29. Skodje, R. T., Rohrs, H. W. and VanBuskirk, J., *Phys. Rev.*, 1989, **A40**, 2894–2916.
30. Parmenter, R. H. and Valentine, R. W., *Phys. Lett.*, 1995, **A201**, 1–8.
31. Schwengelbeck, U. and Faisal, F. H. M., *Phys. Lett.*, 1995, **A199**, 281–286.
32. Faisal, F. H. M. and Schwengelbeck, U., *Phys. Lett.*, 1995, **A207**, 31–36.
33. Misner, C. W., Thorne, K. S. and Wheeler, J. A., *Gravitation*, W. H. Freeman, San Fransisco, 1973.
34. McDonald, S. W. and Kaufman, A. N., *Phys. Rev. Lett.*, 1979, **42**, 1189–91.
35. Berry, M. V., *Proc. R. Soc. London*, 1987, **A413**, 183–198.
36. Lakshmanan, M. and Ganesan, K., *Curr. Sci.*, 1995, **68**, 38–44 and references therein.
37. Casati, G., Chirikov, B. V., Guarneri, I. and Shepelyansky, D. L., *Phys. Rep.*, 1987, **154**, 77–123.
38. Hasegawa, H., Robnik, M. and Wunner, G., *Prog. Theor. Phys. Suppl.*, 1989, **98**, 198–286; Ganesan, K. and Lakshmanan, M., *Phys. Rev.*, 1990, **A42**, 3940–3947; Howard, J. E. and Farrelly, D., *Phys. Lett.*, 1993, **A178**, 62–72.
39. Delande, D. and Gay, J. C., *Phys. Rev. Lett.*, 1987, **59**, 1809–1812; Delande, D., in *Chaos and Quantum Physics* (eds. Giannoni, M. J., Voros, A. and Zinn-Justin, J.), Elsevier, 1991; Friedrich, H. and Wintgen, D., *Phys. Rep.*, 1989, **183**, 37–79.
40. Holle, A., Weibusch, G., Main, J., Hager, B., Rottke, H. and Welge, K. H., *Phys. Rev. Lett.*, 1986, **56**, 2594–2597; Holle, A., Main, J., Weibusch, G., Rottke., H. and Weldge, K. H., *Phys. Rev. Lett.*, 1988, **61**, 161–164.
41. Ames, W. F., *Numerical Methods for Partial Differential Equations*, Academic, New York, 1977, p. 252.
42. Chattaraj, P. K., Rao, K. S. and Deb, B. M., *J. Comp. Phys.*, 1987, **72**, 504–512.

ACKNOWLEDGEMENTS. PKC thanks CSIR, New Delhi, for financial assistance. We are grateful to the referee for constructive criticism.

Received 12 April 1996; revised accepted 7 June 1996

Reduced uptake based zinc resistance in *Azospirillum brasilense* sp7

P. Mangala Gowri and Sheela Srivastava

Department of Genetics, University of Delhi, South Campus, Benito Juarez Road, New Delhi 110 021, India

Zinc resistant *Azospirillum brasilense* sp7 exhibited a low affinity for the metal. A zinc-sensitized, streptomycin-resistant variant and a sensitive mutant of the parent strain showed an increased affinity for zinc. Both the affinity for, and uptake of the metal were in the order of parent strain < sensitised variant < sensitive mutant, suggesting reduced uptake as the mechanism of resistance to zinc. Neither magnesium nor manganese could bestow any protection against zinc toxicity in the sensitive mutant, suggesting a specific pathway for the entry of the metal.

THE presence and buildup of metal pollutants in the soil affects the soil microflora, which encompass the beneficial microorganisms, like the biological nitrogen fixers or BNFs. It has been reported that the total output of BNFs could be reduced in the presence of a metal ion[1]. One such nitrogen fixer is the gram negative *Azospirillum brasilense* sp7, which has gained importance owing to its non-obligate association with grasses. This bacterium expressed a high level (10 mM) tolerance to zinc (Zn) which was constitutively expressed[2]. Continuing to work with the same strain (referred to as the parent strain hereafter), the mechanism of Zn resistance has been studied in the bacterium and is presented in this paper.

