Differential Synthesis of Alkaline Phosphatase in *Rhizobium* Species Isolated from the Tropics

Akhand P. Singh * and J. B. Singh

Laboratory of Bioenergetics, Department of Botany, Banaras Hindu University, Varanasi, 221005, India

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A total of 28 strains of several species of *Rhizobium* were screened for the presence and regulation of the enzyme alkaline phosphatase. In contrast to all other species of *Rhizobium*, the activity of this enzyme was absent in *R. trifolii*. Different levels of alkaline phosphatase were observed in various species of *Rhizobium*. Both, species as well as strain specific differences were found in the level of alkaline phosphatase.

Introduction

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1), which is encoded by the *pho* A gene in *Escherichia coli*, is a periplasmic enzyme, the synthesis of which is induced when the cell is starved of phosphate [1–3]. Transcription of *pho* A is controlled in a complex manner by three positive regulator genes (*pho* B, *pho M* and *pho R*), one of which (*pho R*) acts as a negative regulator [4–7]. In addition to these, the synthesis of alkaline phosphatase in *E. coli* is also controlle by *pho S*, *pho T* and *pst A* gene products, which are involved in the regulation of the transport of inorganic phosphate [8, 9].

Besides *E. coli*, the activity of this enzyme has also demonstrated in other bacterial species such as *Bacillus subtilis* and other *Bacillus* species, *Serratia marcescens*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, *Staphylococcus aureus* [10], *Mycobacterium smegmatis* [11], *Bacteroides ruminicola* [12], and *Rhizobium leguminosarum* [13].

The only species of *Rhizobium* in which the presence of alkaline phosphatase enzyme has been reported before is *R. leguminosarum* [13]. In this paper, we have examined the occurrence and regulation of this enzyme in various field isolates of *Rhizobium* spp. from the tropics (Varanasi, India). For comparison, the standard species of *Rhizobium* were also studied here.

Reprint requests to Dr. Akhand P. Singh, Department of Biological Sciences, Brock University, St. Catharines, Ontario, L2S 3A1, Canada.

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Materials and Methods

Bacterial strains

A total of 28 strains (Table I) were used in this study. The species represented were: *Rhizobium leguminosarum*, *R. meliloti*, *R. phaseoli*, *R. trifolii*, *R. japonicum*, and *R. lupini*. In addition to these, 22 *Rhizobium* spp. isolated from the localities situated on the campus of Banaras Hindu University, which is about 5 kilometers south of Varanasi City (25°, 18′ north latitude and 83°, 1′ east longitude), were also employed in this investigation. The Varanasi city is situated on the upper gangetic plains toward the west bank of the river Ganges. It is approximately 79.2 meters above the sea level. The soils of the test localities are sandy loams consisting of coarse sand (0.92%), fine sand (69.52%), silt (15.2%) and clay (12.80%) and have a pH between 6.8 to 7.8 [14, 15].

Chemicals

The chemicals were of the highest purity available commercially, and were not further purified. Tris-base (Trizma-base), ethylenediaminetetraacetic acid (EDTA), chloramphenicol, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co., St. Louis, Mo., USA.

Media

The following media were used:

a) *High phosphate:* This medium contained: 50 mM Tris-HCl, 2.64 mM KH_2PO_4, 8 mM K_2HPO_4, 3.4 mM NaCl, 0.8 mM $MgSO_4$. $7H_2O$, 0.14 mM $CaCl_2 \cdot 2H_2O$, and 0.25% (w/v) yeast extract (Difco).

b) *Low phosphate:* The composition of this medium was the same as the high phosphate except

that K_2HPO_4 was omitted and the amount of KH_2PO_4 was 0.1 mM.

Glucose (0.25%, w/v) was used as the carbon source in both media (pH adjusted to 7.3).

Isolation of rhizobia

The *Rhizobium* spp., listed in Table I, were isolated from the healthy root nodules of their respective host plants by the method of Vincent [16]. The isolated strains were quite stable over a period of four years or more, and were maintained on mannitol yeast extract agar [16] at 4 °C and transferred routinely to fresh slants at two month intervals.

Growth of cells

Cells were grown in 20 ml of various media in 100 ml Erlenmeyer flasks fitted with a sidearm tube. 0.4% (v/v) of a log phase culture grown in the same medium, was used as inoculum. In all the cases, culture flasks were incubated at 28 °C under aerobic conditions (180 rev/min) in a thermostated waterbath shaker (Adco, India). Growth was recorded as absorbance (650 nm) against a medium blank with a Photochem colorimeter (MK II, India).

