Bacteriological and Pathological Characteristics of Wild Types and Induced Mutants of *Xanthomonas campestris* pv. *oryzae*

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ABSTRACT

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The relationship between bacteriological and pathological characteristics of wild types and induced mutants of *Xanthomonas campestris* pv. *oryzae* was studied. Most strains of the three pathogenic groups found in the Philippines were homogeneous in biochemical and physiological characteristics. Two parent strains, PXO61PT and PXO63-6PT, of different pathogenic groups were treated with *N*-methyl-*N*-nitro-*N*-nitroso-guanidine (NTG) and 14 induced mutants were obtained, which were classified into three types on the basis of virulence to rice and colony color on peptone-sucrose agar medium. Weakly virulent mutants developed shorter lesions than the parent strains on all rice cultivars that were tested. White colony mutants derived from PXO63-6PT were relatively stable in colony color both in vivo and in vitro, and almost as virulent as the parent strain. Those derived from PXO61PT, however, were unstable in colony color, which spontaneously changed to yellow, and were avirulent. Revertants induced from avirulent mutants of PXO61NT3 by NTG treatment produced different reaction types on rice differentials. Most of the general bacteriological characteristics of all mutants were similar to those of the wild-type strains, however, the phage sensitivity of some mutants had changed.

Additional key words: bacterial blight, induced mutant, virulence.

Bacterial blight, caused by *Xanthomonas campestris* pv. *oryzae* (Ishiyama, 1922) Dye, is one of the most important rice diseases in Asia. Information on bacteriological and pathological characteristics of the pathogen (3,5,10,11) and on resistance of rice to the disease (1,12,13) has been obtained. The bacterial isolates differ widely in their bacteriological properties and virulence (8,11). The results of recent studies conducted independently in Japan (1) and at the International Rice Research Institute (8) have shown that the virulence of *X. campestris* pv. *oryzae* could be determined on the basis of specificity of infection in rice possessing specific resistance. Four and five distinct pathogenic groups have been classified on a set of rice differentials in the Philippines and Japan, respectively. We, therefore, revived our interest in whether the pathological characteristics of the strains of different pathogenic groups of *X. campestris* pv. *oryzae* are related to some of their physiological and biochemical characteristics.

In the present study, both the wild types and induced mutants of the bacterial strains were evaluated.

MATERIALS AND METHODS

Induction and isolation of mutant strains. Thirty strains of *X. campestris* pv. *oryzae* maintained in the Department of Plant Pathology, IRRI, were selected for the study. Eighteen of the strains belong to group I, 9 to group II, and 3 to group III of a classification based on virulence to rice with specific resistance (8). One each of the representative wild strains of groups I and II, PXO61 and PXO63-6, were cultured on the streptomycin containing (100 μg/ml) peptone-sucrose agar (PSA) medium (10 g peptone, 10 g sucrose, and 1 g sodium glutamate in 1,000 ml of water, pH 7.0) to obtain streptomycin-resistant mutants, PXO61PT and PXO63-6PT. The parent strains were treated with *N*-methyl-*N*-nitro-*N*-nitroso-guanidine (NTG) to derive mutants. The procedure for mutagenesis is shown in Fig. 1.

Cells of parental strains were grown for 24 hr in peptone-sucrose broth (PSB) at 30 C in shake culture. The bacterial cells were washed twice in the sterile phosphate buffer by centrifugation. The final suspension (adjusted bacterial concentration at approximately 1×10^7 viable cells per milliliter) was treated with 50 μg NTG/ml in 0.067 M phosphate buffer at pH 7.0. After incubation for 20 min at 30 C in a water bath, cells were washed twice in the same buffer to remove NTG. A portion of the suspension was serially diluted,

