Variability in *Xanthomonas oryzae* pv. *oryzae*, the incitant of bacterial blight disease of rice. II. Phage sensitivity of the isolates

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ABSTRACT

Attempts were made to classify different isolates of Xanthomonas oryzae pv. oryzae, the causal organism of bacterial blight disease of rice, through their phage sensitivity and correlate it with their virulence. Fifteen phage strains were isolated from infected leaf samples collected from different states of India. The sensitivity of 52 bacterial strains, collected from 12 rice growing states of the country, to individual phage strains was determined by spot test and plaque test. These could be grouped into 13 lysotypes on the basis of their phage sensitivity by conventional methods, also confirmed through cluster analysis. Irrespective of the methods of grouping, the isolates in each lysotype were exactly the same. Each lysotype consisted of bacterial isolates originating from different states and belonging to different pathotypes as well as virulence factors. On the other hand, the isolates of same origin, pathotypes or virulence factors were randomly distributed over different lysotypes, thus proving non-parallelism between clustering pattern and geographical distribution. There was no relationship between phage sensitivity and virulence of the isolates.

Key words: *Rice, bacterial blight, Xanthomonas oryzae pv. oryzae, phage sensitivity, lysotypes, clustering pattern, virulence*

Bacteriophages infecting *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the incitant of bacterial blight disease of rice, were first isolated by Yoshii *et al*. (1953). Since then, these have been used for ecological studies to estimate bacterial population (Wakimoto, 1957; Wakimoto and Yoshii, 1955; Mizukami and Wakimoto, 1969) and disease forecasting (Muzukami, 1966; Tagami, 1959a,b). Wakimoto (1960) classified the strains of *Xoo* according to their sensitivity to bacteriophages. Kauffman and Pantulu (1972) used the techniques on Indian isolates also. Though majority of the studies failed to establish any relationship between phage sensitivity and virulence (Fujii *et al*., 1974; Kuhara *et al*., 1965; Wakimoto, 1960), Choi *et al*. (1981) found virulence closely related to phage sensitivity. The grouping of bacterial strains into lysotypes would be much more useful if these have a close relationship with the pathogenicity pattern based on host differentials. The present experiment was, therefore, aimed at classification of 52 isolates of *Xoo* into lysotypes based on their sensitivity to Indian phages and comparison of their virulence pattern with the pathotypes identified through a set of host differentials.

MATERIALS AND METHODS

Isolation and purification of bacteriophage. Fifteen phage strains, designated as CRP-1 to CRP-15, were isolated from bacterial blight infected leaf samples of 15 local as well as high yielding rice genotypes collected from various locations in different states of India, namely Andhra Pradesh, Bihar, Orissa and Tamil Nadu. One gram of chopped diseased leaves was suspended in 25 ml of sterile distilled water in 100 ml flasks and kept at 25-28^{o}C for 48h to release the phages. The supernatant was decanted and centrifuged at 300 rpm for 30 min., mixed with chloroform at the rate of 0.5 ml to 50 ml of the suspension and shaken vigorously. A few minutes later, one ml of the supernatant and bacterial suspension, each was mixed and added to 25 ml of melted potato sucrose agar (PSA) medium, mixed thoroughly and plated. The agar plates were incubated

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at 25-28°C for 24h. Isolated plaques were obtained on the bacterial lawn with higher dilutions. Representative, uniformly looking plaques, and also those differing in size, were picked up from each of the samples separately. These were further subjected to typing on the collection of host bacteria in order to establish distinct strains. Based upon their typing pattern against *Xanthomonas oryzae* pv. *oryzae*, the 15 phage strains designated CRP-1 to CRP-15 were differentiated. Purification of the phages was attained by suspending their single plaques in 5 ml of sterile distilled water and plating successively thrice against the bacterial strain. Single plaques of each phage were finally transferred to 5 ml of sterile distilled water in test tubes. These single plaque-isolates constituted the 'stock phage', and were maintained in refrigerator at 4±1^{0}C for further use. The 'stock phage' was propagated periodically, on respective bacterial strains.

Phage sensitivity to bacterial cultures. Susceptibility of all the isolates of *Xanthomonas oryzae* pv. *oryzae* to individual phage strains was determined by (i) spot test and (ii) plaque test. Spot test was carried out by mixing one ml of bacterial suspension with 25 ml of melted cooled PSA and pouring in Petriplates previously marked into four equal sectors by drawing two diagonal lines on the under surface of the plates with a glass marking pen. Individual phage strains were seeded into each of the sectors of the solidified agar surface by picking a loopful of stock phage. The plates were incubated in BOD incubator at 25±3^{0}C for 24h, after which the lytic action of the phage was observed. The test bacterial isolates which gave positive reaction under the spot test, were subjected to plaque test for further confirmation. The reaction of the bacterium was indicated by the development of plaques, which was performed according to the standard procedure for isolated plaque development described above. The time of plaque appearance was recorded, following serial dilution of each strain of stock phage culture mixed with bacterial suspension and plated on 25 ml of PSA in sterilized Petriplates. The phage sensitivity was presented as lysis (+) or no lysis (-).

