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Encapsulation as a response of *Azospirillum brasilense* sp7 to zinc stress

P. Mangala Gowri and S. Srivastava*

***Azospirillum brasilense* sp7 was exposed to 2 mM Zn^{2+} in minimal medium upon which the cells turned black and non-motile within 24 h. A streptomycin-resistant variant did not exhibit this phenomenon and is sensitized to zinc. A prelude to encystation was the elution of a melanin-like pigment into the medium.**

Key words: *Azospirillum brasilense* sp7, melanin-like pigment, streptomycin resistance, stress response, zinc.

Azospirillum brasilense sp7 is a Gram-negative, nitrogen-fixing bacterium found in association with grasses (Boddey & Dobereiner 1988). Because of its economic significance, studying its stress responses is important, as soil microflora are continuously being exposed to deleterious environmental conditions. Metal ions are a source of continuing soil contamination and can interfere with microbial metabolism and induce morphological and ultrastructural changes (Beveridge 1989). As a result, microflora have evolved to evade metal toxicity. We report here the phenomenon of 'melanization' in *A. brasilense* sp7 in response to zinc stress and its absence in a streptomycin resistant, zinc-sensitized variant. Ultrastructural changes are observed in the cells, and are accompanied by the formation of a melanin-like pigment in response to zinc stress.

Materials and Methods

Organism and Growth Conditions

A. brasilense sp7 (from Dr. K.V.B.R. Tilak of the Microbiology Division of the Indian Agricultural Research Institute, New Delhi) was grown at 30°C on minimal medium (MM) (Albrecht & Okon 1980) with sodium succinate and ammonium chloride as the carbon and nitrogen sources, respectively. $ZnSO_4.7H_2O$ was added at the stated concentrations. Complete medium (CM) used was Luria-Bertani (Atlas 1993) broth or agar (1.5% w/v). Liquid cultures were grown at 200 rev/min in Erlenmeyer flasks (250 ml capacity) in a volume of 50 ml.

Wild type *A. brasilense* cells are sensitive to streptomycin. Streptomycin resistance was induced incrementally. Cells grown overnight on CM were subcultured in CM and the antibiotic was added after 2 h. After a further growth of 2 h, the cells were spread on solid CM supplemented with 30 μg streptomycin/ml. Resistant colonies were selected on progressively higher concentrations of the antibiotic by further induction as before. A streptomycin resistant (Sm^r) variant MS12 was isolated on 200 μg of the antibiotic/ml on which it was subsequently maintained.

Inoculum for experiments on melanization was prepared by growing a loopful of cells in CM overnight, and subculturing in MM for a further 16 h. A 10% inoculum was used for experiments. The growth was monitored as total soluble protein which was extracted by disrupting 3 ml culture using an ultrasonic processor. BSA was used as the standard for estimation by the Bradford method.

Pigment Analysis

Tests for melanin were conducted on the culture supernatant, intact and broken (sonicated) blackened cells, for which protocols of Lingappa *et al.* (1963) and Ivins & Holmes (1980) were followed. Cells were disrupted as described above.

The culture supernatant was scanned in a Shimadzu UV260 recording spectrophotometer for absorption maxima in the wavelength range 200 to 700 nm. Distilled water or MM was used as the blank.

Ultrastructural Studies

Cells for transmission electron microscopy were prepared by the method described by Bhagat & Srivastava (1994). Both *A. brasilense* sp7 and the Sm^r variant (MS12) were grown without and with Zn^{2+} (2 mM). The cells were finally embedded in beam capsule with Araldite + accelerator (tridimethylaminomethyl phenol, DMP-30) and polymerized at 50°C for 24 h, followed by 60°C for 48 h. Ultrathin sections were cut with a microtome, stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope (CM-10 Phillips, Netherlands).

The authors are with the Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India; fax: 91 11 6886427, 91 11 6885270. * Corresponding author.