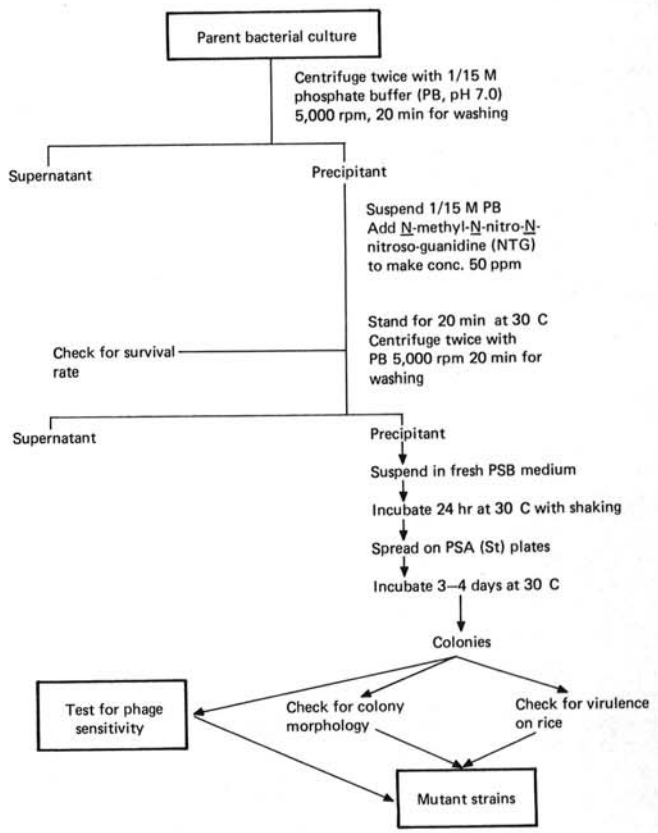

Fig. 1. Procedural flowchart for induction and identification of *Xanthomonas campestris* pv. *oryzae* mutants.

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subsequently spread on the surface of PSA plates, and incubated at 30 C to check the rate of survival. Approximately 15% of the treated cells survived. The remaining suspension was transferred to fresh PSB medium and incubated overnight for multiplication of mutants. Cultures were then diluted, spread on PSA plates containing streptomycin at 100 μg/ml, and incubated for 3–4 days at 30 C.

The colonies different in color from the parental strain frequently were selected and some were picked at random for further tests. Besides colony color mutants, several hundred colonies similar to parent strains were picked to check for mutants with altered virulence. A white colony mutant, PXO61NT3, was cultured on PSA plate medium or treated with NTG to obtain spontaneous or induced revertant mutants.

Pathogenicity tests. The pathogenicity of wild-type and mutant strains was tested on rice differentials by the leaf clipping method of inoculation. Inoculum was prepared by suspending a 48- to 72-hr-old culture in sterilized water at 10^9 cells per milliliter. Virulence of the strains and mutants was evaluated on 35- to 40-day-old plants of rice cultivars IR8, IR20, and IR1545-339, which have specific resistance. The virulence was assessed on the basis of lesion length initiated by individual strains 14 days after inoculation (8).

TABLE 1. Wild-type, parent-type, and induced-mutant strains of *Xanthomonas campestris* pv. *oryzae*

Strain[a]	Relevant phenotype[b]		
	Virulence	Streptomycin resistance	Pigmentation
PXO61 (wild type)	V	S	+
PXO61PT (parent type)	V	R	+
PXO61NT3	AV	R	−
PXO61NT3RV-1	AV	R	+
PXO61NT3RV-2	V	R	+
PXO61NT3RV-3	V	R	+
PXO63-6 (wild type)	V	S	+
PXO63-6PT (parent type)	V	R	+
PXO63-6NT2	V	R	−
PXO63-6NT3	V	R	+
PXO63-6NT5	WV	R	+
PXO63-6NT8	V	R	−
PXO63-6NT8I-1	V	R	−
PXO63-6NT8I-2	V	R	−
PXO63-6NT11	V	R	−
PXO63-6NT12	V	R	−
PXO63-6NT15	WV	R	+
PXO63-6NT17	WV	R	+

[a]NT = NTG induced; RV = revertant; I = reisolated from inoculated plant.
[b]V = virulent; WV = weakly virulent; AV = avirulent; S = sensitive; R = resistant; + = positive; − = negative.

