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Koga, Kazuharu
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

Furuya, Naruto
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

Wang, Zhiwei
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

Wakimoto, Satoshi
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

他

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Pathogenicity of the Tn5 Inserted Mutant Strains of *Pseudomonas glumae*

Kazuharu Koga*, Naruto Furuya, Zhiwei Wang, Satoshi Wakimoto'* and Nobuaki Matsuyama

Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University 46-01, Fukuoka 812, Japan

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Tn5 was inserted into the genome of *Pseudomonas glumae*, the plant pathogenic bacterium causing grain rot and seedling rot of rice, by conjugation with an *Escherichia coli* strain carrying pJB4JI, the plasmid vector for Tn5. Kanamycin resistant transconjugants appeared at the frequency of 2.73 x 10^{-5} per recipient. Of 250 Tn5 mutant strains obtained, 3 strains completely lost their pathogenicity to rice seedlings and 28 strains were attenuated in virulence. The inserted Tn5 element was detected in the chromosome of the avirulent transconjugants by Southern blot analysis. Avirulent transconjugants multiplied similarly to the virulent original strain in media, while the virulent strain multiplied to reach about 10 times higher level as compared to the avirulent transconjugants on/in the grains of the inoculated seedlings. The original strain could decompose all kinds of the four substrates, pectin, pectic acid, carboxymethylcellulose (CMC) and casein, while most of the avirulent and attenuated strains could not decompose some of them. These results suggest that the degrading enzymes are playing some important roles in the expression of pathogenicity.

INTRODUCTION

Pseudomonas glumae Kurita and Tabei was first reported as the causal bacterium of the grain rot disease of rice (Tominaga, 1971; Goto et *al.*, 1987). This disease was known to occur not only in Japan but also some other rice growing asian countries. In 1976, Uematsu *et* al. reported that this bacterium caused rice seedling rot in the nursery boxes under the controlled conditions of high humidity and high temperature (Uematsu *et al.*, 1976a and 1976b). The symptoms of this disease are characterized by rotting of rice seedlings and growth inhibition accompanied with chlorosis or necrosis on the leaves or leaf sheaths.

In plant pathogenic bacteria, the factor(s) such as phytotoxin, plant cell wall degrading enzymes, extracellular polysaccharide and lipopolysaccharide produced by bacteria are known to be involved in the mechanisms of the expression of symptoms (Braun, 1955; Collmer and Keen, 1986; Ferguson and Johnston, 1980; Morris *et al.*, 1977 and Stack *et al., 1980).*

Recent advances in the technology of DNA recombination have accelerated

* Present address: Leaf Tobacco Research Laboratory, Japan Tobacco Inc., Tochigi 323, Japan.
** Present address: Tokyo University of Agriculture, Tokyo 156, Japan.

genetic analyses of the mechanisms of pathogenicity expression in some plant pathogenic bacteria. An important and versatile tool in this approach is the transposon, which is randomly transposable into bacterial DNA at single-site inducing nonleaky polar mutations with a specific phenotype such as drug resistance. Transposon mutagenesis greatly facilitates the cloning of specific genes or gene regions of chromosome (De Bruijn and Lupski, 1984).

The mechanism of the pathogenicity of *P. glumae* to rice seedlings has not been clarified. The purpose of this study is obtaining induced mutant strains which lost pathogenicity by insertion of the transposon Tn5 into chromosomal DNA of P. *glumae* to clarify the mechanism of pathogenicity to rice seedlings.

MATERIALS AND METHODS

Bacterial strains and plasmid

A virulent mutant of *Pseudomonas glumae* Ml, which was induced from *P. glumae* Kyu82-34-2 strain by UV irradiation, and *Escherichia coli* HB101 (Boyer and Roulland -Dussoix, 1969), which is bearing plasmid pJB4JI (Beringer et *al.,* 1978) were used in this study.