The minimal nutrient medium (MM) and culture conditions for *A. brasilense* sp7 were as mentioned earlier[2]. A streptomycin-resistant variant, MS12, was derived with a reduced maximum tolerable concentration[3] (MTC) of Zn of 2 mM. This mutation seems to be of a multifold importance in azospirilla.

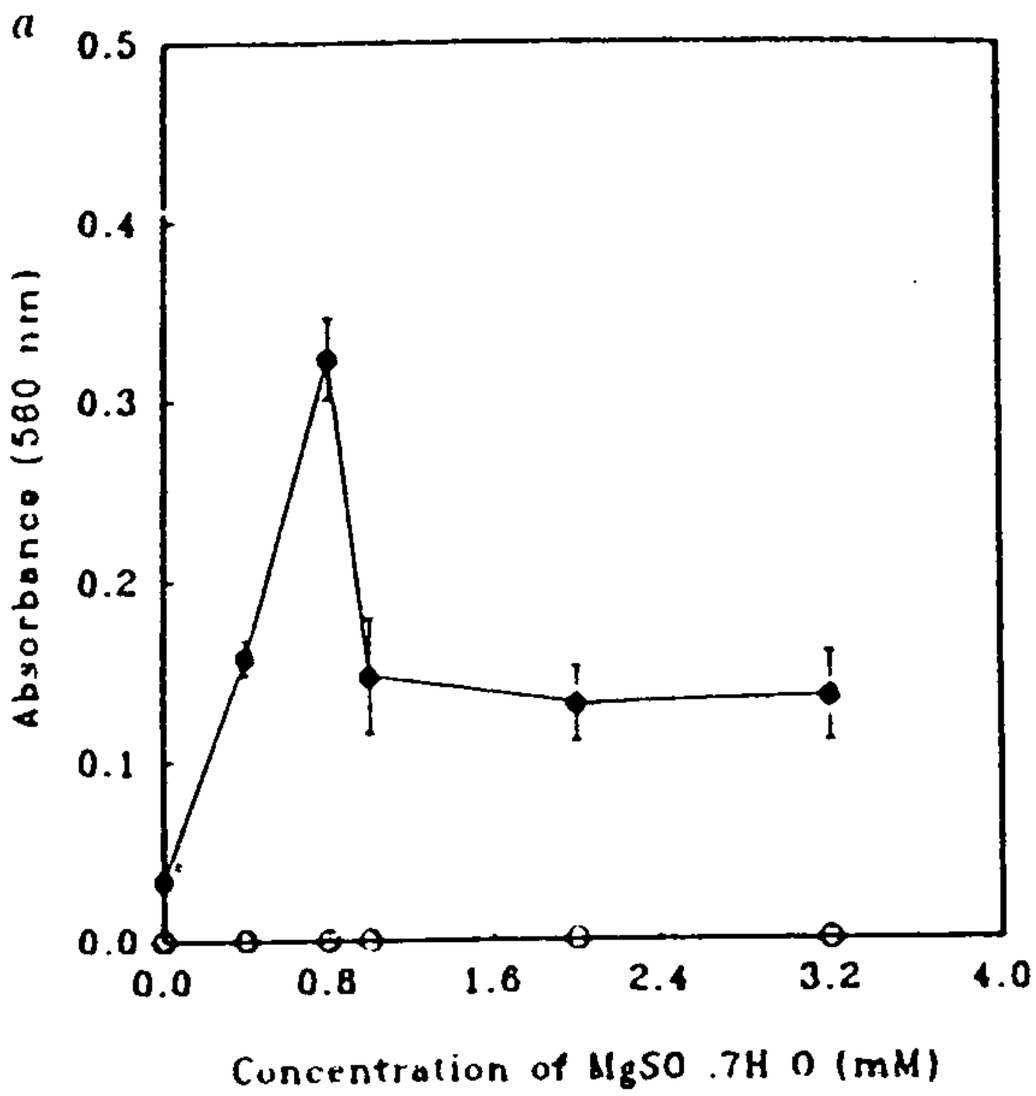
A. brasilense sp7 has been known to be recalcitrant to mutagen treatments. For the members of the Azotobac-