Assay of alkaline phosphatase

Since phosphate esters are usually poorly transported into bacterial cells [17], some indication of the location of the phosphatase could be achieved by comparing the levels of enzyme activity of toluene-treated and untreated derepressed cells. Bacteria made permeable with toluene had nearly identical activities of alkaline phosphatase to untreated bacteria, suggesting that the enzyme was accessible to the substrate and presumably, therefore, external to the cytoplasmic membrane [13, 18, 19].

Cultures grown previously in either high phosphate or low phosphate media were harvested at the end of log phase growth, and the cells were washed twice with 0.05 M Tris-HCl buffer by centrifugation at $10000 \times g$ for 10 min. The cells were resuspended to the desired cell density in the same buffer but containing 50 µg/ml chloramphenicol. The cells were toluenized and alkaline phosphatase activity [20] and protein content [21] determined.

One unit of enzyme activity is defined as that amount which catalyzes the hydrolysis of 1 nmol of substrate/min. The specific activity is defined as units/mg of protein.

Results and Discussion

The specific growth rate constant (K) for various species of *Rhizobium* in high and low phosphate media is given in Table I. Analysis of these data by a paired t-test shows that the growth rate in low phosphate medium is significantly lower than in the high phosphate medium ($P < 0.01$), presumably due to the limitation of inorganic phosphate in the former medium. The data for alkaline phosphatase levels under above conditions are also given in Table I. Analysis of these data showed that there was a direct correlation between the alkaline phosphatase (APase) levels of the various strains, with exception to *R. trifolii*, and their growth rates in the low phosphate medium ($P < 0.01$). As reported previously [20], the activity of APase was absent in *R. trifolii*. The lack of this enzyme in this strain was not due to the presence of a low molecular weight endogenous inhibitor. This possibility was ruled out on the grounds that the cell free extract of *R. trifolii*, when added to the cell free extract of APase containing strains of *Rhizobium*, did not inhibit the activity of this enzyme in the latter strains. In contrast to the fast-growing species, the slow growing species of *Rhizobium* had lower levels of this enzyme (Table I), although no differences were observed in their ability to accumulate inorganic phosphate from the growth media [22].

Different levels of this enzyme were noticed in different species of *Rhizobium*. For example, a phosphate repressible enzyme synthesis occurred in *R. japonicum* and *Rhizobium* spp. RCJ 1, RCM 1, RCT 1, RLS 1, and RPS 1; whereas the synthesis of APase seems to have been constitutive in strains, RAP 1, RSA 1, and RSG 1 (Table I). In some strains, the synthesis of this enzyme was semiconstitutive. Like *Escherichia coli* B and K-12 strains, the basal level (activity in high phosphate medium) of this enzyme was also different in various species of *Rhizobium* (Table I). Consistent with the findings on *E. coli* B and K-12 [23], differences in the genetic backgrounds of above species of *Rhizobium* may affect the basal level of APase. In addition to the species specific variation, strain specific variations were also observed in the levels of APase (Table I). A similar strain specific variation in rhizobia has also been noticed in the activities of invertase and β-galactosidase [24], and in the utilization of inorganic phosphate [25].

Table I. Specific growth rates (K), and alkaline phosphatase (APase) activity in different species of *Rhizobium* cultured in high and low phosphate media, respectively.