Grouping of the isolates. The 52 isolates of *Xoo* were grouped into lysotypes, on the basis of their phage sensitivity reactions by conventional as well as numerical taxonomic methods (Sneath and Sokal, 1973), following the unweighted average pair group of methods using the SPSS package. Such groupings were also compared with pathotyping of the respective isolates determined by pathogenicity patterns as well as virulence factors.

RESULTS AND DISCUSSION

None of the bacterial isolates were found to be susceptible to all the phage strains or no phage strain could lyse all the bacterial isolates (Table 1). Among the different bacterial groups showing sensitivity to phage, lysotypes-I and II consisting of 3 isolates each, exhibited phage sensitivity against 14 phage strains with the only exception of CRP-6 for lysotype-I and CRP-12 for lysotype-II, which showed negative reaction. Lysotype-III, consisting of 15 bacterial isolates (CRXoo 1, 3, 5, 12, 13, 14, 15, 18, 21, 22, 23, 24, 30, 43 and 45), showed sensitivity to 13 phage strains, except CRP-12 and 13. Similarly, lysotype-IV consisting of three bacterial isolates, showed sensitivity to 10 phage strains, except CRP-4, 5, 13, 14 and 15. Lysotype-V consisting of 5 bacterial isolates (CRXoo 7, 17, 28, 35 and 50) showed sensitivity to 13 phage strains, except CRP-8 and 14. The two isolates CRXoo 38 and 41 constituted lysotype-VI, exhibiting sensitivity to 12 phage strains, except CRP-10, 11 and 14. CRXoo 48 alone formed an independent group of lysotype-VII, showing sensitivity to 10 phage strains, except CRP-1, 10, 11, 14 and 15. Lysotype-VIII, consisting of four isolates, exhibited sensitivity to 12 phage strains, except CRP-1, 4 and 11. Six isolates namely, CRXoo 10, 16, 25, 36, 37 and 39 showing sensitivity to 11 phage strains, except CRP-1, 2, 5 and 6, formed lysotype-IX. Lysotype-X consisting of two isolates (CRXoo 40 and 46) exhibited sensitivity to eight phage strains, except CRP-3, 5, 6, 7, 9, 13 and 15. CRXoo 27, 29, 34 and 51 showing sensitivity to 10 phage strains, except CRP-6, 8, 9, 10 and 13, formed lysotype-XI, while the three bacterial isolates namely CRXoo 6, 20 and 42 showing sensitivity to nine phage strains, except CRP-3, 4, 9, 10, 11 and 12 formed lysotype-XII. The single isolate CRXoo 44, exhibiting sensitivity to 10 phage strains, except CRP-3, 7, 8, 11 and 12 formed independent lysotype-XIII. Thus, on the basis of the variability in sensitivity to 15 phage strains, the 52 bacterial isolates could be grouped into 13 lysotypes. Such grouping of the bacterial isolates was also confirmed through numerical analysis represented by a dendrogram (Fig. 1), which revealed 13 clusters. The constituent bacterial isolates of each

Table 1. Grouping of isolates of *Xanthomonas oryzae* pv. *oryzae* into lysotypes, based on phage sensitivity

Lysotype	Phage strains															No. of phage strains typing each lysotype	Bacterial isolates with common phage reactions
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
I	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	14	CRXoo 26,31, 52
II	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	14	CRXoo 8, 19, 33
III	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	13	CRXoo 1,3, 5, 12, 13, 14,15,18,21,22, 23, 24, 30,43, 45
IV	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	10	CRXoo 2, 9, 47
V	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	13	CRXoo 7, 17, 28, 35, 50
VI	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	12	CRXoo 38, 41
VII	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	10	CRXoo 48
VIII	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	12	CRXoo 4,11, 32, 49
IX	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	11	CRXoo 10,16, 25, 36, 37, 39
X	+	+	-	+	-	-	-	+	-	+	+	+	-	+	-	8	CRXoo 40, 46
XI	+	+	+	+	+	-	+	-	-	-	+	+	-	+	+	10	CRXoo 27,29, 34, 51
XII	+	+	-	-	+	+	+	+	-	-	-	-	+	+	+	9	CRXoo 6, 20, 42
XIII	+	+	-	+	+	+	-	-	+	+	-	-	+	+	+	10	CRXoo 44
	10	12	10	10	10	9	11	10	10	9	8	9	9	9	10		

