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NITROGENASE DEREPRESSED MUTANTS IN *AZOSPIRILLUM*

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IT is not only with *Azospirillum* but with almost all diazotrophs that the presence of combined nitrogen represses the key enzyme nitrogenase with the result that even a genetically engineered strain of nitrogen fixer ceases to fix nitrogen[1,2]. In this communication the development of mutants in *Azospirillum* derepressed for nitrogenase is reported.

Following the enrichment technique of Day and Dobereiner[3], a large collection of *Azospirillum* was made from the root tissues of cereals, minor millets, fodder grasses and also from a few weed plants. The pure cultures of *Azospirillum* were maintained on yeast extract glucose agar stabs. For selecting the efficient cultures for nitrogen fixation, the cultures were screened for nitrogen fixation following the methods of Humphries[4]. Isolates that fixed more than 20 mg of nitrogen per g of carbon were selected and the isolates were assayed for acetylene reduction activity (ARA) in a Perkin Elmer Gas Chromatograph (Model F.33) fitted with flame ionization detector. The conditions of the assay were essentially the same as reported earlier[5].

The influence of combined nitrogen on the nitrogenase activity of the selected cultures was studied by incorporating graded levels of ammonium chloride in the nitrogen-free malate medium. It was of interest to observe that only one culture, TMV6#2, an isolate from the roots of gingelly crop showed nitrogenase activity even at higher levels of ammonium chloride in the growth medium. Therefore we designated this as a spontaneous mutant. In order to obtain a large number of mutants, the parent culture, Pt.1 was exposed to the chemical mutagen, methyl methane sulphonate (MMS) at 100 μg per ml. The procedures followed were the same as described by Clowes and Hayes[6].

The procedure adopted for the selection of the clones was based on the dye reduction technique[7]. This involved the streaking of the clones on indicator media. The mutant clones would be distinctly abnormal in colony colour on the indicator media. Malate medium containing 20 mM of ammonium chloride added separately with (i) bromothymol blue and bromothymol purple, (ii) eosine-methylene blue, and (iii) triphenyl tetrazolium chloride served as indicator media in this study. The clones that exhibited aberrant colony colour were picked up and purified by repeated streaking on the same media and maintained on yeast extract glucose agar stabs.

The ARA of the mutants along with the parent culture was examined as detailed earlier. The assay

Table 1 *Effect of NH_4^+ and NO_3^- on the ARA* activity of the mutants of Azospirillum*

Mutants	Acetylene reduction activity: Under N_2	+NH_4Cl (20 mM)	Per cent reduction	+KNO_3 (20 mM)	Per cent reduction
Pt.1	190.96	Nil	100.00	Nil	100.00
AZ.MMS#2	166.98	68.60	58.92	98.00	41.31
AZ.MMS#3	180.32	74.28	58.81	102.39	43.22
AZ.MMS#4	146.47	39.26	73.20	84.71	42.16
TMV.6#2	176.78	56.66	67.94	158.12	10.56

Data represent mean of three determinations; * Activity expressed as nmol of ethylene produced hr^{-1} mg^{-1} protein.

was performed in medium containing 20 mM of ammonium chloride. Finally, the differences if any, on the ammonia excretion pattern were also determined for the mutants following the Conway microdiffusion assay technique[8].

The results indicate that all the isolates chosen for the study recorded reasonably high levels of nitrogenase activity in the range of 82 to 194 nmol of ethylene/hr/mg protein. The culture, Sp.7 from Brazil recorded lower ARA than the local isolates. At all concentrations of ammonium chloride in the growth medium there was no ARA detectable. This is obviously due to repression by ammonium ions. However, the spontaneous mutant, TMV6#2 is unique in that it did not suffer from repression of nitrogenase activity even at 20 mM of ammonium chloride in the medium. It has an added advantage of being pink in colour.

All the mutant clones selected by the dye reduction method exhibited nitrogenase activity in the presence of ammonium chloride and potassium nitrate (table 1) which was much lower than it was under elemental nitrogen. In this respect the mutant, AZ.MMS-3 did register high ARA.