Rhizobium species and their host plants	Source or localities	Specific growth rate (K) in		APase specific activity in	
		High phosphate	Low phosphate	High phosphate	Low phosphate
R. leguminosarum KL	1	0.180	0.158	2.90	11.76
R. meliloti KM	1	0.160	0.145	3.10	13.37
R. phaseoli AHU 1133	1	0.185	0.158	2.00	11.42
R. trifolii 4S	1	0.090	0.080	0.00	0.00
R. japonicum AHU 1130	1	0.120	0.093	0.00	6.00
R. lupini AHU 1132	1	0.120	0.093	2.85	12.00
Rhizobium sp. RAP 1 (for *Abrus precatorius*, Linn.)	2	0.245	0.220	74.00	85.00
Rhizobium sp., RAH 1 (for *Arachis hypogea*, Linn.)	3	0.287	0.271	24.00	112.60
Rhizobium sp., RBF 1 (for *Butea frondosa*, Roxb.)	4	0.193	0.178	13.00	36.80
Rhizobium sp., RCJ 1 (for *Crotalaria juncea*, Linn.)	1	0.185	0.162	16.70	20.30
Rhizobium sp., RCM 1 (for *Crotalaria medicaginea*, Lamk.)	5	0.133	0.128	0.00	15.55
Rhizobium sp., RCT 1 (for *Clitoria ternatea*, Linn.)	2	0.215	0.187	0.00	30.00
Rhizobium sp., RCC 1 (for *Cajnus cajan*, Spreng.)	6	0.280	0.260	8.20	18.00
Rhizobium sp., RCA 1 (for *Cicer arietinum*, Linn.)	3	0.173	0.142	8.00	32.70
Rhizobium sp., RDS 1 (for *Delbergia sisso*, Roxb.)	2	0.110	0.094	5.00	15.00
Rhizobium sp., RDL 1 (for *Dolichos lablab*, Linn.)	2	0.231	0.199	12.40	50.00
Rhizobium sp., RLS 1 (for *Lathyrus sativus*, Linn.)	3	0.180	0.165	0.00	24.50
Rhizobium sp., RLE 1 (for *Lens esculenta*, Linn.)	3	0.215	0.185	11.50	35.00
Rhizobium sp., RPS 1 (for *Pisum sativum*, Linn.)	2	0.165	0.157	0.00	30.20
Rhizobium sp., RMS 1 (for *Medicago sativa*, Linn.)	3	0.178	0.165	11.50	70.00
Rhizobium sp., RPL 1 (for *Phaseolus lunatus*, Linn.)	2	0.188	0.159	9.50	40.00
Rhizobium sp., RPM 1 (for *Phaseolus mungo*, Linn.)	2	0.188	0.160	4.40	42.00
Rhizobium sp., RPR 1 (for *Phaseolus radiatus*, Linn.)	2	0.175	0.160	3.20	46.80
Rhizobium sp., RSA 1 (for *Sesbania aegyptiaca*, Pers.)	7	0.200	0.190	44.50	78.50
Rhizobium sp., RSG 1 (for *Sesbania grandiflora*, Pers.)	7	0.241	0.210	43.30	76.50
Rhizobium sp., RTF 1 (for *Trigonella foenum*, Graceum, Linn.)	2	0.190	0.180	9.86	18.86
Rhizobium sp., RTP 1 (for *Tephrosia purpuria*, Linn.)	7	0.179	0.165	10.00	36.50
Rhizobium sp., RVC 1 (for *Vigna catiang*, Walp.)	2	0.213	0.198	7.80	38.20

Source and localities: 1, Department of Biology, Kagoshima University, Japan; 2, BHU Botanical Garden; 3, BHU Agricultural Farm; 4, Varuna River Bank; 5, Road side of BHU Campus; 6, Broacha Hostel Field; 7, BHU Aurvedic Garden.

The field isolates of various *Rhizobium* spp., reported here showed higher levels of phosphate irrepressible APase activity compared to the standard strains (Table I). At present, the reason for this difference is not clear. It is possible that due to high contents of phosphate (0.1 to 10 mM) in the root nodules [26], the efficient strains may have evolved *in vivo*, with the capacity of producing higher levels of APase through the process of natural selection. In this regard, it is interesting to note that the synthesis of this enzyme is also found to be constitutive in rumen bacteria [12], further indicating that some kind of selection does exist in nature.

The completely derepressed levels of APase in symbiotic *Rhizobium* spp. and in rumen bacteria [12] are always lower than those of the free-living *E. coli* [1–3]. The invariably low levels of enzyme in *Rhizobium* spp. could be correlated with the availability of high phosphate levels in the root nodules as discussed above, compared to relatively low amounts of phosphate present in the habitate of free-living bacteria. It has been reported [26, 27] that in phosphate depleted environment, APase plays a vital role in the phosphate nutrition of microbes in general and rhizobia in particular. Rhizobia as bacteroids in the root nodules (where phosphate is freely available) do not encounter phosphate starvation, compared to free-living bacteria, and hence synthesize low levels of APase. Free-living bacteria exhibit high levels of APase under conditions of phosphate starvation. The present observations for species of *Rhizobium* other than those of *R. trifolii* are also analogous to that of low APase activity in rumen bacteria *in situ* [12].

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