TABLE 2. Virulence of mutant strains derived from PXO63-6 strain of *Xanthomonas campestris* pv. *oryzae* on differential cultivars of rice

Strain[a]	Lesion length[b] (cm)		
	IR8	IR20	IR1545-339
PXO63-6	21.3 a	18.2 a	1.5 a
PXO63-6PT	18.3 bc	18.0 a	2.5 a
PXO63-6NT2	11.5 e	7.3 f	2.0 a
PXO63-6NT3	14.6 d	9.1 ef	1.7 a
PXO63-6NT5	3.1 f	2.1 g	1.0 a
PXO63-6NT8	17.2 bcd	13.4 cd	0.9 a
PXO63-6NT8I-1	20.1 ab	16.5 ab	2.1 a
PXO63-6NT8I-2	16.7 cd	14.6 bc	1.0 a
PXO63-6NT11	14.2 de	10.8 de	1.2 a
PXO63-6NT12	16.4 cd	12.2 cd	1.1 a
PXO63-6NT15	4.0 f	4.4 g	0.9 a
PXO63-6NT17	1.9 f	1.9 g	0.5 a

[a]NT = NTG induced; I = reisolated from inoculated plant.
[b]Means followed by a common letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

Sensitivity to phages. All colonies isolated were examined for phage sensitivity as a check for the possible inclusion of contaminants. Four isolates of phage that belong to OP_1 group (S. Wakimoto, *unpublished*), were collected in the Philippines. They were enriched with *X. campestris* pv. *oryzae* isolate PXO63-6, and the centrifuged supernatant containing phages (approximately at 10^8 PFU/ml) was used as phage preparation. The bacterial strains were tested for phage sensitivity by the spot plate technique.

Physiological and biochemical characteristics. The methods described by Reddy and Ou (10) were used for the tests of gelatin liquefaction; litmus milk reaction; reduction of nitrate; production of indole, ammonium, and H_2S; mode of utilization of glucose (O-F test); acid production from carbohydrates; Voges-Proskauer test; methyl-red test; oxidase activity; and utilization of citrate and tartrate. Enzyme activities of pectinase, cellulase (Cx) and protease were tested by the solid agar method using the basal medium (5 g peptone, 1 g Na-glutamate, 1 g $NH_4H_2PO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 15 g agar in 1,000 ml of water) containing 0.5% (w/v) pectin, 0.5% (w/v) carboxymethyl cellulose (CMC), and 1% (w/v) casein, respectively.

Stability of mutant strains in in vivo and in vitro. The stability of mutant strains in growth, colony color, streptomycin resistance, and virulence, both in vivo and in vitro was evaluated. In the in vitro experiment, the effect of sucrose concentration in PSA medium on the color of white colony mutants was tested. The strains grown on PSA slant (sucrose concentration, 2%) for 48 hr at 30 C, were transferred to the PSA plates with five different sucrose concentrations (0.5–5.0%) and incubated at 30 C. Growth and colony color were observed periodically until 14 days after incubation. Stability of mutant strains in vivo was tested by periodic reisolation of the bacteria from 40-day-old IR8 rice plants inoculated with 48-hr-old mutants. Growth, colony color maintenance, and streptomycin resistance were checked on PSA plates with streptomycin within 6 days after isolation. Reisolated bacteria also were tested for virulence by being inoculated to IR8, with the original mutant strain as a control.

TABLE 3. Changes in pigmentation, virulence and pathogenicity of induced revertant strains of *Xanthomonas campestris* pv. *oryzae*

Strain[a]	Pigmentation	Lesion length[b] (cm)		
		IR8	IR20	IR1545-339
PXO61PT	+	20.3 a	7.4 b	2.5 a
PXO61NT3	−	0.0 d	0.0 c	0.0 b
PXO61NT3RV-1	+	0.0 d	0.0 c	0.0 b
PXO61NT3RV-2	+	15.2 b	15.3 a	3.5 a
PXO61NT3RV-3	+	7.9 c	9.1 b	2.5 a

[a]NT = NTG induced; RV = revertant; I = reisolated from inoculated plant.
[b]Means followed by a common letter are not significantly different by least significant difference test, $P = 0.05$

TABLE 4. Phage sensitivity of mutant strains of *Xanthomonas campestris* pv. *oryzae*

Strain[a]	Phage strain				Lysotype
	1	4	19	22	
PXO61	+[b]	−	−	−	D
PXO61PT	+	+	−	−	C
PXO61NT3	+	+	+	−	B
PXO61NT3RV-1	+	+	−	−	C
PXO61NT3RV-2	−	−	−	−	E
PXO61NT3RV-3	−	−	−	−	E
PXO63-6	+	+	+	+	A
PXO63-6PT	+	+	+	+	A
PXO63-6NT2	−	−	−	−	E
PXO63-6NT3	−	−	−	−	E
PXO63-6NT5	+	+	+	+	A
PXO63-6NT8	+	+	+	+	A

[a]NT = NTG induced; RV = revertant.
[b]+ = sensitive; − = resistant.

