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Reversibility of oxygen induced inactivation of nitrogenase in some enterobacteria

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Aerobic and microaerobic diazotrophs possess numerous oxygen restriction strategies to protect nitrogenase from inactivation by oxygen without interfering with energy generation through oxidative phosphorylation. Protection by conformational change in nitrogenase was first detected and described in *Azotobacter*. This strategy once considerd unique for *Azotobacter* has been shown in this study to occur in *Citrobacter freundii* (Braak) Werkman and Gillen and *Klebsiella pneumoniae* subspecies *rhinoscleromatis* (Trevisan) Migula also. However, in these enteric bacteria the entire enzyme is not protected probably due to the absence of any respiratory protection similar to that found in the aerobe, *Azotobacter*.

Nitrogenase (E.C.1.18.1.6), which catalyses the reduction of dinitrogen to ammonia is extremely sensitive to oxygen and exposure to oxygen invariably causes irreversible destruction of the enzyme. But in all aerobic and microaerobic diazotrophs the required energy for metabolic activities is derived through oxidative phosphorylation and therefore these diazotrophs cannot totally exclude oxygen from their metabolic systems during growth. Hence, these organisms have evolved numerous strategies to protect nitrogenase[1]. It is likely that in many of these bacteria, more than one strategy will be operational at any time for protection of nitrogenase and for maintenance of the ATP flux through oxidative phosphorylation for dinitrogen fixation. A number of reviews also have appeared on oxygen restriction mechanisms in diazotrophs [2-4].

In one of the strategies first reported for *Azotobactor chroococcum*[5] and later proposed for a species of *Klebsiella*[6] and the microaerobe *Xanthobacter*[7] exposure to oxygen results in the nitrogenase undergoing a conformational change where the enzyme remains structurally stable but catalytically inactive. When the oxygen stress is removed this enzyme reverts back to its active state. Such a mechanism has not been reported for any other diazotroph till now. This communication presents data to show revival of nitrogenase activity in *Citrobacter freundii* and *Klebsiella pneumoniae* subspecies *rhinoscleromatis* in response to reintroduction of anaerobiosis after exposure to high oxygen flux.

Citrobacter freundii, a naturally occurring diazotrophic strain of *Escherichia coli*, *Klebsiella pneumoniae* subspecies *rhinoscleromatis*, *Edwardsiella tarda* and *Enterobacter agglomerans* anaerogenic biogroup 1 used in this study were originally isolated and characterized at this laboratory by Balram *et al*[8]. Nitrogen-free medium of Raju *et al*[9]. with slight modifications was used in the study. Growth was measured as turbidity at 540 nm. Nitrogenase activity was determined by the acetylene reduction assay technique[10]. Protein was estimated by the method of Lowry *et al*[11].

The organisms were grown as normal nitrogen fixing 250 ml volume cultures under an atmosphere of dinitrogen in 1 litre Erlenmeyer flasks with provision for continuous sparging with gases and facility for collecting samples without disturbing the setup. Turbidity and nitrogenase activity were determined at 22 hr after initiation of cultures. At 24 hr, after collecting samples for turbidity and nitrogenase measurements, the cultures were first sparged with sterile oxygen for 10 min at the rate of 25 ml min^{-1} and then with sterile dinitrogen (30 ml min^{-1}) for 15 min to remove all oxygen from cultures. Tetracycline found suitable to inhibit synthesis of fresh nitrogenase protein (Postgate–personal communication) was added to all cultures at a final concentration of 50 mg/l and then the cultures were maintained under an atmosphere of dinitrogen. Turbidity and nitrogenase were measured in the cultures immediately after the treatment (i.e. at 24.5 hr), then at 26 hr and further at 2 hr intervals for a period of 10 hr.

All the test diazotrophs used in this study showed good growth at 24 hr (Fig. 1). *E. agglomerans* anaerogenic biogroup 1 showed the highest turbidity followed by *E. coli*, *E. tarda*, *C. freundii* and *K. pneumoniae* subspecies *rhinoscleromatis*. All these

diazotrophs also possessed nitrogenase activity before sparging the cultures with oxygen (Table 1). The specific activity was highest in *E.agglomerans* anaerogenic biogroup 1 at 24 hr (97 n moles C_2H_4 mg protein^{-1} hr^{-1}), *C. freundii* had the lowest activity of 11 n moles C_2H_4 mg protein^{-1} hr^{-1}.

Exposure to oxygen even for a short period of 10 min produced different effects on nitrogenases from the different diazotrophs studied here. The immediate and common effect was a drastic reduction in nitrogenase activity as compared to the activities in the respective 24 hr old cultures before oxygen treatement (Table 1). Reintroduction of anaerobiosis in cultures of *E.tarda. E.coli* and *E.agglomerans* anaerogenic biogroup1 did not activate the nitrogenase even after 10 hr of removing oxygen from cultures. Infact the low activity observed in the cultures immediately on removal of the high pO_2 flux declined rapidly and in about 6 to 8 hr the entire activity vanished. This indicates the absence of protective measures in these bacteria resulting in total inactivation of nitrogenase activity by oxygen. In other words the inhibition of nitrogenase in these microbes is irreversible.