All the mutants including the parent culture excreted ammonia in the culture medium during nitrogen fixation. The mutant, AZ.MMS-3 recorded the maximum amount of ammonia excretion. The wild strain, Pt.1 also excreted ammonia but only low amounts (figure 1). It has been suggested that repression of nitrogenase sets in due to the presence of excess of ammonium ions in the medium which deenergises the cytoplasmic membrane[9]. However, Shanmugam *et al*[10] opined that in the mutants there might be genetic alterations in the ammonia assimilatory enzyme, glutamine synthetase (GS). The derepressed mutants of *Klebsiella pneumonia* strain S.K.25 was found to excrete high amounts of ammonia in the culture medium[11]. This is believed to enhance derepression of nitrogenase. It is presumed that when the cell-pool ammonia is maintained low due to excretion, the cell continues to fix nitrogen and hence there is no repression. In the present study the spontaneous mutant, TMV6#2 and the mutant, AZ.MM-3 excreted large quantities of ammonia. The stability of the mutants was checked for fifteen generations and the mutants were quite stable.

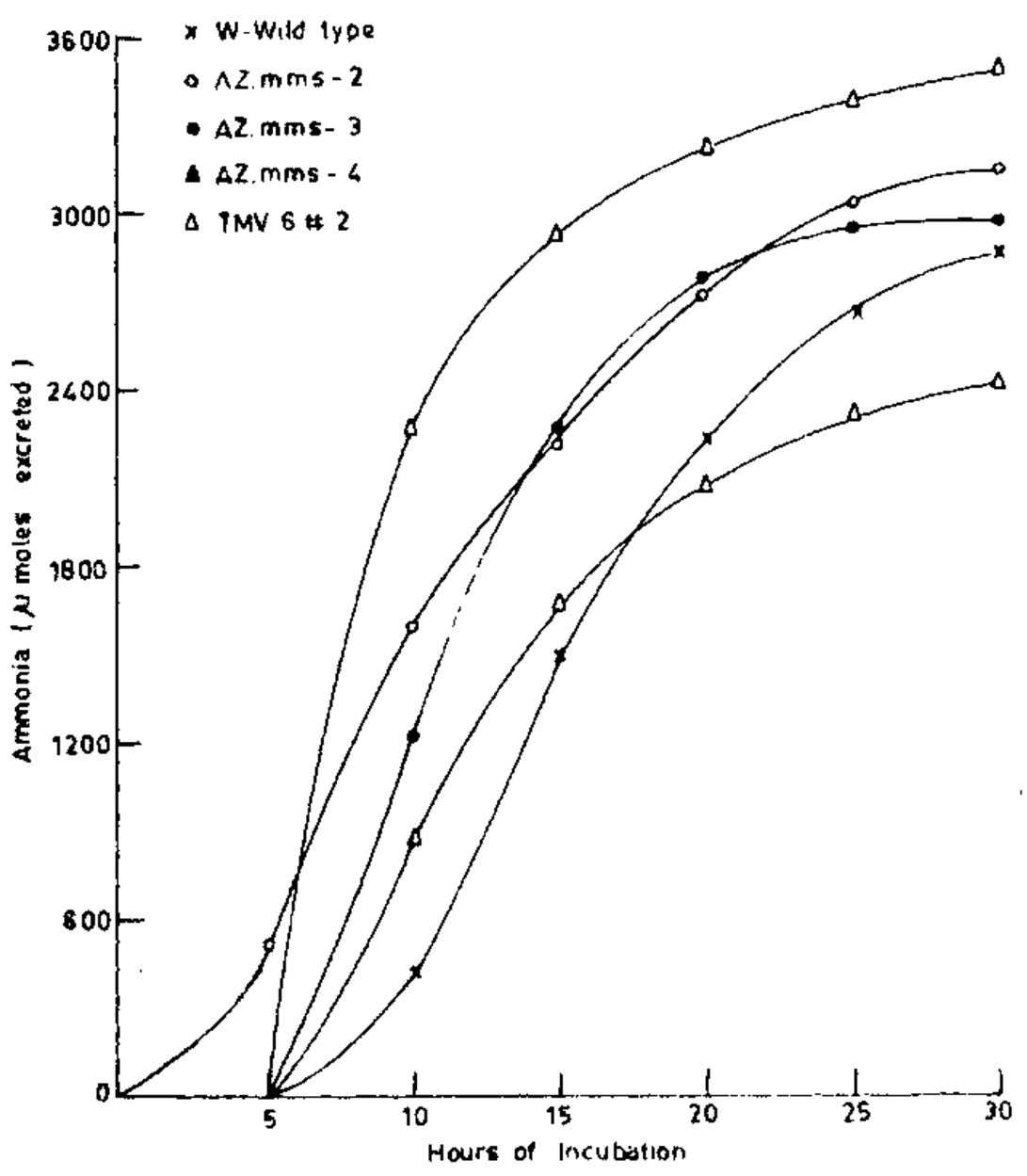

Figure. 1. Excretion of ammonia by the mutants of *Azospirillum* in the medium.

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ULCERATIVE FORM OF *AEROMONAS HYDROPHILA* INFECTION OF *CATLA CATLA*

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AEROMONADS are considered important fish pathogens. Of these, *Aeromonas salmonicida* is best defined and its role in pathogenicity is well known. Shotts *et al*[1] have shown that *A. hydrophila* and *A. shigelloides* are associated with mortality among fish, turtles and alligators. Aquarium tropical fishes are also susceptible to *Aeromonas* infection and this was studied by Richard *et al*[2] who isolated 15 strains of *A. hydrophila* after several deaths occurred in two aquaria. Infectious dropsy, a condition where there is an abnormal accumulation of fluid in the whole body or localized in some organs of European carps causing severe epidemics in temperate areas, is well recorded[3,4] but Gopalkrishnan[5] was the first to observe the disease in Indian carps and found *Catla catla* to be the most susceptible followed by *Cirrhina mrigala* and *Labeo rohita* in that order. He recorded instances of entire populations getting wiped out by the epidemics in many stocking tanks in West Bengal. He observed abnormal and rounded bulging of the belly with grey fluid accumulation, exophthalmos and in terminal stages, septicaemia.

