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Some Characters of Chickpea-Nodulating Rhizobia Native to Thal Soil

Shahida Nasreen Khokhar, Muzaffer A. Khan and [1]Mohammad Fayyaz Chaudhri
Applied Microbiology, National Agricultural Research Center, Park Rd. Islamabad, Pakistan
[1] Department of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan

Abstract: Fifteen selected isolates of chickpea-root nodules, from Thal area, having dry loamy sand, were studied for their cultural, biochemical and physiological characters of ecological & agricultural significance. TAL 1148 was taken as reference strain. All the characters studied varied among the isolates. Colony size varied from 1mm to 4mm, while texture from dry gummy to watery/slimy. Ten isolates had mean doubling time (MDT) of 4hrs or less, while four had MDT between 7 and 10 hrs., with no correlation to acid or alkali production. Four of the isolates were thermo-tolerant, while six were found to be highly resistant to draught, having osmotic potential between −22.2 and −31.02 bars. High resistance (up to 800 ug/ml) to Streptomycin was recorded in isolates from Kalurkot. All the isolates differed in their symbiotic effectiveness as well as in relative cell surface charge in terms of methylene blue cation adsorption. Four of the isolates were found capable of solubilizing phosphate *In vitro*. The isolates differed in their total cell protein profiles. Most of the isolates shared proteins with TAL 1148 in the range of 25 to 45 KD, but differed in proteins above 45 KD. The work reports some unusual characters of rhizobia, which may be exploited in the laboratory as well as in the field.

Key words: Rhizobia, chickpea, TAL 1148, protein profile, Thal soil

Introduction

Chickpea, the largest grown grain-legume in Pakistan has been reported to respond variably to inoculation (Aslam *et al.*, 2000; Rupela & Beck, 1990). Inconsistency in response has sometimes been attributed to the variation in bacterial number and highly competitive though ineffective native population of rhizobia (Keatinge *et al.*, 1995). They may possess extremely novel characters in addition to their symbiotic characters, like lignolytic and cellulytic ability (Mateos *et al.*, 1992), presence of bacterio-chlorophyll in *Rhizobium trifolii* (Evans *et al.*, 1990). In order to develop a strategy to exploit maximum inoculation response and also to explore unusual characters if any, knowledge of the native rhizobial population is urgently required. Identification and characterization of symbiotically effective and ecologically persistent strains has been suggested to be taken as research priority (Keatinge *et al.*, 1995). The present study is focused on isolation and determination of some physiological characters of chickpea-nodulating rhizobia from dry loamy sand of Thal (having an arid climate and summer temperature up to 50 °C), with the objective of exploring the diversity in characters, which may be helpful in their saprophytic survival.

Materials and Methods

Fifteen isolates, (PAC-19/1a, PAC-19/1 (SK-8, THAL 8), PAC-19/2 (Sc4), PAC-19/3(BN8), PAC-20, PAC-21, PAC-22, PAC-4, PAC-23, PAC-24, PAC-25, PAC-26, PAC-27, PAC-28) were recovered from the nodules of chickpea from farmers field at twelve sites in Northern Thal, TAL 1148 (acquired from NifTAL Hii) was used as reference strain. Following characteristics were determined.

Colony characters: The size and gumminess of the colonies on Yeast extract Mannitol agar (YMA) medium containing Congo red (Somasegaran & Hoben, 1985) was recorded.

Acid/alkali production was recorded on YMA containing 0.01% bromothymol blue as pH indicator.

Mean Doubling Time (MDT) was determined by inoculating known number of cells of each strain into 100 ml aliquots of Yeast Mannitol broth at 27 °C. The cultures were incubated on a wrist action shaker. Viable counts were checked after suitable regular intervals, varying from 6-24h. MDT was calculated according to Somasegaran & Hoben (1985).

Effect of elevated temperature was measured in terms of MDT determined in YM broth as mentioned above at 40 °C.

Streptomycin -resistance of the isolates was evaluated by plating 0.1ml of each of the rhizobium cultures (having a population of 10^7cells/ml), on YMA plates containing streptomycin at the rate of 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 μL /L. The plates were incubated at 27-30 °C. Level of resistance was determined according to Beck *et al.* (1993).