teriaceae, a soil treatment method[4] has been suggested to overcome a similar difficulty[5]. The cells of the parent strain were, therefore, subjected to soil treatment to obtain a strain amenable to mutagenesis[6] (i.e. with 5 times less DNA than the parent strain owing to the reduced size of the treated cells). The derived strain was subjected to sequential *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis (50 and 100 μg/ml) for 10 min in citrate buffer (pH 5.5). The mutagen was removed by centrifugation and the cell pellet suspended in normal saline was spread after appropriate dilutions. This resulted in three Zn^S (MTC 0.5 mM) mutants able to grow only in the presence of casamino acids. One of them, ZS2, was selected for further studies. This mutant retained the ampicillin resistance of the parent strain, and did not revert to either prototrophy or Zn resistance (frequency less than 1.6×10^{-10}).

The strains obtained were maintained as pure cultures raised from a single colony and the purity of the strains was checked periodically by testing antibiotic resistance markers, plasmid profiles, motility, cell morphology and growth on a defined medium.

In order to study the interaction of Zn with two commonly occurring divalent cations in the medium, Mg and Mn, competition experiments were conducted in the strain ZS2. 1 M stock of metal salts was prepared. Against a constant 1 mM Zn concentration, a range of Mg and Mn concentrations was tried. All the cations were added in the required amounts in the sterile MM (+0.2% casamino acids) before inoculation. Initial (0 h) and final (24 h) growth was recorded in reference to the absorbance at 560 nm. Uptake experiments were performed with ^{65}Zn (Bhabha Atomic Research Centre, Trombay, Bombay, India) at a concentration of 0.01 µCi/ml (0.005 µCi/µmol) with a final, 2 mM Zn concentration. The assay proper was done in MM (or +casamino acids for ZS2) as described by Tynecka *et al.*[7], except that the washing was done with MM containing 5 times Zn concentration used for assay. For determination of K_m and V_{max}, uptake was recorded after 30 min of ^{65}Zn addition, with a range of external Zn concentrations. Protein was extracted by subjecting 3 ml of culture to sonication in Vibronics Ultrasonic Processor P2 with the small probe. Three pulses of 15 sec each with an equal interval between the pulses were given. The amounts of protein were estimated by Bradford's protocol[8] with bovine serum albumin as the standard.

No growth of ZS2 was observed in the presence of Zn with increasing concentrations of either Mg or Mn (Figure 1). Other divalent cations like cadmium, cobalt, copper, etc. could not be tested as the parent strain did not resist these metals. Also Mg^{2+} and Mn^{2+} are known to be the main competitors for Zn^{2+} in bacteria[9–11]. However, the response of ZS2 to Zn in the presence of Mg/Mn indicated that perhaps Zn has its specific pathway, as reported for *Escherichia coli* K12 (ref. 12).