Media and growth condition

The original strain Ml of *P. glumae* was grown on YPA plate (yeast extract 5 g, peptone 10 g, NaCl 5 g, agar 15 g per 1 liter of distilled water, pH 7.0) at 30°C. *E. coli* was grown on LB plate (Sambrook et *al.,* 1989) (bacto-trypton 10 g, yeast extract 5 g, NaCl 10 g per 1 liter of distilled water, pH 7.0) containing 50 ppm of kanamycin and 100 ppm of streptomycin at 37°C. Tn5-inserted transconjugants were grown at 30°C on YPA plate containing kanamycin and streptomycin at the same concentration. Minimal medium (K_2HPO_4 7 g, KH_2PO_4 3 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4 . 7H_2O$ 0.1 g, dextrose 5 g, agar 15 g per 1 liter of distilled water) containing kanamycin 50 ppm and streptomycin 100 ppm was used to select transconjugants. To grow inocula, YPDA slant (yeast extract 3 g, peptone 0.6 g, dextrose 3 g, agar 15 g per 1 liter of distilled water, pH 7.2) was used.

Tn5 mutagenesis

The donor strain, *E. coli* HB101, carrying the suicide plasmid pJB4JI::Tn5 was grown overnight in 5 ml of LB broth. The recipient strain, *P. glumae* Ml, was grown in 5 ml of YP broth for 48 hrs. The donor and recipient cells were mixed in sterilized tube (donor cells : recipient cells = 5:1), directly dropped on YPA plate and incubated at 30°C for 24 hrs. The cells were plated on the minimal medium containing kanamycin and streptomycin to select Tn5-inserted transconjugants. This procedure was practiced twice. The transconjugants were identified as *P. glumae* by serological reaction with anti-P. *glumae* serum preserved in the Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University (Wakimoto *et al.,* 1987).

Pathogenicity test

Transconjugants were cultured on YPDA slant at 30°C for 48 hrs and suspended into sterilized distilled water (ca. 10^9 cfu/ml). The rice grains *Oryza sativa* L. cv.

Asominori, after removing their husks, were disinfected by soaking in 70% ethanol and sodium hypochlorite solution (3% active chlorine). The disinfected grains were washed in sterilized distilled water and sown on 0.5% water agar in test tube (2 grain/tube) and inoculated with 0.2 ml of bacterial suspension. The virulence of the bacteria was evaluated at 10th day after inoculation.

Preparation of DNA

Genomic DNA was extracted from the cells of *P. glumae* cultured in minimal medium by the modified method of Silhavy et al. (1987). Extracted DNA was stored in TE buffer (Sambrook et al., 1989) at 4°C. Plasmid pJB4JI DNA was extracted from *E. coli* by the method of Maniatis *et al.* (1989). After restriction with Hind111 (Boehringer Mannheim), the plasmid DNA was separated by 0.7% agarose gel electrophoresis and purified Tn5 fragment by Gene clean™ II (Funakoshi Medical Inc.) to use as a probe in Southern blot analysis.

Southern blot hybridizations

Genomic DNA fragments digested with *Eco*RI (Boehringer Mannheim) were separated by 1% agarose gel electrophoresis in Tris-acetate buffer (Sato *et al.*, 1989) and transferred to Hybond™-NT (Amersham Inc.). For DNA labelling and DNA hybridization, ECL gene detection system (Amersham Inc.) was used according to the manufacturer's specification.

Pigment productivity

The pigment productivity of *P. glumae* on YPDA slant and in minimal medium was tested. The YPDA slant inoculated with the bacteria was incubated at 30°C and the minimal medium inoculated was shaken at the same temperature. The pigment productivity was observed at 48th hr of incubation with naked eye.

Multiplication of *P. glumae* in media and on/in rice grains

The multiplication of *P. glumae* was tested in sterilized distilled water, YP broth and minimal medium. The bacteria cultured on YPDA slant at 30°C for 48 hrs were suspended in sterilized distilled water and inoculated into sterilized distilled water at the initial concentration of ca. 10^5 cfu/ml, and YP broth and minimal medium at the initial concentration of ca. 10^7 cfu/ml. After inoculation, the inoculated sterilized distilled water was incubated under still culture condition at 30°C, and the YP broth and minimal medium were shaken at the same temperature. Water agar in test tube was used to grow rice seedlings to be inoculated. The bacterial cells cultured on YPDA slant at 30°C were suspended in sterilized distilled water. Each 10 ml of water agar (agar conc. 0.5%) supplemented with bacterial suspension at the concentration of ca. 10^7 cfu/ml was divided into test tubes. The rice grains were disinfected by soaking in 70% ethanol and sodium hypochlorite solution (3% active chlorine) and sown on the water agar in test tubes. The rice seedlings were periodically taken out, leaves and roots were discarded, and grains were homogenized and suspended in 5 ml of sterilized distilled water. The suspension was plated on the minimal medium containing antibiotics to count viable *P. glumae* cells.