RESULTS

Induced mutants and their virulence. The white-colony mutants were successfully derived from both parent strains, PXO61PT and PXO63-6PT, by NTG treatment. More mutants were produced from the latter than from the former. Fourteen mutants, including three revertant strains, were isolated. They were classified on the basis of virulence to rice and colony morphology on PSA medium into three types: weakly virulent with yellow colony; virulent with white colony; avirulent with white colony (Table 1). Weakly virulent mutants with yellow color derived from PXO63-6 produced shorter lesions than the parent strain regardless of the rice variety tested. White colony mutants derived from PXO63-6 were almost identical to the parent strain in virulence (Table 2). On the other hand, an avirulent mutant, with white colony PXO61NT3, was unstable in its colony color; ie, it spontaneously reverted from white to yellow during incubation. All the 20 isolates of the spontaneous revertant strains tested remained avirulent, like PXO61NT3RV-1. However, two virulent revertant strains of yellow colony (PXO61NT3RV-2 and PXO61NT3RV-3) were induced by NTG treatment, at a mutation rate of 2.5% from PXO61NT3. The former was virulent on IR8 and IR20, but less virulent on IR1545-339 (Table 3). Although virulence was restored in the latter, it was very weak on all the differentials (Table 3).

Phage-sensitivity of the mutant strains. On the basis of the sensitivity to four phage isolates of *X. campestris* pv. *oryzae*, the mutant strains were divided into five lysotypes: A, B, C, D, and E (Table 4). The mutant strains derived from PXO61 varied more widely in lysotype than those from PXO63-6. The mutant strains from PXO63-6 showed simple response to phages and were either resistant or susceptible to all phage isolates. No phage strain could lyse all strains of *X. campestris* pv. *oryzae*. No relationship was observed between lysotype and pathogenicity or virulence.

Physiological and biochemical characteristics of the wild-type and mutant strains. Four and 10 mutants derived from the parent strains PXO61PT and PXO63-6PT, respectively, of *X. campestris* pv. *oryzae* were compared for bacteriological characteristics. All the wild-type strains were Gram-negative, had weak motility in Hugh-Leifson's medium, and did not grow in minimal medium. All the strains of pathogenic groups I, II, and III showed similar characteristics in most of the bacteriological tests (Table 5). All the wild and mutant strains were rod-shaped with single or sometimes bipolar flagella, showing no distinct morphological differences among strains. They were strictly aerobic, oxidative in the O-F test, and negative in the methyl-red, Voges-Proskauer, and nitrate-reduction tests.

None of the strains produced indole and all produced ammonium. H_2S was slightly produced by the bacteria of group I, II, and mutants, but not by those of group III.

With the method used, casein and gelatin were not hydrolyzed. Tests for the enzyme activities of cellulase (Cx), pectinase, and oxidase were positive, variable, and negative, respectively. Almost all isolates used citrate slightly, but did not use tartrate. No differences were detected among the three groups in acid production from carbohydrates. That is, reactions were positive for glucose, xylose, arabinose, sucrose, glycerin, sorbitol, and inositol and negative for maltose, lactose, raffinose, starch, and dextrin

TABLE 5. Physiological and biochemical characteristics of the wild type and induced mutant strains of *Xanthomonas campestris* pv. *oryzae*