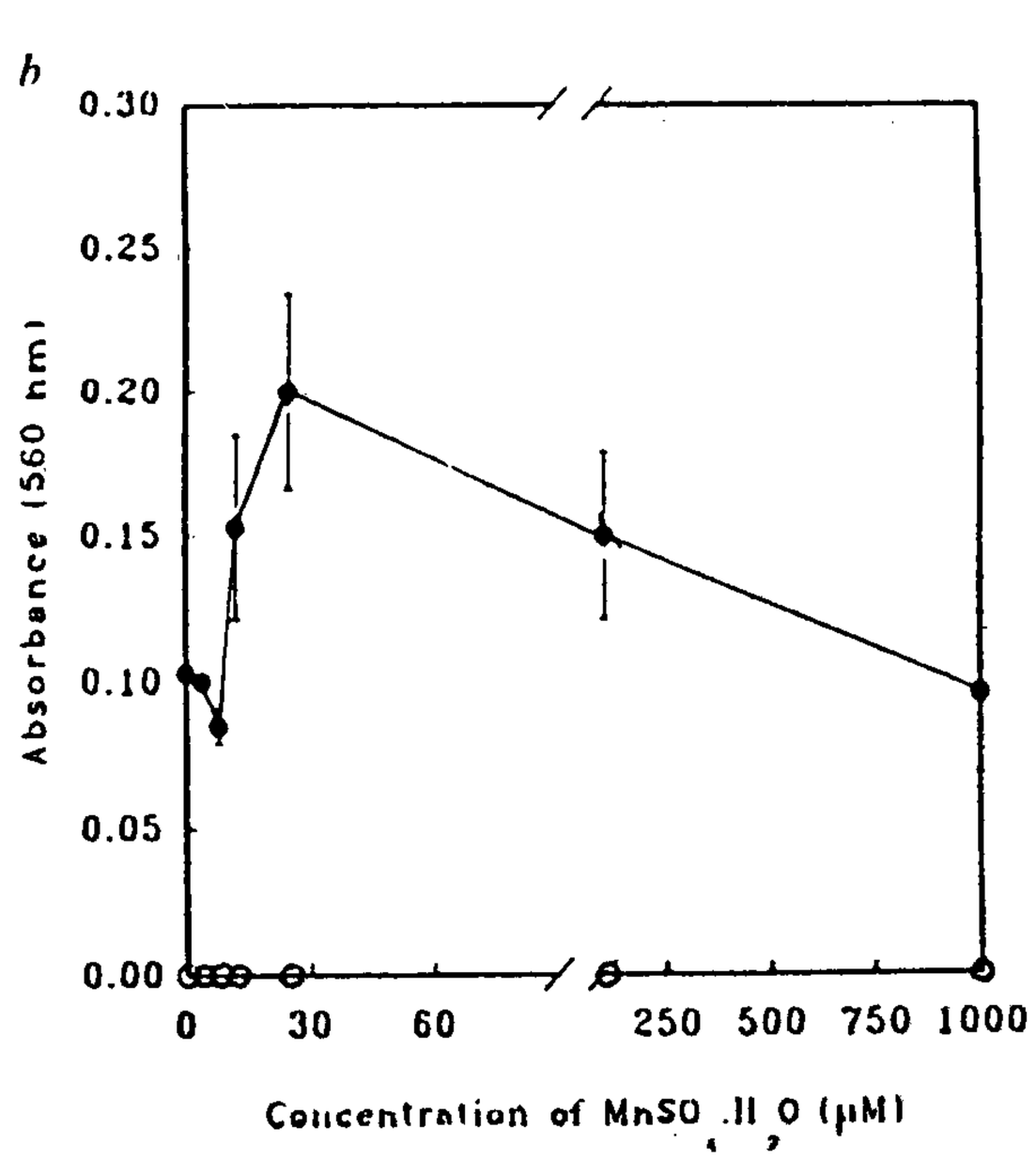


Figure 1*a*, *b*. Effect of divalent cations on the growth responses of ZS2 to Zn. Growth of ZS2 in the absence (●) and presence (O) of 1 mM Zn. Mean values ±S.E.M. are plotted. Varying concentrations of (*a*) $MgSO_4 \cdot 7H_2O$ and (*b*) $MnSO_4 \cdot H_2O$ used.

The uptake pattern by *A. brasilense* sp7, variant MS12 and ZS2 is depicted in Figure 2. The behaviour of