The ulcerative form of *A. hydrophila* infection, which is a milder form appears to be not frequently encountered in Indian major carps. We observed in *C. catla*, this form of infection wherein dropsy was not an accompanying feature and the predominant signs were white cutaneous lesions on the snout, loose scales at the base of the fins and mild disintegration of the fin margins (figure 1).

The infected fish which was observed in the College pond was brought to the laboratory for studying the etiology of the condition. Sterile cotton swabs were used for collecting material from the superficial regions. This was done by gently rubbing

Figure 1. Infected *Catla catla* with lesions in the snout and pectoral fins.

the swabs on lesions over the snout, gills, branchial region, margin of pectoral, pelvic and tail fins. Some loose scales were picked up using sterile forceps.

Primary inoculations were made on blood agar containing 5% rabbit blood in nutrient agar and MacConkey's agar. The scales were gently embedded on the agar surface. The plates were then incubated at room temperature (29°C ± 2°C) for 24 hr and incubation continued for 48 hr in plates where colonies had not developed well. All cultures from lesions were predominated by large typical, β-haemolytic colonies. These were further purified, put on nutrient agar slants and identified as follows[6].

Gram staining and hanging drop were done and after confirming them as gram negative bacilli exhibiting active motility, other biochemical tests such as oxidase test, O/F test on Hugh's and Leifson's medium, carbohydrate fermentation test, ability to produce indole, MR–VP test, starch and gelatin hydrolysis and ability to produce H_2S from cysteine were performed. Acid and gas production was recorded for glucose whereas other carbohydrates such as arabinose, cellobiose, dulcitol, galactose, lactose, maltose, mannitol, rhamnose, sucrose, xylose, sorbitol and salicin were observed merely for acid production. Table 1 shows the reactions of the isolates.

All the isolates were resistant to the pteridine compound 2-4 diamino 6-7 diisopropyl pteridine