C.freundii and *K.pneumoniae* subspecies *rhinoscleromatis* showed recovery of nitrogenase activity with in 2 hr of removal of oxygen (Table 1) and measurable amounts of nitrogenase activity were observed throughout the duration of the period of further study. In *C.freundii* the revived activity was only about 50% of the activity observed prior to oxygen treatment. This augmented activity was retained till the 10th hr. In *K.pneumoniae* subspecies *rhinoscleromatis* the increase in activity in the first 2 hr itself was about twice that found immediately on removal of oxygen. Within about 8 hr the activity showed more than three fold increase. Though the activity remained slightly lower thereafter, there were no indications of any sharp fall even after 12 hr of oxygen removal (Table 1). Since synthesis of new enzyme protein is not possible in presence of tetracycline, the nitrogenase activity observed in these two diazotrophs on reversion to anaerobic conditions could only due to the revival of the enzyme which was inhibited by oxygen earlier. In other words suppression of the enzyme activity in these microbes is reversible. When the data were analysed statistically highly significant difference across the time point was noted for all the five test organisms (ANOVA) and the mean difference was significant at 0.05 level. Taking 22 hr nitrogenase activity as control the activity decreased considerably after oxygen treatment from 24.5 to 36 hr for *E.coli, E.tarda* and *E.agglomerans* anaerogenic biogroup 1 while moderate decrease in activity was noted in *C.freundii* and *K.pneumoniae* subspecies *rhinoscleromatis* by Dunnett multiple comparisons test.

A "switch off–switch on" mechanism of nitrogenase activity has been suggested for *Azotobacter*

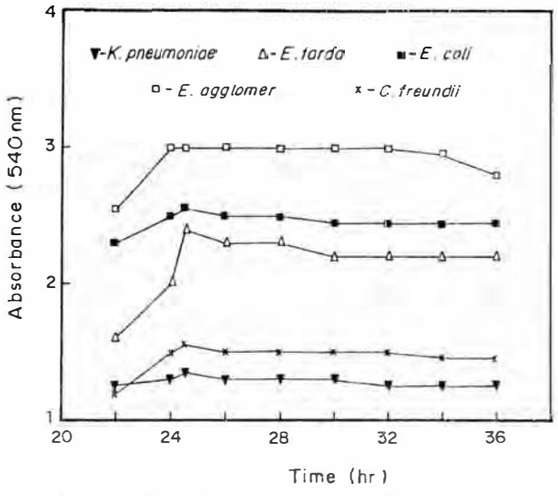

Fig. 1—Effect of oxygen on growth of test organism

Table 1—Effect of oxygen on nitrogenase activity (n moles ethylene produced mg protein^{-1} min^{-1}

Organism	Age(hr)										
	22	24	Oxygen sparging for 10 min	Nitrogen sparging for 15 min	24.5	26	28	30	32	34	36
Edwardsiella tarda	30	47			3	3	—	—	—	—	—
Escherichia coli	20	39			2	3	2	—	—	—	—
Citrobacter freundii	6	11			4	4	5	5	5	5	3
Klebsiella pneumoniae	23	28			4	7	9	10	12	9	10
Enterobacter agglomerans	94	97			2	2	2	2	2	—	—

The results are the mean value of three replications and subjected one way ANOVA and Dunnett multiple comparisons test

chroococcum[12] during exposure to and subsequent isolation from high oxygen flux. *Azotobacter* being an aerobic diazotroph has the highly potent oxygen restriction mechanism of enhanced respiratory activity. This enables this bacterium to effect conformational alterations in the nitrogenase molecules in such a way that the entire enzyme system reverts back to full N_2 fixing potential immediately on removal of the excess pO_2 from the vicinity of the N_2 fixation sites. However, the two enterobacteria which exhibit revival of nitrogenase activity fix dinitrogen only under anaerobic or microaerobic conditions and hence there is no mechanism for respiratory protection of nitrogenase. This results in a portion of the existing nitrogenase system getting irreversibly damaged before the conformational alterations are effected. This appears to be the reason for the occurrence of low nitrogenase activity when anaerobiosis is restored. Again in absence of synthesis of new enzyme due to tetracycline, the nitrogenase activity observed could only be due to revival of the enzyme. Such a reversible conversion of nitrogenase into a protected inactive state has also been reported in *A. vinelandii*.[13]. Taken together, the present results allow us to draw the conclusion that the activity of nitrogenase recorded after the removal of oxygen stress is only due to the reversibility of nitrogenase activity, which was in a protected inactive state during oxygen stress. This is a first report of the occurrence of such reversibility of nitrogenase in these enterobacteria.

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