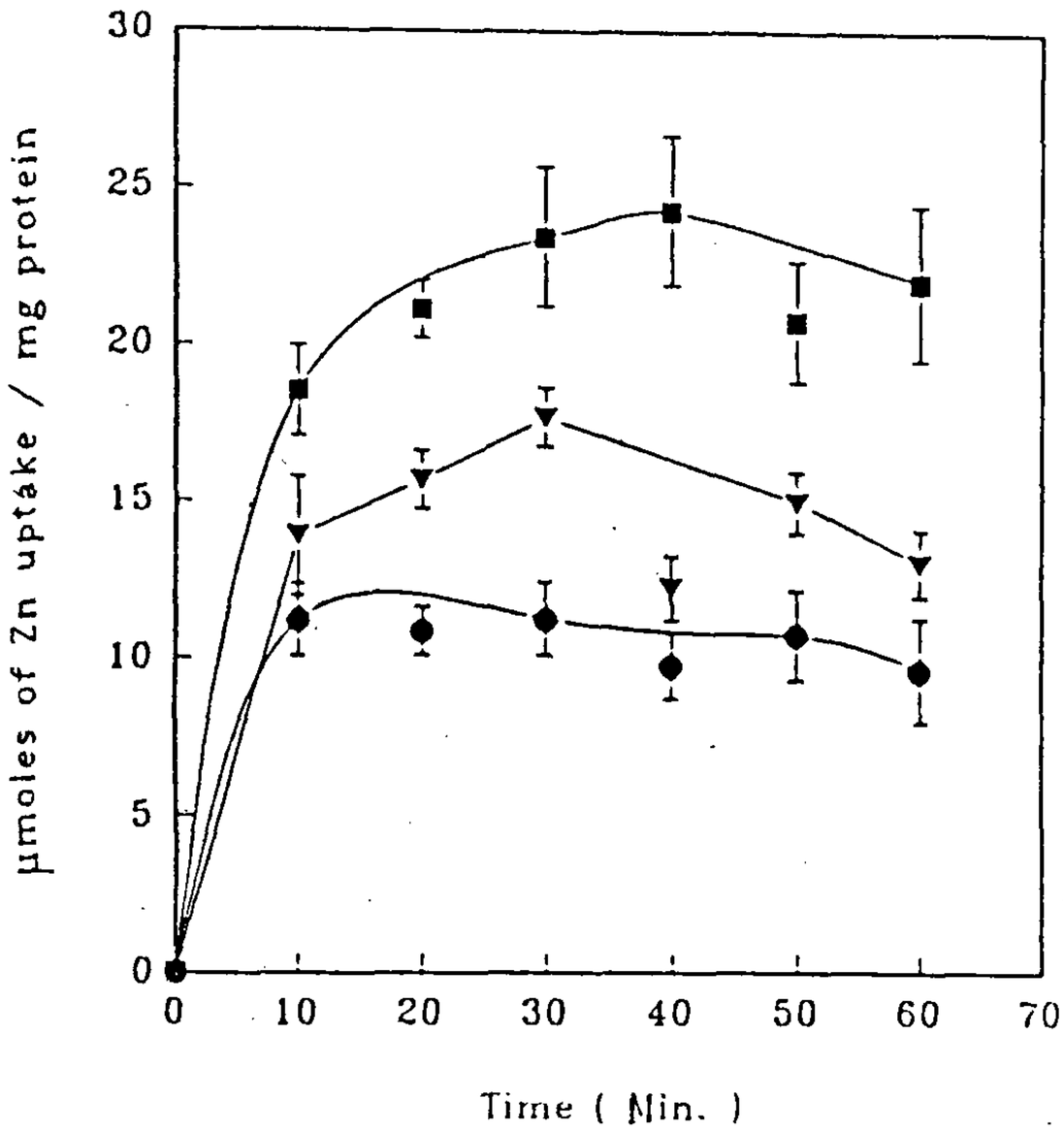


Figure 2. ^{65}Zn uptake in the parent strain (●), variant (▼) and the Zn^S ZS2 (■). Bars indicate ±S.E.M.

