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Symbiotic Properties of Phage Resistant Mutants of *Rhizobium* sp. of *Vigna aconitifolia*

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Abstract: Spontaneously arising phage resistant mutants were isolated from a streptomycin resistant mutant of *Vigna aconitifolia Rhizobium* MR-41, susceptible to two different lytic viruses, V_1 producing turbid plaques and V_2 producing clear plaques. Forty mutants resistant to V_1 (group I) and the other 40 mutants resistant to V_2 (group II) were examined for their *in vivo* nitrate reductase activity. In both the groups mutants ranged from complete lack of nitrate reduction to 25-fold higher reduction. Nodules produced by the mutants of the two groups were often significantly higher than the parent. Two mutants of group I and 6 mutants of group II were significantly better than parent in symbiotic effectiveness. None of the mutants of the two groups was ineffective.

Key words: *Rhizobium*, phages, resistant mutants, symbiotic effectiveness, nitrate reductase.

Members of genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* are ecologically and economically important since they induce dinitrogen-fixing nodules on roots of the leguminous plants (Sprent, 1989). In the rhizosphere they are subject to the influence of several biotic and abiotic factors limiting their growth (Dixon and Whealer, 1986). Rhizobiophage is one of the important biotic factors (Barnet, 1979). Phages that are infectious to rhizobia of most of the legume crops have been reported and their presence may readily be shown in the soils cultured to legume hosts (Dhar and Ramkrishna, 1987). While the presence of phages may cause a selection pressure on sensitive strains of rhizobia, their continued co-existence with the sensitive host genotype is interesting (Kleczkowaska 1971; Patel, 1978; Hashem and Angle, 1988). Under such selection pressure, the phage-resistant forms may obviously have better fitness in the rhizosphere. Since spontaneous mutations do occur conferring resistance against phage infection in susceptible strains of rhizobia, in such variants the knowledge of associated changes in their symbiotic properties is important. In some of the studies conducted earlier, such variants are often shown to be symbiotically ineffective (Kleczkowaska, 1971; Barnet, 1979). However, more host phages system of *Rhizobium* of different legumes need to be studied to arrive at a generalized conclusion. The present investigation was conducted to study symbiotic properties of phage resistant mutants isolated from two different lytic phages parasitic on *Rhizobium* MR-41 of *Vigna aconitifolia*.

Table 1. In vivo specific nitrate reductase activity of different phage resistant mutants of Rhizobium MR 41 of Vigna aconitifolia sensitive to two lytic phages V_1 and V_2