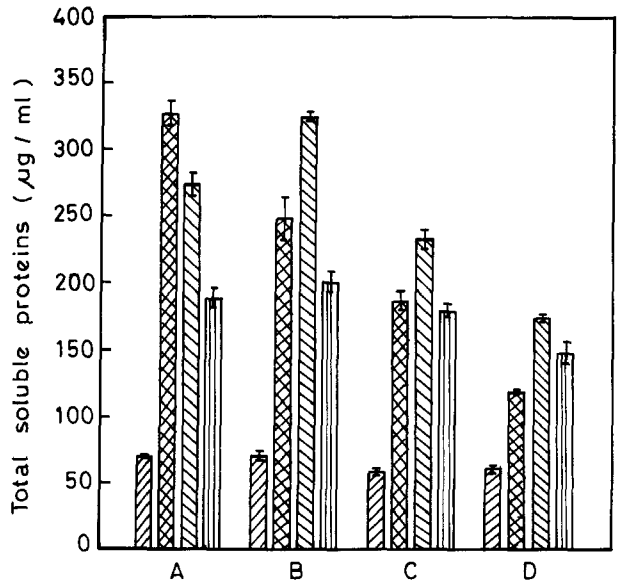


Figure 1. A comparison of growth (μg protein/ml) of *A. brasilense* sp7 and its variant MS12 on Zn^{2+} (2 mM) after ▨ 0 h, ▩ 8 h, ▩ 16 h and ▥ 24 h. (A) — strain sp7 on MM; (B) — strain sp7 on MM + Zn; (C) — variant MS12 on MM; and (D) MS12 on MM + Zn.

Table 1. Viability and synthesis of exopolysaccharides (EPS) by Sm^s and Sm^r *A. brasilense* sp7 in the presence of Zn^{2+}.

Strains	% Survival ± SEM	EPS (% w/v)*
A. brasilense sp7	16 ± 1.1	(+) 19
Variant MS12	3.3 ± 0.04	(−) 22

Experiments were performed in triplicate. *Control = Growth/EPS synthesis in the absence of Zn = 100%. 100% survival c.f.u./ml): *A. brasilense* sp7 — $4.66 \pm 3.55 \times 10^{10}$; Variant MS12 — $5.30 \pm 4.10 \times 10^{10}$. 100% EPS (mg/l): *A. brasilense* sp7—47.5 ± 2.5; Variant MS12 — 76.5 ± 2.5. (+) and (−) indicate increase and decrease in EPS amount with respect to the relevant controls.

Estimation of Exopolysaccharides

Exopolysaccharides were extracted by the protocol of Bitton & Freihofer (1978) and estimated by the anthrone method (Jayaraman 1981).

Results and Discussion

The resistance to Zn^{2+} in *A. brasilense* sp7 and its constitutive nature has been reported earlier (Mangala Gowri & Srivastava 1994). We further elucidate the response of the bacterium to Zn^{2+} in the medium. The cells, upon exposure to the metal in MM, turned black and became non-motile within 24 h of growth. Along with this feature, a change in the medium colour was visible after 8 h of inoculation, due to the elution of a brownish black pigment into the culture medium. The intensity of pigment colour was proportional to the Zn^{2+} concentration in the medium.

The colour of the pigment prompted the testing for melanin. Although melanin formation in *A. brasilense* sp7 has been observed by Sadasivan & Neyra (1987), tests conducted with the culture supernatant and the cells (both intact and sonicated) did not indicate true melanin to be present. While melanin solubilizes in hot alkali, the pigment in *A. brasilense* sp7 did not. Also, the absorption maximum of a typical bacterial melanin is in the wavelength range 400 to 600 nm, while in the case of *A. brasilense* sp7, it appeared in the range 300 to 400 nm. The water solubility of the pigment bore resemblance to 'pyomelanin' reported in *Pseudomonas aeruginosa* (Yabuuchi & Ohyama 1972) and a similar water-soluble diffusible pigment in *Vibrio cholerae* (Ivins & Holmes 1980). Thus the pigment in *A. brasilense* sp7, is best termed 'melanin-like'.

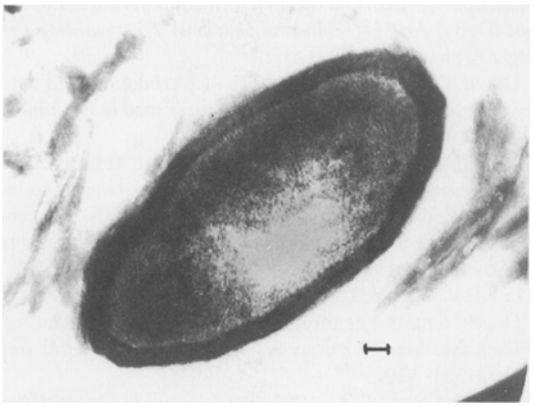
The cells, though lacking the characteristic motility by 24 h of growth, continued to grow in the presence of Zn^{2+} (Figure 1).

The role of exopolysaccharides (EPS) in metal chelation is well known. Floc-forming bacteria are good metal chelators (Brown & Lester 1982; Al-Shahwani *et al.* 1984; Geesey & Jang 1989). In *A. brasilense* sp7, the amount of EPS in the Zn^{2+}-grown culture increased over its control ($-Zn^{2+}$) (Table 1). Our earlier studies have shown that the cells do not accumulate significant amounts of Zn^{2+} in MM (Mangala Gowri & Srivastava 1994). The higher amount of EPS in *A. brasilense* sp7 in the presence of Zn^{2+} could thus be a protection against environmental stress, as reported in *Klebsiella aerogenes* (Bitton & Freihofer 1978; Al-Shahwani *et al.* 1984).