Activity of bacterial enzymes related to degrading plant cell wall

To test the enzyme activities of the transconjugants degrading cell-wall, pectin, pectic acid (Wako Pure Chem. Indust. LTD.), carboxymethylcellulose (CMC), casein (Kyushu Katayama Chem. Inc.) were used as substrates. The bacterial strains cultured in 5 ml of YP broth at 30°C for 48 hrs were dropped on the plate of the media containing substrate (10μl) and incubated at 30°C for 24 hrs. The plates were then flooded with 1% (w/v) aqueous solution of cetyltrimethylammonium bromide for detecting degradation of pectin (Jayasanker and Graham, 1970), pectic acid (Jayasanker and Graham, 1970) and casein (Tsuchiya et al., 1983). For testing degradation of CMC (Andre et al., 1984), 0.1% (w/v) aqueous solution of congo red was flooded and blended with 1 M NaCl. When the bacterial strains produced degrading enzymes, transparent zone was formed around the colony. The diameters of the transparent zone (T) and the colony (C) were measured. The enzyme activities were shown by (T -C)/C.

RESULTS

Isolation of Tn5 mutants

When the bacterial cultures of *P. ghmae* strain Ml and *E. coli* strain HB101 containing the plasmid pJB4JI were mixed, *P. ghmae* transpositional mutants, characterized by kanamycin resistance, arose at a frequency of approximately 2.73 $x\,10^{-5}$ per recipient cells. Two hundred and fifty transconjugants were isolated. All transconjugants were confirmed to be *P. ghmae* by reaction with the anti-P. *glumae* serum which was previously cornfirmed to react specifically with *P. glumae*.

Pathogenicity

Of **250** transconjugants (tentatively TR numbered), 219 strains were as virulent as the original Ml strain, 28 strains were attenuated in some degree in virulence and 3 strains completely lost their pathogenicity.

Southern blot analysis of *Eco*RI-digested genomic DNA from mutants indicated the presence of Tn5 (Fig. 1).

Pigment productivity

On YPDA slant, all of the Tn5 inserted TR strains tested with only exception of TR5 strain produced yellowish-green pigment. However, only Ml strain produced the pigment in minimal medium (Table 1).

Multiplication of P. *glumae* in media and on/in rice grains

In sterilized distilled water, both strains of Ml and TR5 multiplied showing the same pattern (Fig. 2). The bacteia also multiplied almost similarly both in YP broth and minimal medium, but later TR5 strain reached higher level than Ml strain (Fig. 3 and 4). On/in rice grains, the bacteria multiplied to reach plateau at the 4th day after inoculation. The population level of the virulent strain Ml was about 10 times higher than that of avirulent strain TR5 (Fig. 5).

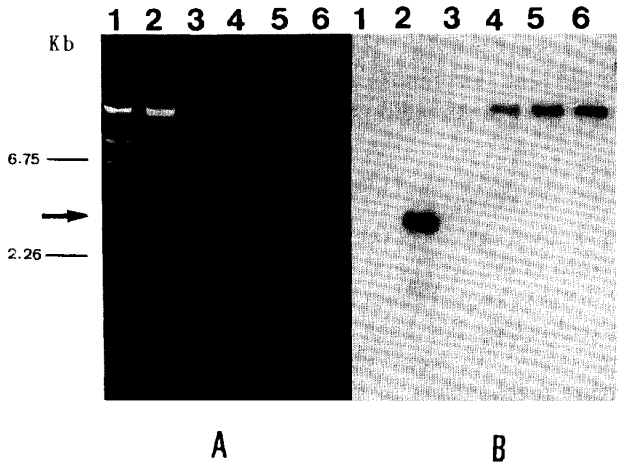

Fig. 1. Restriction enzyme and Southern blot analysis of genomic DNA isolated from Tn5 inserted mutants of *P. glumae*.

The genomic DNA fragments digested with *Eco*RI were separated by 1% agarose gel electrophoresis, transferred onto nyron membrane and hybridized with Tn5 fragment.