Osmotic potential of rhizobial cells was determined by plating active cell cultures (grown in YM broth) on agar (Harrower and Nagy ,1979) by adding KCl to standard medium (Na_2HPO_4, 0.75g; KH_2PO_4, 0.75g; $MgSO_4.7H_2O$, 0.12g; NaCl, 0.1g; NH4NO3, 0.4g; glucose, 1.8g; yeast extract, 0.1g; malt extract, 1.0g; agar, 15-18g; distilled water, 1L) in amounts of no KCl, 7.46g, 14.91g, 22.37g, 37.28g, 44.74g and 52.19g to obtain the gradients of osmotic potential from −1.5b,-4.58b,-13.43b,-17.82b, -22.21b, -26.62b, -31.02b respectively. The lowest osmotic potential which showed growth of the particular rhizobia equally (in terms of colony forming units) as on the control, was considered and recorded as the osmotic potential of that rhizobium.

Symbiotic effectiveness was determined by inoculating germinating seeds of chickpea cv C-44 with one mL of test culture containing 10^8-10^9 cells, in sterilized sand culture. The plants were harvested at one and half months' age and records were made on the nodulation, shoot dry weight and N-content of shoot.

Phosphate-solubilization was determined by growing the test-cultures, to mid log phase in YM broth. A loopful of each of the test rhizobia was spotted onto the modified Petri's medium ($Ca(NO_3)_2.4H_2O$, 0.4g; $MgSO_4.7H_2O$, 0.15g , rock phosphate, 10.0g ; $Ca_3(PO4)_2$, 5.0g; agar, 18.0g; dist.water, 1.0L) onto the plates. The plates were incubated at 30 °C for 2-10 days so as to obtain a growth of one mm diameter. The efficiency of each culture was evaluated by taking the ratio of diameter of clear zone to that of colony, as described by Nguyen *et al* (1992).

Relative cell surface charge was determined in terms of the adsorption of positively charged methylene blue cations onto the negatively charged sites on rhizobium cell surface. A

colorimetric method was developed to determine cell surface charge. The test rhizobia were grown in YM broth up to a cell population of 10^9 $ml^{-1}$and cells were harvested by centrifugation and resuspended in phosphate buffer (pH = 7.25), to obtain cell population 10^7 mL^{-1}. To 5.0 ml of the cell suspension of each test rhizobium, 20 μL of 0.3% methylene blue stain were added. The reaction was allowed to occur at 40 °C for fifteen minutes. The cells were harvested by centrifugation at 5000 x g and supernatant was transferred to cuvettes to read the absorbance of residual stain by spectronic-21. Methylene blue solution without any rhizobial cells processed above were taken as control. The absorbance of residual stain in the supernatant was interpreted as the negative index of cell surface charge. The cell surface charge was taken as inverse of relative absorbance and expressed in terms of relative absorbance (RA).

Whole cell protein profile of rhizobia was carried out by SDS-PAGE. Cell free extracts were prepared by harvesting cells in mid log phase from broth cultures, washing them with Tris-HCl buffer (pH = 7.6) and disrupting the cells by temperature shock (freezing followed by boiling). Sample buffer 0.5 ml (SDS, 0.92g; beta-mercaptoethanol, 2ml; Glycerol, 4ml; tris buffer, 0.3g; bromophenol blue, 2ml.) was added to equal volume of extract and stored at −20 °C before analysis. All solutions were prepared after Laemmli (1970) and the gel was cast according to the recipe between the plates of Mini-Protean II dual slab gel cell. The samples (50 μl each) were loaded. Egg albumin, and trypsinogen were run as molecular markers. The gel was run at initial voltage of 70 followed by 100 V for three hours at 15 °C. The gels were stained in 0.1 % coomasine blue and destained in destain I (methanol, 50ml; glacial acetic acid, 10 ml, volume made to one liter with dist. water) and destain II (Methanol, 50 ml ; glacial acetic acid, 75 ml ; volume made to one liter) before photography. Rf value of each protein was measured and thereby molecular weight in KD was estimated.

Results and Discussion

The rhizobial isolates varied in their gum production (Table 1) on YMA and maximum colony size varied from 1mm to 4mm from isolate to isolate.