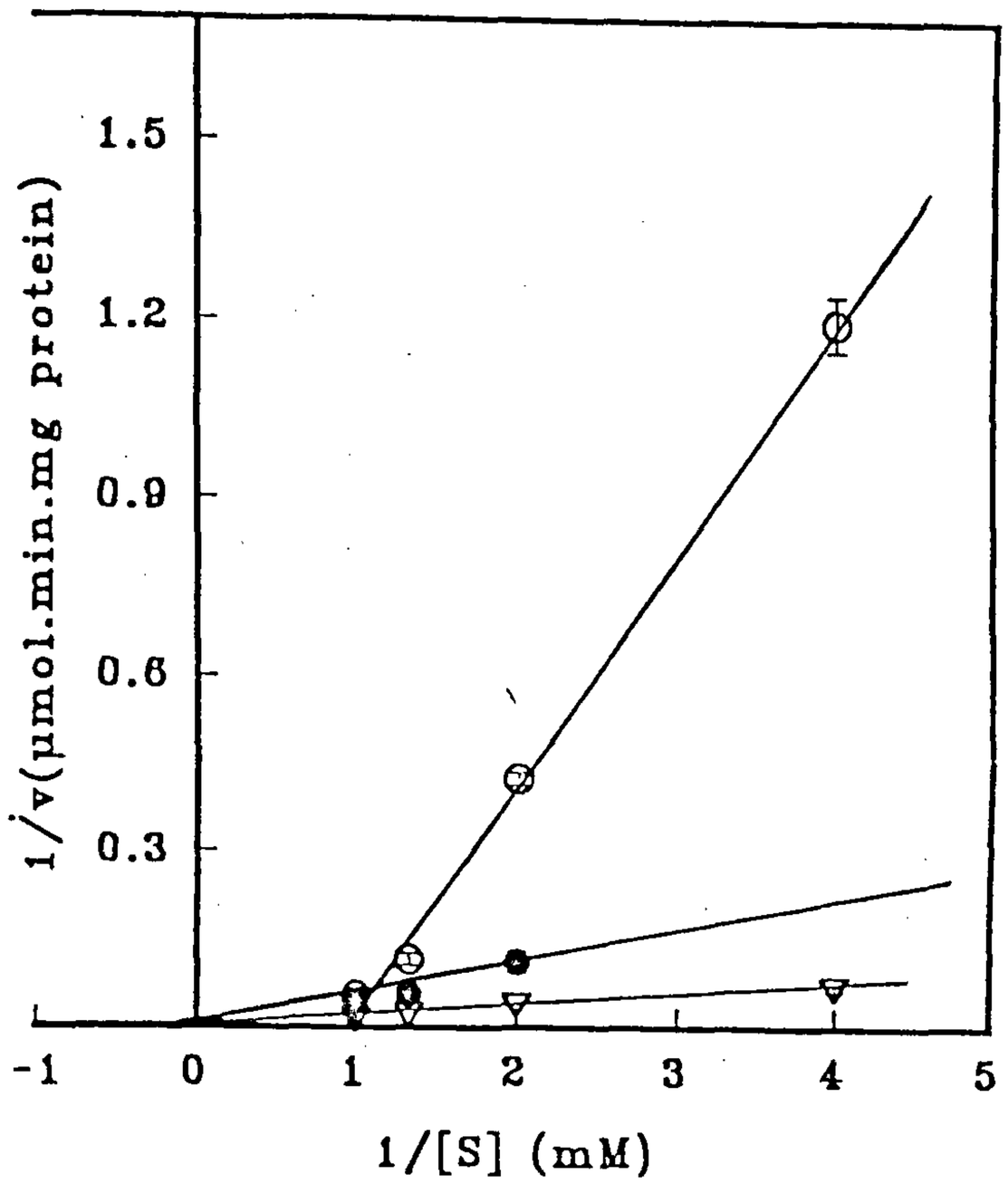


Figure 3. Double reciprocal plot for Zn uptake in strains sp7 (○), MS12 (●) and ZS2 (▽).

strains MS12 and ZS2 is suggestive of the reduced uptake of Zn^{2+} being the mechanism of resistance in the parent strain. The degree of sensitivity, qualitatively, was directly proportional to the amount of Zn intake. That uptake of the metal in *A. brasilense* sp7 did not involve its accumulation has been proved earlier[2]. Reduced uptake of a metal, as the mechanism of resistance, is known in several bacterial species, e.g. for Cd^{2+} in *Staphylococcus aureus*[7,13], *Bacillus subtilis*[14], for Cd^{2+} and Zn^{2+} in *Pseudomonas putida*[15], and for CrO_4^{2-} in *P. aeruginosa*[16]. The response of *A. brasilense* sp7 thus conforms to this general trend in several bacteria.