(A) Agarose gel electrophoresis (B) Southern hybridization. Lane 1: molecular weight marker lambda DNA digested with *Hin*dIII, lane 2: plasmid pJB4JI::Tn5 DNA digested with *Hin*dIII, lane 3-6: the chromosomal DNAs from *P. glumae* strains Ml and transconjugants (TR5, TR220, TR221) were digested with *Eco*RI. Arrow indicates Tn5 inserted fraction.

Table 1. Relationship between pigment productivity and pathogenicity in the randomly selected Tn5-inserted mutant strains of *P. glumae*.

Strains	Pigment productivity of bacteria grown		Pathogenicity
	on YPDA medium	in minimal medium	
Ml	+	+	+++
TR5	−	−	
TR220	+	−	
TR221	+	−	
TR8	+	−	+
TR50	+	−	+
TR179	+		+
TR184	+		+
TR131	+	−	+
TR129	+		++
TR153	+		++
TR199	+		++
TR204	+	−	++
TR136	+	−	+++
TR143	+		+++

Enzyme activities

All strains decomposed pectin. However, some of the TR strains such as TR5 and TR50 strains did not decompose pectic acid, CMC and casein. TR8, TR131, TR184, TR220 and TR221 strains did not decompose only CMC (Table 2).

DISCUSSION

P. glumae is known as a pathogenic bacteria causing bacterial grain rot (Tominaga, 1971; Goto *el* ***al., 1987)*** and seedling rot of rice (Uematsu et al., 1976a and 1976b). However, little is known about the mechanisms of symptom expression by the bacterium. Gene technology has been widely applied to clarify the mechanisms of pathogenicity expression with many plant pathogenic bacteria (Kerr, 1987; Nester, 1984) and the rotting mechanisms of rice seedlings by *P. glumae* were studied by applying Tn5-insertion in this experiment.

Of 250 transconjugants obtained, 219 (87.6%) were as virulent as wild strain, 28 (11. 2%) were attenuated in virulence, and 3 (1.2%) were avirulent. Thus, transconjugants were different in virulence. Salch ***et al. (1988)*** reported that the 2,686 kanamycin resistant strains were obtained by conjugation between *E. coli* WA803 (pGS9::Tn5) and ***P.*** *syringae* pv. ***tabaci.*** With regard to the pathogenicity of these mutant strains against tobacco leaves, 8 strains (0.3%) showed no symptoms and 10 strains were

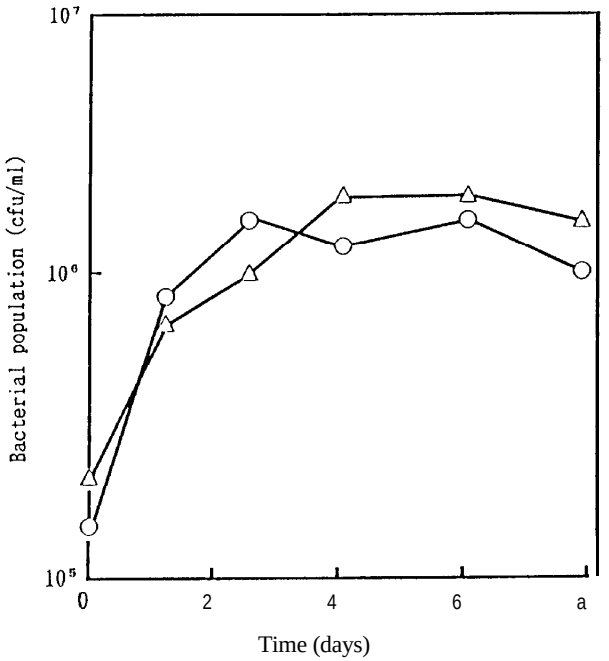

Fig. 2. Growth of *P. glumae* in sterilized distilled water at 30°C.
—○—: strain Ml -A-: strain TR5

attenuated (Salch and Shaw, 1988). The frequency of avirulent transconjugants in the case of ***P. glumae*** was much higher than in the case of ***P. syringae*** pv. ***tabaci.*** In ***P. solanacearum***, wilt induction to host plants had been attributed in part to the production of slime which composed mostly of extracellular polysaccharide (EPS) (Husain and Kelman, 1958). However, virulent strain of ***P. solanacearum*** which lacks of EPS productivity was found in Tn5 inserted strains (Xu et al., 1990) and plant cell wall degrading enzyme was found to be related to pathogenicity (Roberts *et al.*, 1988).