Mean doubling time varied from 1.8 to 4h for nine isolates while from 7.0 to 10.0 h for the remaining three (Table 1). Of the fast growers, three isolates showed neutral reaction on bromothymol blue(BTB)- containing YMA medium, three produced alkali while others produced acid. Of the slow growers only one was alkali-producer while other two showed neutral reaction. All chickpea-rhizobia have been reported to be acid producers by Ruiz-Argueso *et al.* (1988). Previously Tan & Broughton (1981) reported that pH decrease is a characteristic of fast growers. However, the results obtained here suggest that growth rate of Chickpea-rhizobia is not necessarily associated with acid or alkali production. Acid production may be a desirable character for soils of Pakistan which are mostly alkaline having pH up to 8.5. Though care must be taken in assigning ecological significance to acid production in laboratory medium, the potential of acid production may be an advantage to desert microorganism existing in soil of high pH.

Intrinsic Antibiotic Resistance is a character helpful in identifying particular isolate in the laboratory, in combination with other characters (Rupela *et al.*, 1982). It is also helpful in predicting saprophytic competence of the isolate in the soil. During present study all the isolates were characterized for streptomycin −resistance. Three isolates from Kalurkot exhibited resistance to high doses of streptomycin sulphate (Table 1). This shows that the native rhizobia are well-adapted to *Streptomyces*, the most common microbe in desert soil. Resistance to such high dose as shown by PAC-19/3 has not previously been reported.

Growth rate at 40 °C was widely varied. Though seven isolates suffered 3-5 fold increase in their doubling time (Table 2), four isolates from Kalurkot remained unaffected in their mean doubling time. This predicts the existence of a gene pool for selection of rhizobia for regions where high summer temperature lowers the survival of inoculated strains.

Osmotic potential of a cell is an index of it's ability to withstand the physical drought. Rhizobial inoculants in Pakistan are expected to undergo such situations frequently when water potential of soil falls as low as −0.3 bars. The rhizobial isolates were thus characterized for their osmotic potential. The isolated chickpea-rhizobia showed great diversity in their osmotic potential. Highest osmotic potential (−3.1MP) recorded was in case of TAL 1148 and PAC-19/1 and PAC-25 (Table 2) while PAC-19/1a (S_2a) exhibited an osmotic potential of −1.5 bars. Previously chickpea −bradyrhizobia have been reported to lose their viability at −3.0MP (Vargas & Bezdicek, 1991), while *Rhizobium meliloti* (fast grower) were unable to grow at −1.5MP in a PEG containing medium (Busse & Bottomley, 1989). The diversity in cell osmotic potential shows that strains of rhizobium even from the same soil-climatic conditions may differ in their ability to multiply and survive at different water potential. Careful selections are therefore recommended to

Table1: Cultural growth characters of isolates from chickpea root-nodules from different sites of Thal.

Isolate	Source site	Colony features	MDT at 27°C(hrs.)	pH reaction	Streptomycin resistance (µg/ml)
PAC-19/1a	Kalurkot	Glossy, 1 mm.	2	N	100
PAC -19/1	Do	Slimy, watery confluent growth.	2	A	200
PAC -19/2	Do	Slightly gummy, 1mm	7-8	Al	400
PAC -19/3	Do	Highly gummy, 2-3mm	9	N	800
PAC -20	Zamaywala	Slightly gummy, 1mm	3	N	0
PAC -21	Rodi	Glossy, 1mm	3	A	0
PAC -22	Fazal	Glossy , 1mm	2.5	N	0
PAC -4	Dullewala	Highly gummy , glossy, 2-4mm	4	Al	100
PAC -23	Rakh mahuta	Glossy, 1mm.	2	Al	0
PAC -24	Khanser-1	do.	1.8	A	0
PAC -25	Khanser-2	do	3.7	A	0
PAC -26	Bhattianwala	do	3.2	Al	0
PAC -27	Dagar kotli	do	2.5	N	0
PAC -28	Rakh ghulaman	do	10	N	50

A: Acid production Al: Alkali production N: Neutral reaction

Table 2: Osmotic potential of the isolates and their Growth response at elevated temperature.