Classes	Nitrate reductase activity*		Mutants	
	Level	Rate (µ mol NO_2 released/ mg protein/h)	Group I	Group II
Class I	Comparable or slightly higher	1.50 - 4.54	MR 41 - Sm^r V_1-1	MR 41 - Sm^r V_2-1
			MR 41 - Sm^r V_1-2	MR 41 - Sm^r V_2-2
			MR 41 - Sm^r V_1-3	MR 41 - Sm^r V_2-3
			MR 41 - Sm^r V_1-4	MR 41 - Sm^r V_2-4
			MR 41 - Sm^r V_1-5	MR 41 - Sm^r V_2-5
			MR 41 - Sm^r V_1-6	MR 41 - Sm^r V_2-6
			MR 41 - Sm^r V_1-6	MR 41 - Sm^r V_2-7
			MR 41 - Sm^r V_1-7	MR 41 - Sm^r V_2-8
			MR 41 - Sm^r V_1-8	MR 41 - Sm^r V_2-9
			MR 41 - Sm^r V_1-9	MR 41 - Sm^r V_2-10
			MR 41 - Sm^r V_1-10	MR 41 - Sm^r V_2-11
			MR 41 - Sm^r V_1-12	MR 41 - Sm^r V_2-12
			MR 41 - Sm^r V_1-16	MR 41 - Sm^r V_2-13
			MR 41 - Sm^r V_1-18	MR 41 - Sm^r V_2-14
			MR 41 - Sm^r V_1-19	MR 41 - Sm^r V_2-15
			MR 41 - Sm^r V_1-22	MR 41 - Sm^r V_2-16
			MR 41 - Sm^r V_1-23	MR 41 - Sm^r V_2-17
			MR 41 - Sm^r V_1-24	MR 41 - Sm^r V_2-18
			MR 41 - Sm^r V_1-26	MR 41 - Sm^r V_2-19
			MR 41 - Sm^r V_1-28	MR 41 - Sm^r V_2-20
			MR 41 - Sm^r V_1-29	MR 41 - Sm^r V_2-21
			MR 41 - Sm^r V_1-30	MR 41 - Sm^r V_2-22
			MR 41 - Sm^r V_1-31	MR 41 - Sm^r V_2-23
			MR 41 - Sm^r V_1-32	MR 41 - Sm^r V_2-24
			MR 41 - Sm^r V_1-33	MR 41 - Sm^r V_2-25
			MR 41 - Sm^r V_1-34	MR 41 - Sm^r V_2-26
			MR 41 - Sm^r V_1-35	MR 41 - Sm^r V_2-27
			MR 41 - Sm^r V_1-36	MR 41 - Sm^r V_2-28
			MR 41 - Sm^r V_1-37	MR 41 - Sm^r V_2-29
			MR 41 - Sm^r V_1-38	MR 41 - Sm^r V_2-30
			MR 41 - Sm^r V_1-40	MR 41 - Sm^r V_2-31
				MR 41 - Sm^r V_2-32
				MR 41 - Sm^r V_2-33
				MR 41 - Sm^r V_2-34
				MR 41 - Sm^r V_2-35
				MR 41 - Sm^r V_2-36

Table 1. contd.:

Classes	Nitrate reductase activitiy*		Mutants	
	Level	Rate (μ mol NO_2 released/ mg protein/h)	Group I	Group II
				MR 41 - Sm^r V_2-37
				MR 41 - Sm^r V_2-38
				MR 41 - Sm^r V_2-40
Class II	Nil	Nil	MR 41 - Sm^r V_1-11	MR 41 - Sm^r V_2-4
			MR 41 - Sm^r V_1-14	MR 41 - Sm^r V_2-18
			MR 41 - Sm^r V_1-15	
			MR 41 - Sm^r V_1-20	
			MR 41 - Sm^r V_1-21	
			MR 41 - Sm^r V_1-27	
			MR 41 - Sm^r V_1-39	
			MR 41 - Sm^r V_1-13	
			MR 41 - Sm^r V_1-17	
Class III	20-25 fold higher	32-40	MR 41 - Sm^r V_1-13	MR 41 - Sm^r V_2-23
			MR 41 - Sm^r V_1-17	

* The activity of the parent was 1.6 μ mol NO_2 released/mg protein h^{-1}.

Material and Methods

The *Rhizobium* MR-41 of *Vigna aconitifolia* used in the present investigation is an isolate of this laboratory. The parent and various mutant derivatives were grown and maintained in yeast mannitol medium (Vicent, 1970) at 28±2°C. Bacterial growth was determined as colony forming units (CFU) per ml. To determine nitrate reduction by active cells, yeast mannitol nitrate medium (YMN) of Schwinghamer (1960) was used. Phages sensitizing strain MR-41 were isolated as described earlier (Dhar and Ramkrishna, 1987). Purified lysate of phage V_2 produced clear plaques with rough margins of 1-2 mm in diameter within 48 h, while phages V_1 produced turbid plaques of similar dimension within 72 h of incubation. Spontaneously arising phage-resistant mutants were isolated from a symbiotically effective streptomycin-resistant spontaneous mutant of *Rhizobium* MR-41, designated as MR-41-Sm^r-1. Five ml of mid-exponential phase bacterial suspension of MR-41-Sm^r-1 were exposed to purified lysates of the two phages separately (M.O.I.=0.1). The host phage suspensions were incubated at 28±2°C to observe complete lysis and regrowth thereafter (approximately 5-6 days). A loopful of regrown suspension was streaked on to YM agar plates for colony isolation. Forty colonies, in respect of each phage, were subjected to two passages of clonal