Our results suggest that many gene regions may be related to the symptom expression. However, a few gene regions which are critical for pathogenicity expression by ***P. glumae*** may exist, because 3 transconjugants completely lost their pathogenicity by Tn5 insertion.

The multiplication of the avirulent strain ***(P. glumae*** transconjugant TR5) was

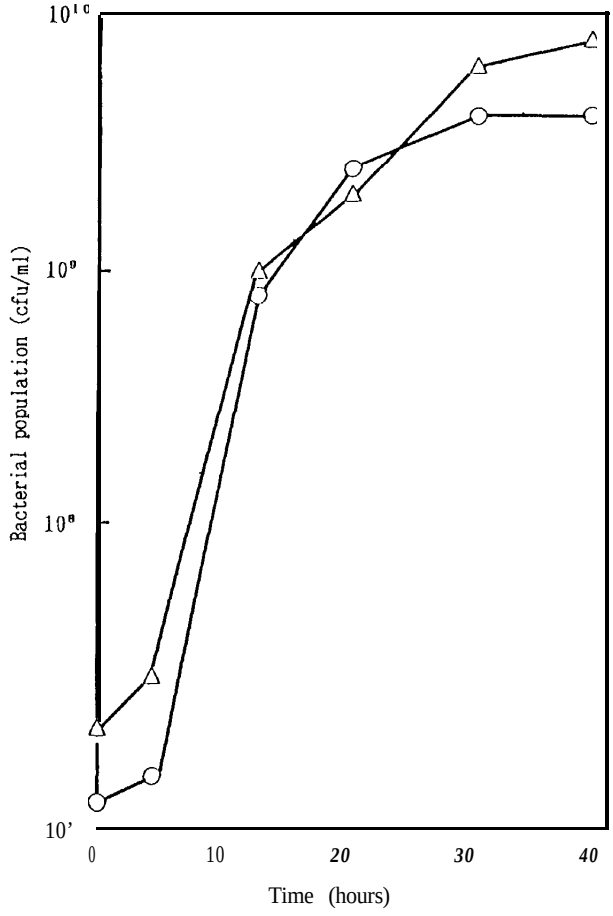

Fig. 3. Growth of ***P. glumae*** in YP broth under shaking condition at 30°C.
—○—: strain Ml —△—: strain TR5

compared with that of the virulent strain (*P. glumae* Ml) in media and on/in the grains of rice seedlings. Both avirulent and virulent strains multiplied showing almost the same patterns in the media. On/in the grains, however, the virulent strain multiplied to reach the level about 10 times higher than that of the avirulent strain. The difference of both strains in virulence may be due to the difference in multiplication inside host tissues.

Multiplication of pathogenic bacteria in plant tissue is considered to be depended upon nutritients released from host cells to intercellular spaces. Therefore, the enzyme activities which were considered to be related to degrading plant cell-wall were examined to know their roles in pathogenicity. As a result, the highly virulent strains decomposed all of the four substrates, pectin, pectic acid, CMC and casein, while attenuated strains lost some of the degrading activities of pectic acid, CMC or