Isolate	Osmotic potential (bars)	Times increase in MDT at 40°C
PAC -19/1a	-1.5	1
PAC -19/1	-31.02	1
PAC -19/2	-22.2	1
PAC -19/3	-22.2	1
PAC -20	-17.8	4
PAC -21	-13.43	3
PAC -22	-31.02	3
PAC -4	-17.82	2
PAC -23	-17.82	4
PAC -24	-17.82	4
PAC -25	-31.02	5
PAC -26	-17.82	4.5
PAC -27	*	4
PAC -28	-31.02	2
TAL 1148	-31.02	1

* was unable to grow on Harrower & Nagy agar.

inoculate arid and semi-arid ecologies.

Knowing the effectiveness of indigenous population is important because it is a determining factor in the success of inoculation (Thies *et al.*, 1991). The isolates differed significantly in their effectiveness in terms of nodule dry mass, shoot dry mass and N-content of shoot (Table 3). This illustrates the variability in symbiotic effectiveness of isolates collected from a low fertility sand of a dry region. PAC-20 , though was able to produce nodule mass comparable to TAL 1148, yet was not able to accumulate nitrogen comparable to the reference strain. PAC-19/3 possessed highest effectiveness, in terms of total nitrogen accumulated in shoot. All others produced significantly lower nodule mass, as well as shoot mass and total N-content. These results indicate that inspite of the fact that majority of the population has poor effectiveness with C-44 (the commonly cultivated variety in the region), high performance indigenous isolates like PAC-19/3 can be recovered & exploited for soil environment through inoculation. This also suggests the promise of isolates for use in large scale production of inoculants. Selections from indigenous micro flora are generally more adapted and competitive than any exotic inoculant (Friederick *et al.*, 1990).

Phosphate-solubilizing activity is a property of great agricultural significance. It enables the microorganisms to solubilize fixed form of soil phosphate, which can thus increase phosphate available for the crop as well as for microbe itself. Among the present isolates, PAC-19/1 (SK-8) was strongly phosphate solubilizing while TAL 1148, PAC-22 and PAC-26 were mild phosphate solubilizers (Table 4). All remaining isolates have zero efficiency. Phosphate solubilization in TAL 1148 and PAC-22 seems not to be due to acid-production. Available phosphate promotes extracellular polysaccharide (EPS) formation in microorganisms (Bushby, 1982), which is considered to play significant role in saprophytic survival of bacteria under draught stress and against predators or toxins. These PO_4-solubilizing rhizobia may improve the survival of other co-inoculant rhizobia by promoting their EPS production.

Surface properties of rhizobial cells are also important from the viewpoint of their adhesion to clay particles, a phenomenon that has been regarded to play important role in their survival under dry conditions (Gannon *et al.*, 1991) through their transport ability. This character has also been suggested having some role in bacterial transport through soil with moving water (Gannon *et al.*, 1991) through their transport abilities. Rhizobia possess cell surface negative charge (Bushby, 1990). Cell surface negative charge was determined in terms of relative extent of methylene blue cations adsorbed from the methylene blue solution, onto the cells. Relative absorbance was taken as inverse of cell surface negative charge. The results (Table 5) indicate the presence of different magnitude of surface negative charge on the isolates. The chickpea-nodulating rhizobia thus possess different capacities to get fine clay particles adsorbed onto their surface. Rhizobia like TAL 1148, PAC-19/3, PAC-23 and PAC-25 had larger surface charge and were expected to be better protected in a dry soil having positively charged or uncharged fine clay particles (Khokhar & Khan, 1994). This spectrometric test can provide a quick tool for characterizing the isolate in combination with other characters.

Total Cell protein profiles of selected indigenous isolates are displayed in Fig. 1. PAC-19/3 and PAC-22 resisted washing of polysaccharides by normal procedure and so profiles could not be obtained. Based on the protein pattern, three zones of separation were recognized. Zone 1 included polypeptides having molecular weights ranging from 94 to 65KD. Zone 2 included polypeptides having molecular weights ranging from 53 to 45 KD. Zone 3 ranged from 45 to 14 KD. However differences were observed in the number and presence or absence of specific polypeptides. The reference strain TAL 1148 shared most of the proteins in zone 3 with all indigenous isolates. The total cell protein profiles thus suggest that the patterns are isolate-specific and can serve as fingerprints. It can hence be concluded that chickpea-rhizobia isolated from same soil-climatic region are diverse. Previously diversity in rhizobia has been reported for several legumes (Keatinge *et al.*, 1995; Samba *et al.*, 1999; Wang *et al.*, 1999). The information obtained here is useful for identifying the isolates under laboratory conditions. Since the farmers' fields, where isolates originate, were never inoculated ,

Table 3: Effectiveness of the isolates.