While the uptake stabilized within 10 min of Zn exposure in *A. brasilense* sp7, both the variant and the sensitive mutant exhibited influx till about 30–40 min of exposure. At the maximum levels of uptake, the fold accumulation of the metal by the strain sp7, MS12 and ZS2 was found to be 3.08, 4.60 and 6.90, respectively, given the intracellular volume of water as 2.2 µl/mg proteins for *A. brasilense* sp7[17].

Bacteria exhibiting obstructed or restrained uptake of the metal as the resistance mechanism show a low affinity for the metal. A consequent increased affinity in sensitive strains has been regularly observed[7,14]. The K_m and V_{max} values calculated from Lineweaver Burk plots for all the three strains in this study followed the same

Table 1. The affinity for Zn^{2+} of the three strains and rates of metal uptake in *A. brasilense*

Strain	K_m (mM)	V_{max} (µmol/min/mg protein)
A. brasilense sp7	–	–
MS12	10.0	3.3
ZS2	2.85	13.3

pattern (Table 1) as known in other systems. The sensitive strains followed the Michaelis–Menten saturation kinetics, while the Zn-resistant parent strain failed to exhibit the saturation, similar to the observation in *B. subtilis*[14,18]. The reciprocal plot constructed for the latter was, thus, unusual (Figure 3). We have concluded that *A. brasilense* sp7 exhibited a lack of affinity for Zn and this results in rendering the organism resistant. We assign this lack of affinity towards Zn^{2+} due to a typical response exhibited by sp7 cells in the presence of the metal. As reported by us earlier, the cells get enlarged, show an increased level of exopolysaccharides over control and release a melanin-like pigment[3].

Though the data on metal resistance in azospirilla are available, they are preliminary and sparse[19,20]. In this

investigation, efforts have been made to unravel the mechanism of resistance to Zn in this hitherto unexplored organism as a step towards effective biofertilizer application. This study also fulfils the need to understand the effect of arbitrary application of metal-containing pesticides on numerous non-target microorganisms. The efficacy of Zn^r *A. brasilense* when applied to maize seeds in *in vitro* studies has already been established in our laboratory[21].

1. Sawhney, V., Sheoran, I. S. and Singh, R., *Indian J. Exp. Biol.*, 1990, **28**, 883–886.
2. Mangala Gowri, P. and Srivastava, S., *Indian J. Exp. Biol.*, 1994, **32**, 626–628.
3. Mangala Gowri, P. and Srivastava, S., *World J. Microbiol. Biotechnol.*, 1996, **12**, in press.
4. Lopez, J. G. and Vela, G. R., *Nature*, 1981, **289**, 588–590.
5. Dimri, G. P. and Das, H. K., *Proc. Indian Natl. Sci. Acad.*, 1992, **B58**, 243–252.
6. Mangala Gowri, P. and Srivastava, S., submitted.
7. Tynecka, Z., Gos, Z. and Zajac, J., *J. Bacteriol.*, 1981, **147**, 305–312.
8. Bradford, M. M., *Anal. Biochem.*, 1976, **72**, 248–254.
9. Abelson, P. H. and Aldous, E., *J. Bacteriol.*, 1950, **60**, 401–413.
10. Nies, D. H. and Silver, S., *J. Bacteriol.*, 1989, **171**, 4073–4075.
11. Perry, R. D. and Silver, S., *J. Bacteriol.*, 1982, **150**, 973–976.
12. Laddaga, R. A. and Silver, S., *J. Bacteriol.*, 1985, **162**, 1100–1105.
13. Chopra, I., *J. Gen. Microbiol.*, 1971, **63**, 263–267.
14. Laddaga, R. A., Bessen, R. and Silver, S., *J. Bacteriol.*, 1985, **162**, 1106–1110.
15. Horitsu, H., Yamamoto, K., Wachi, S., Kawai, K. and Fakuchi, A., *J. Bacteriol.*, 1986, **165**, 334–335.
16. Cervantes, C. and Ohtake, H., *FEMS Microbiol. Lett.*, 1988, **56**, 173–176.
17. Riou, N. and Le Rudulier, *J. Gen. Microbiol.*, 1990, **136**, 1455–1461.
18. Burke, B. E. and Pfister, R. M., *Can J. Microbiol.*, 1986, **32**, 539–542.
19. Gallori, E., Casalone, E., Colella, C. M., Doly, S. and Polsinelli, M., *Res. Microbiol.*, 1991, **142**, 1005–1012.
20. Rai, R., *J. Agric. Sci. Camb.*, 1986, **106**, 279–285.
21. Mangala Gowri, P. and Srivastava, S., in *Environmental Biotechnology: Principles and Practice* (eds Moo-Young, M., Anderson, W. A. and Chakrabarty, A. M.), Kluwer, Dordrecht, 1995, pp. 135–143.