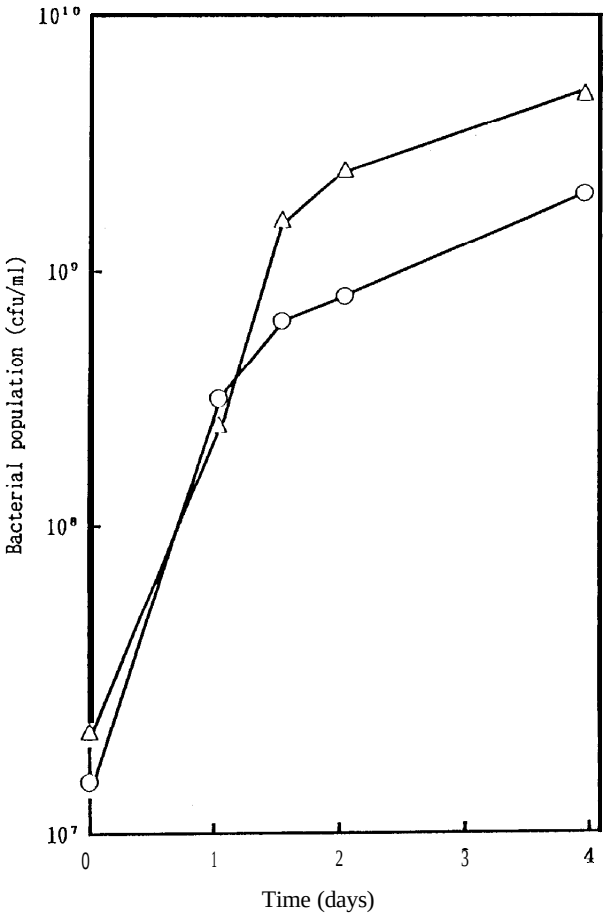

Fig. 4. Growth of *P. glumae* in minimal broth under shaking condition at 30°C.
—○—: strain Ml -A-: strain TR5

casein. These results suggest that the enzyme activities are playing some roles in the pathogenicity to rice seedlings. Recently, some phytotoxins were reported as a possible factor to cause rice seedling rot (Sato et al., 1989).
The productivity of phytotoxins from the Tn5 inserted mutants shoud be tested in future.

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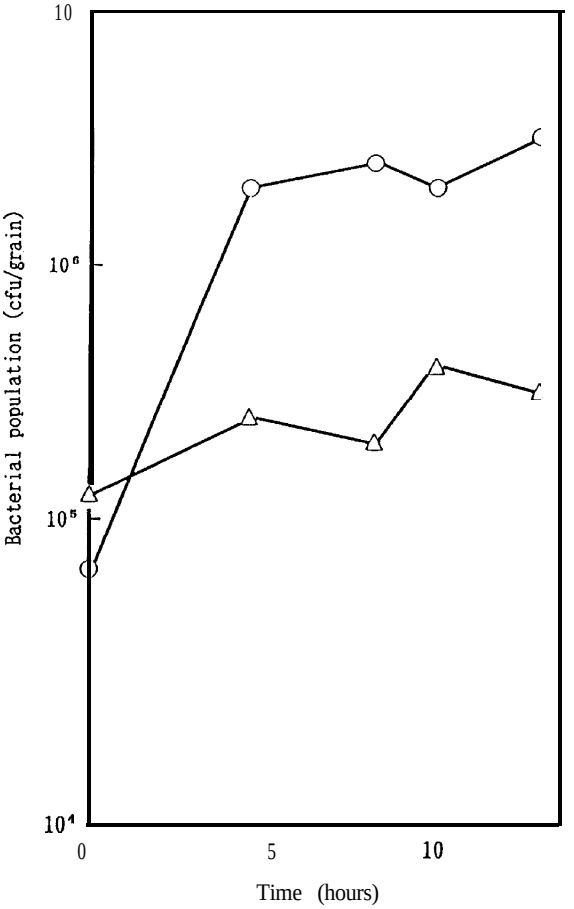

Fig. 5. Multiplication of *P. glumae* cells on/in the grains of rice seedlings, —○—: strain Ml —△—: strain TR5

Table 2. Relationship between enzyme activities related to decompose host cell-wall and pathogenicity in the randomly selected Tn5-inserted mutant strains of *P. glumae*

Strains	Substrates				Pathogenicity
	Pectin	Pectic acid	CMC	Casein	
M1	1.57[a]	0.59	0.67	1.02	+++
TR5	1.32	0	0	0	−
TR220	1.06	0.34	0	0.61	−
TR221	1.03	0.40	0	0.77	−
TR8	1.32	0	0	1.02	+
TR50	0.66	0	0	0	+
TR179	1.67	0.56	0.67	0.72	+
TR184	0.95	0.37	0	0.45	+
TR131	1.90	0.75	0	0.81	+
TR129	1.20	0.63	0.42	0.69	++
TR153	1.53	0.40	0.31	0.95	++
TR199	1.45	0.60	0.63	0.79	++
TR204	1.29	0.50	0.50	0.79	++
TR136	1.64	0.50	0.50	0.79	+++
TR143	1.56	0.40	0.32	0.73	+++

a) (T-C)/C ratio, T: diameter of transparent zone, C: colony diameter

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