Table 2. Symbiotic effectiveness of phage resistant mutants of Rhizobium MR-41 of Vigna aconitifolia

Class	Mutant	Nodule per plant	Nodule fresh weight (mg)	Nodule dry weight (mg)	Plant fresh weight (g)	Plant dry weight (mg)	N-content (mg)
Parent	MR 41 - *Sm*^{r} V_1-1	14.00	22.00	3.70	0.425	85.00	9.39
Group I	MR 41 - *Sm*^{r} V_1-4	29.88	40.00	7.96	0.780	159.66	18.50
	MR 41 - *Sm*^{r} V_1-17	40.00	44.33	9.63	0.725	157.00	17.16
	Mean (of all 40 mutants)	25.09	30.66	6.03	0.574	123.75	13.42
Group II	MR 41 - *Sm*^{r} V_2-7	26.33	164.66	21.20	1.134	149.33	17.00
	MR 41 - *Sm*^{r} V_2-9	29.00	106.33	19.26	0.848	147.33	16.58
	MR 41 - *Sm*^{r} V_2-13	25.00	89.00	18.53	0.780	135.33	12.00
	MR 41 - *Sm*^{r} V_2-22	38.33	83.66	13.96	0.974	157.66	17.08
	MR 41 - *Sm*^{r} V_2-24	29.33	135.33	26.96	1.184	182.66	17.45
	MR 41 - *Sm*^{r} V_2-31	29.66	65.67	12.83	1.068	202.00	21.58
	Mean (of all 40 mutants)	20.99	57.39	10.45	0.748	140.51	14.98
	CD at 5%	10.75	17.29	2.95	0.286	51.15	6.98
	CV (%)	29.18	24.60	22.47	26.95	24.34	31.08
	Correlation coefficient With N-content/plant	+0.68**	+0.882**	+0.178	+0.363**	+0.398**	

** Significant at P<0.01.

purification on YM agar plates. Finally the purified clones (including the parent, as MR-41-*Sm*^{r}-1) were cultured on YM broth medium containing streptomycin @ 500 $\mu g\ ml^{-1}$ and appropriate phage for confirming their mutant character. For determining nitrate reduction by parent and the mutants, the free nitrite content released in the medium was assayed colorimetrically (Snell and Snell, 1949). The enzyme activity was expressed as μ mol of nitrite released per mg of protein per hour. Protein was determined as described by Lowery *et al.* (1951).

For culture of plants in nitrogen-free sterile sand and measurement of symbiotic effectiveness at 40 days of growth, the procedure described by Jat and Ramkrishna (1999) was followed. Symbiotic effectiveness of inoculated plants was judged by comparing total nitrogen content of treated vs. control plants. Nitrogen content was determined by microkjeldahl distillation method of Paech and Tracey (1956). The data were analyzed as per completely randomized design.

Results and Discussion

Spontaneously arising phage-resistant mutants had appeared at a frequency of 2×10^{-8} and 6×10^{-8} for phages V_1 and V_2, respectively. All the 80 phage-resistant mutants (40 each of the two phages), isolated and tested in the present study, showed that their growth in YM medium in presence

of streptomycin and the appropriate phage was comparable (data not shown). Thus, the mutants were stable and were true derivatives of the parent.

In laboratory experiment, the phage was used as a selection pressure for identification of phage-resistant mutant which may have spontaneously originated. Such a selection pressure may be built under rhizospheric soil also because the event of nodule bursting releases metabolically active rhizobia and allow multiplication of virulent phages (Dhar and Ramkrishna, 1987). While the phage-susceptible bacteria is eliminated, the phage-resistant forms have apparent advantage of being free from the selection pressure imposed by phage.