ACKNOWLEDGEMENTS. We thank Mr Rajiv Chawla, JTPA, UDSC, New Delhi, for help rendered in the preparation of this manuscript. PMG acknowledges the financial assistance from CSIR, New Delhi.

Received 8 February 1996; revised accepted 12 June 1996

Ammonia volatilization and denitrification losses from nitrogen fertilized flooded soil as affected by the addition of iron pyrites

D. Blaise*, A. Amberger and S. von Tucher

Institute of Plant Nutrition, TU-Munich-Weihenstephan, D-85350 Freising, Germany

*Central Institute for Cotton Research, GPO Bag 225, Nagpur 440 001, India

Results of a laboratory study indicated that iron pyrites reduced nitrous oxide (N_2O) and NH_3 evolution from flooded soil fertilized with KNO_3 or urea. Total gaseous ($N_2O + NH_3$) loss was reduced by 47.3% with the addition of pyrite compared to the urea alone treatment.

GASEOUS N loss through NH_3 volatilization and denitrification is responsible for the low N use efficiency of rice under flooded conditions[1]. Nitrification[2] and urease inhibitors[3] and slow-release fertilizers[4] are proposed as strategies to reduce N loss and enhance NUE. Nitrification inhibitors reduce losses through denitrification[5,6] but have been found to accentuate NH_3 loss[7]. Iron pyrites, a mineral occurring in plenty as sedimentary deposits in parts of Bihar, India, have been found to inhibit nitrification[8] and reduce NH_3 loss from surface-applied urea[9]. Since pyrites retard nitrification, it could possibly reduce denitrification. Hence, an incubation study was conducted in the laboratory to find out the effect of pyrite on denitrification and NH_3 volatilization from urea applied to a flooded soil.

Surface soil (0–15 cm) from cultivated fields of Duernast, Freising, Germany, having pH 6.8 (soil:$CaCl_2$ 1:2.5), organic C 1.12%, 4.1 mg NH_4^+–N and 15.6 mg NO_3^-–N kg^{-1} soil, total N 0.12% was used in the study. Air-dry soil (200 g) was placed in 500 ml jars. The soil was flooded to a depth of 2 cm and pre-incubated for two weeks. To enhance the reduced conditions, glucose solution equivalent to 500 mg C kg^{-1} soil was applied. The mineral-N status of soil after pre-incubation was 6.3 mg NH_4^+–N and 0.1 mg NO_3^-–N kg^{-1} soil. After the pre-incubation, the soils were treated with N at the rate of 100 mg kg^{-1} soil in the form of KNO_3 and urea solution, separately, with or without pyrite. Pyrites, obtained from Pyrites and Phosphates Chemicals Limited (PPCL) New Delhi, was added equivalent to 100 mg S kg^{-1} soil. Each treatment combination was replicated thrice. A no N control was also maintained. The jars were covered with a lid provided with a rubber septum. The sides of the lid and rubber septum were sealed with silicone to prevent gas leaks. After 1, 2, 3, 5 and 7 days, gas samples were taken and analysed for N_2O–N by gas chromatography (Varian 3400) using an electron capture