Isolates	Number of nodules	Dry mass of nodules	Dry mass of shoot (g)	%-N	Total N of the shoot (mg)
PAC -19/1a	21	23.2abc	0.63 bc	1.1 b	8.9 de
PAC -19/1	23	26.7abc	0.66 b	1.5 b	9.9 d
PAC -19/2	18	21.8abc	0.65 b	2.0a	13.0 c
PAC -19/3	24	50.0a	0.98a	2.4a	23.5a
PAC -20	27	41.3a	0.56 cd	1.4 b	7.8 ef
PAC -21	14	24.4abc	0.50 de	1.2 b	6.0 fg
PAC -22	22	20.6 bc	0.50 de	1.0 b	5.0 gh
PAC -4	11	18.7 bc	0.53 de	0.9 bc	4.8 ghi
PAC -23	17	16.5 c	0.42 f	0.6 bc	2.5 j
PAC -24	19	21.3 bc	0.46 ef	0.7 bc	3.0 ij
PAC -25	20	23.3abc	0.45 ef	0.6 c	2.6 j
PAC -26	19	25.7abc	0.51 de	0.7 bc	3.4 hij
PAC -27	18	19.6 bc	0.46 ef	0.4 c	1.8 j
PAC -28	15	10.1 c	0.41 f	0.4 c	1.6 j
TAL 1148	18	56.2a	0.94a	1.9a	17.9 b

*Data /plant recorded at the age of 6-weeks.

** Means followed by the common letters are not significantly different at P = 0.05 by DMRT.

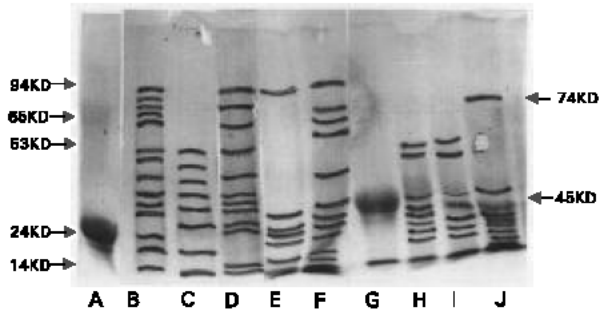

Fig. 1: Whole cell protein of chickpea-rhizobia from Northern Thal
Lane A: Trypsinogen, B: PAC-21, C: PAC-19/3
D: PAC-19/1 E: PAC-27, F:PAC-26,
G: Egg Albumin, H: PAC-23, I:PAC-25,
J: TAL 1148

Table 4: Phosphate-solubilization by chickpea-rhizobia.

Rhizobium isolates	Solubilization Efficiency
PAC-19/1a	0
PAC-19/1	2
PAC-19/2	0
PAC-20	0
PAC-21	0
PAC-22	1
PAC-23	0
PAC-24	0
PAC-25	0
PAC-26	1
PAC-27	0
PAC-28	0
TAL 1148	1

Table 5: Relative cell surface charge of chickpea-rhizobia.

Rhizobium isolates	Relative absorbance
TAL 1148	0.17
PAC-19/1	0.74
PAC-19/2	0.57
PAC-19/3	0.28
PAC-21	0.53
PAC-22	0.95
PAC-23	0.42
PAC-24	0.74
PAC-25	0.32
PAC-26	0.71
PAC-27	0.57

Control (PO_4-buffer) 1.00(Relative absorbance ∝ 1/cell surface charge).

the presence of yet undefined new species can not be overlooked. The present collection of chickpea-rhizobia, which represents a sample population of northern Thal, thus comprises of a physiologically heterogenous group of rhizobia. They present some unusual characters like phosphorus-solubilization, high cell osmotic potential and relative cell surface charge, which may support their saprophytic survival in dry soil and make them more acceptable as biofertilizers. Though it remains to be a follow-up research to study their competitiveness in terms of nodule occupancy, the present information on indigenous population would be helpful in developing successful inoculation strategy for chickpea in northern Thal.

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