The mutants resistant to phages V_1 and V_2 were arbitrarily designated as group I and II mutants, respectively. There was no noticeable change in the colony characteristics of the isolated mutants. Mutants of both the groups were screened for their ability to reduce nitrate while growing in YMN media that contained KNO_3 as a constituent. In both the groups, three classes of mutants could be identified. First, mutants having comparable or 2 to 3-fold higher nitrate reductase activity than the parent; second, those mutants in which there is complete lack of the activity; and third in which the mutants exhibited 20 to 25-fold more activity when compared with the parent (Table 1). It appears that both the groups of phage-resistant mutant share the same classes of alteration in nitrate reductase activity. This observed physiological change may be attributed to pleiotrophic effect of mutation that confers phage resistance. Therefore, it seems that the two groups of mutants shared similar mutations and at least 3 different sites in the genome. In the present study no attempt was made to observe the resistance of group I mutants against the phage V_2 or the resistance of group II mutants against phage V_1.

Results revealed that there were significant differences, arising from inoculation with these mutants, on nodules per plant, nitrogen content per plant and other symbiotic traits studied. The group II mutants generally formed larger nodules than those of group I mutants. On the basis of N content/plant, six mutants of group II were found to be significantly better than the parent whereas, in group I, only two mutants were statistically superior to the parent. These superior mutants of the two groups were also associated with significantly higher values of other traits studied (Table 2). Within each group, there were no noticeable differences for symbiotic characteristics between three types of mutants classified on the basis of nitrate reduction. While there was remarkably high proportion of mutants with high effectiveness, no ineffective mutant was found in either group. These results are not consistent with those reported earlier that virulent phages play a role in increasing the proportion of ineffective strains in a population (Barnet, 1979; Kleczkowaska, 1971). The genetic background of the host bacteria employed for isolation of phage resistant mutants may be responsible for such variable results.

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References

Barnet, Y.M. 1979. Properties of *Rhizobium trifolii* isolates surviving exposure to specific bateriophages. *Canadian Journal of Microbiology* 25: 979-986.

Dhar, B. and Ramkrishna, K. 1987. Morphology and general characteristics of phages of chickpea rhizobia. *Archieves of Microbiology* 147: 121-125.

Dixon, R.O.D. and Whealer, C.T. 1986. *Nitrogen Fixation in Plants: Tertiary Level Biology*. Blackie, USA, Champan and Hall, New York.

Hashem, F.M. and Angle, J.S. 1988. Rhizobiophage effects on *Bradyrhizobium japonicum* nodulation and soybean growth. *Soil Biology and Biochemistry* 20: 69-73.

Jat, R. and Ramkrishna, K. 1999. Symbiotic effectiveness of different antibiotic resistant mutants of moth bean (*Vigna aconitifolia*) *Rhizobium* MR 67. *Annals of Arid Zone* 38: 31-35.

Kleczkowaska, J. 1971. Genetical changes in *Rhizobium* bacteria and in their bacteriophages during co-existence. *Plant and Soil* (Special Issue), pp. 47-56.

Lowery, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurements with the folin reagent. *Journal of Biological Chemistry* 193: 265-275.

Paech, K. and Tracey, M.V. 1956. *Modern Methods of Plant Analysis*, Volume I. Springer Verlag, Berlin.

Patel, J.J. 1978. Symbiotic effectiveness of phage resistant mutants of two strains of lotus rhizobia. *Plant and Soil* 49: 251-257.

Schwinghamer, E.A. 1960. Studies of induced variation in rhizobia I. Defined media and nodulation test technique. *Applied Microbiology* 8: 349-352.

Snell, F.D. and Snell, C.T. 1949. *Colorimetric Methods of Analysis*, 3rd Ed. II, pp. 802-804. D. Van Nostrand Co. Inc., New York.

Sprent, J.I. 1989. Which steps are essential for the formation of functional legumes nodules? *New Phytology* 111: 129-153.

Vincent, J.M. 1970. *A Manual for Practical Study of Root Nodule Bacteria*. I.B.P. Handbook No. 15, Blackwell Scientific Publications, Oxford and Edinburgh.