Lysotypes typed by each phage : + = Lysis, - = No lysis

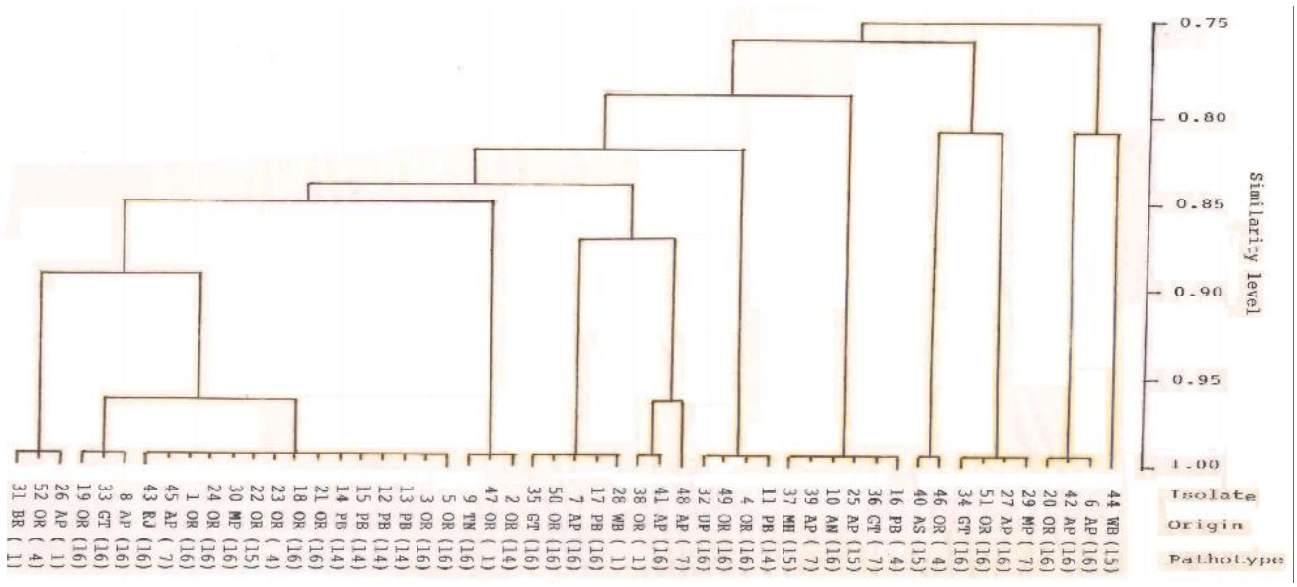

Fig.1. Dendrogram showing the similaity and successive clustering of 52 isolates of Xanthomonas *Oryzae* pv. *oryzae* based on their phage sensitivity.

cluster, obtained through numerical analysis, was exactly similar to those obtained through conventional method.

Clustering pattern compared with geographic distribution, pathotyping and virulence factors. The compiled data on origin of the isolates, pathotypes and the number of virulence factors present in each of the 13 clusters of isolates (Table 2) revealed that each lysotype consisted of isolates originating from different states of the country. On the other hand, isolates originating from the same state were grouped under different lysotypes. These results indicated a non-parallelism between geographic distribution and clustering pattern of the isolates. Such findings of non-parallelism were further confirmed through pathotyping as well as number of virulence factors present in each of the isolates. Thus, it is apparent that lysotypes, pathotypes and virulence factors are unrelated.

The present findings on grouping of *Xoo* isolates into lysotypes on the basis of typing pattern of different phage strains corroborate earlier findings reported from India (Kauffman and Pantulu, 1972), Japan (Wakimoto, 1960; Miyajima, 1980), China (Chu *et al*., 1982), Thailand (Nilpanit *et al*., 1984) and Malayasia (Singh *et al*., 1970). The application of method of numerical taxonomy (Sneath and Sokal, 1973) in the present experiment, to group the virulent phenotypes, resulted in 13 lysotypes at a similarity level of 0.99 on the basis of phage sensitivity of the isolates to 15 phage strains. It is interesting to note that, besides the similarity in grouping of the isolates into 13 lysotypes by conventional method, the constituent isolates of the respective clusters were exactly the same. This conclusion based on the exact mathematical analysis *i.e.* cluster analysis, is in full agreement with those resulting from the conventional grouping of the isolates through visual comparison.