Test[a]	Pathogenic group I (18 strains)	Pathogenic group II (9 strains)	Pathogenic group III (3 strains)	Mutant strain (17 strains)
Gelatin liquefaction	−	−	−	−
Casein hydrolysis	−	−	−	−
Litmus milk reaction				
(alkaline)	+	+	+	+
Reduction of nitrate	−	−	−	−
Production of:				
Indole	−	−	−	−
H_2S	±	±	−	±
Ammonium	+	±	+	+
O-F test (oxidative)	+	+	+	+
Voges-Proskauer test	−	−[c]	−	−
Methyl-red test	−	−	−	−
Cellulase activity (Cx)	+	+	+	+
Pectinase activity	−	−	±	−
Oxidase activity	−	−	−	−
Citrate utilization	±	±	±	±[b]
Tartrate utilization	−	−	−	−
Acid production from:				
Glucose	+	+	+	+
Galactose	+	+[b]	+	+[c]
Fructose	+[b]	+	+	+
Mannose	+[b]	+	+	+
Xylose	+	+	+	+
Arabinose	+	+	+	+
Sucrose	+	+	+	+
Maltose	−	−	−	−
Lactose	−	−	−	−
Raffinose	−	−	−	−
Starch	−	−	−	−
Dextrin	−	−	−	−
Inulin	±	−	−	+
Glycerin	±	±	±	±[b,c]
Mannitol	±	−	±	±
Sorbitol	±	±	±	±
Inositol	±	±	±	±

[a]Test results: + = positive, ± = weakly positive, and − = negative.
[b]Strains derived from PXO61PT were negative.
[c]Some variations exist in individual strains.

(Table 5). Differences in reaction were noted for inulin and mannitol for group I, II, and mutants. A slight variation in individual strains also was found for galactose, fructose, and mannose. However, not one of the characteristics was comparable with virulence of the strains.

Some other properties of mutant strains. There was no significant difference in growth rate between PXO63-6PT (parent strain) and PXO63-6NT8 (white colony mutant) in PSB medium. Neither growth rate nor colony color was affected by concentration of sucrose in the medium.

Periodic reisolation of bacteria from rice leaves inoculated with mutant strains of PXO63-6PT and PXO63-6NT8 indicated that the properties of pigmentation and resistance to streptomycin remained stable at least 20 days during growth in vivo. The cells of PXO61NT3 (avirulent with white colony color), however, did not multiply in plant tissues.

DISCUSSION

X. campestris pv. *oryzae* varies widely in virulence (8) and in bacteriological characteristics (11). It also has been reported, however, that isolates collected from different geographical locations in Asia possess similar biochemical characteristics (10). In the case of *Pseudomonas solanacearum*, the biochemical characteristics of biotypes were as distinct (4) as the colony morphology of virulent and avirulent mutants on the tetrazolium chloride medium (7). In the present study, most of the different virulent strains of *X. campestris* pv. *oryzae* from the Philippines had similar bacteriological characteristics, as reported by Reddy and Ou (10). Although close relationships between biochemical properties and pathogenicity in *X. campestris* pv. *oryzae* have been reported by some workers (2,9,14,15), we could not reconfirm them. An attempt to isolate auxotrophic mutants, as demonstrated by Yamasaki et al (14), was unsuccessful. The relationship between the nutritional requirements and virulence of mutant strains obtained is under study. In our preliminary trials, the white or avirulent mutants were not successfully restored with pigmentation or virulence by mixed culturing with respective parent strains.

Wild-type strains were divided into five lysotypes based on sensitivity to phages. The mutant strains changed their lysotype remarkably and some mutants lost sensitivity to all phage strains. This change suggests that structures of receptor site or some biochemical factors related to phage reproduction might have been altered by mutation.

The mutant PXO61NT3, derived from the parent strain PXO61PT, lost its virulence and pigmentation. However, the white colony mutants derived from PXO63-6PT were not significantly different in virulence from their parent strain. Furthermore, the spontaneous revertant PXO61NT3RV-1, obtained from PXO61NT3, changed colony color from white to yellow but remained avirulent (Table 3). It appears therefore that pigmentation is not necessarily associated with pathogenicity in this bacterium. Similar results were reported for color mutant of *Corynebacterium facians* (6). The revertants, PXO61NT3RV-2 and PXO61NT3RV-3, induced from avirulent mutant PXO61NT3 were different from the parent strain PXO61PT in virulence to IR8 and IR20, suggesting the shift of pathogenicity from group I to group II. The information may indicate that the virulence of group II strains designated at IRRI had an origin similar to that of group I.

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