An increased amount of EPS, lack of motility and 'melanin-like' pigment formation indicated that the phenomenon observed could be a prelude to encystation. Ultrastructurally, the cells look enlarged, with a thicker outer layer when exposed to Zn^{2+} (Figure 2a, b). These observations gain support from the already existing literature on *A. brasilense* (Lamm & Neyra 1981; Sadasivan & Neyra 1987; Del Gallo *et al.* 1989). It also has been reported that an increased EPS synthesis (encapsulation) leads to encystation, both in *A. brasilense* and *Azotobacter* (Eklund *et al.* 1966; Berg *et al.* 1980; Sadasivan & Neyra 1985). These studies have identified ageing and nutritional stress as factors inducing encystation in azospirilla. In the present investigation, however, these characteristics were observed in actively growing cells within 24 h, as compared with incubation for 1 week (Sadasivan & Neyra 1987) and cannot be correlated with ageing.

The streptomycin-resistant variant (MS12) derived by incremental induction, exhibited a decreased tolerance to Zn^{2+}. The maximum tolerable concentration of Zn^{2+} in strain MS12 was 2 mM as compared with 10 mM in the strain sp7 (Mangala Gowri & Srivastava 1994). Black cells

(A)



(B)

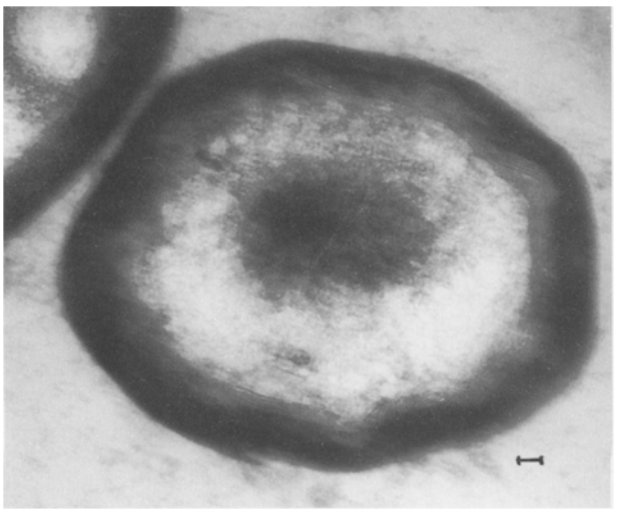


Figure 2. Transmission electron micrographs of *A. brasilense* sp7 cells. (A) control (grown on MM); (B) Zn exposed cells. Note the thickening of the cell covering in (B) and also the enlarged cell size. Marker Bar = 1 μm.

were not formed and no pigment was eluted. MS12 cells also showed reduced growth in the absence as well as presence of Zn^{2+} (Figure 1). Compared with the 16% survival of strain sp7 in the presence of Zn^{2+} in MM, the viability of the variant was drastically decreased (3.32% survival). There was also a reduced EPS production in the Sm^r strain in the presence of Zn^{2+} as compared with the $-Zn^{2+}$ condition (Table 1). Ultrastructurally, no apparent change was observed in MS12 cells in the presence of the metal.

The increased sensitivity of the variant MS12 to Zn^{2+} thus corresponded with the simultaneous loss of a typical morphological and ultrastructural response observed in the Sm^s sp7 strain. Resistance to streptomycin has been known to occur spontaneously within cereal roots and its importance seems to be multifold (Baldani & Dobereiner 1980) in this bacterium. Induced streptomycin resistance brings about several structural changes in the RNA and membrane (Foster 1983). The differential response to Zn^{2+} in this instance in MS12 could be explained on the basis of an altered physiological response brought about by streptomycin resistance, thus bringing in a negative cross-resistance with the metal.

Sadasivan & Neyra (1987) suggested that other triggering factors, besides ageing and nutritional stress, need to be identified for encystation. Our results suggest that the presence of a metal, such as Zn^{2+} in the medium provides one such factor that hastens the phenomenon that would eventually lead to encystation. Streptomycin resistance probably provides a selective advantage in penetration of root hair by azospirilla (Dobereiner & Baldani 1979) and Sm^r mutants have been reported to enhance growth and nitrogen fixation (Rai *et al.* 1984). Thus, while on the one hand, Sm-resistance seems to be providing a selective advantage to azospirilla, on the other, as observed during this investigation, the Sm^r strain lacks the advantage of encapsulation and Zn^{2+} tolerance. That this loss of a phenotypic trait did not result in any appreciable effect on its nitrogen-fixing ability, but rather led to an enhanced efficiency under certain conditions has also been determined in our laboratory (Mangala Gowri & Srivastava 1995).

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