A critical appraisal of these findings reveal that the variability in both, the bacterium as well as the phage is much greater among the Indian isolates as compared to those reported from other countries. Such a variation among Indian isolates could be attributed to diverse agro-climatic conditions under which thousands of rice cultivars of diverse genetic background are grown following different cultural practices as well as the free exchange of seed material among the states and some sneaking unguarded entry of foreign strains. These

Table 2. Clustering pattern of 52 isolates of *Xanthomonas oryzae* pv. *oryzae* based on numerical analysis with origin, compared with conventional grouping of isolates

Cluster/	Isolates		Origin of isolates	Cluster	No.of	Pathotype
Lysotype	Conventional method	Numerical analysis		mean (cm)	v-factors	
I	CRXoo 26, 31, 52	CRXoo 26, 31, 52	Andhra Pradesh, Bihar, Orissa	10.60	11	1, 4
II	CRXoo 8, 19, 33	CRXoo 8, 19, 33	Andhra Pradesh, Gujarat	5.39	4	16
III	CRXoo 1, 3, 5, 12,13, 14,15, 18,21, 22, 23, 24, 30,43, 45	CRXoo 1, 3, 5, 12,13, 14,15,18,21, 22, 23, 24, 30, 43, 45	Andhra Pradesh, Madhya Pradesh,Orissa, Punjab, Rajasthan	6.29	10	4,7,14,16
IV	CRXoo 2, 9, 47	CRXoo 2, 9, 47	Orissa, Tamil Nadu	8.22	11	1, 14, 16
V	CRXoo 7, 17, 28, 35, 50	CRXoo 7, 17, 28, 35, 50	Andhra Pradesh, Gujarat, Orissa, Punjab, West Bengal	6.81	11	1, 16
VI	CRXoo 38, 41	CRXoo 38, 41	Andhra Pradesh, Orissa	8.79	11	1, 16
VII	CRXoo 48	CRXoo 48	Andhra Pradesh	7.99	7	7
VIII	CRXoo 4, 11, 32, 49	CRXoo 4, 11, 32, 49	Orissa, Punjab, Uttar Pradesh	5.78	7	14, 16
IX	CRXoo 10,16,25,36,37,39	CRXoo 10,16,25,36,37,39	Andaman & Nicobar Islands, Andhra Pradesh, Gujarat, Maharashtra, Punjab	7.47	10	4,7,15,16
X	CRXoo 40, 46	CRXoo 40, 46	Assam, Orissa	7.86	10	4,15
XI	CRXoo 27, 29, 34, 51	CRXoo 27, 29, 34, 51	Andhra Pradesh, Gujarat, Madhya Pradesh, Orissa	6.04	8	7,16
XII	CRXoo 6, 20, 42	CRXoo 6, 20, 42	Andhra Pradesh, Orissa	5.39	4	16
XIII	CRXoo 44	CRXoo 44	West Bengal	7.90	7	15

situations also could have contributed significantly for the non-parallelism between the geographic distribution and clustering pattern of the isolates observed in the present investigation.

A comparison of the lysotypes with the pathotypes of respective isolates revealed that different pathotypes were randomly distributed among the lysotypes. No single pathotype showed a similar reaction to all the 15 phage strains, thus corroborating the findings of Kauffman and Pantulu (1972). The number of virulence factors were also randomly distributed among the lysotypes, depending upon the constituent isolates. Although there was a strong relationship between the number of v-factors and the mean lesion length among the isolates (unpublished data of the authors), it was not prominent among the lysotypes due to random distribution of the isolates within lysotypes (Table 2). The grouping of the isolates even at similarity levels of <0.99 did not influence these interpretations in any way, excepting the reduction in the number of clusters accompanied by increased number of isolates in each cluster, with decreasing similarity levels. Thus the phage sensitivity of the isolates could not be correlated with virulence. The present findings are in agreement with those reported by Wakimoto (1960), Kuhara *et al*. (1965) and Fujii *et al*. (1974). On the contrary, Choi *et al*. (1981) reported close relationship between phage sensitivity and virulence. However, Vera Cruz & Mew (1988) reported that bacterial isolates belonging to race-4, were sensitive to group-D phages, while those of race-6 sensitive to none of the phage groups and reactions of the race groups –1, 2 & 3 were overlapping.

The classification of 52 isolates of *Xoo* into 13 lysotypes tested through their sensitivity to 15 bacteriophages, the non-parallelism between the geographic distribution and clustering pattern of the isolates into lysotypes and non-existence of distinct relationship between lysotypes and virulence groupings through pathotypes as well as v-factors, clearly indicated wide variation among the bacterial strains, and also that lysotypes are not comparable with those of pathotypes identified based on a set of differential host genotypes or even the v-factors present in respective bacterial isolates.

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