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Development of Host Range Mutants of *Xanthomonas campestris* pv. *translucens*

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***Xanthomonas campestris* pv. *translucens* is the causal agent of bacterial leaf streak of cereal grains and grasses, and individual strains within the pathovar differ in their host range among the cereals. Coinoculation of a wide-host-range and a narrow-host-range strain resulted in the wide-host-range reaction. Transposon and chemical mutagenesis of the wide-host-range strain Xct4, pathogenic on barley, wheat, rye, and triticale, resulted in variants with reduced host range. When pathogenicity was inactivated independently for barley, wheat, triticale, and rye, wild-type symptoms were retained on the other members in the host range. Testing of some host range mutants on additional varieties of the cereals indicated some cultivar specificity. In addition, mutants nonpathogenic on combinations of the hosts or on all hosts were isolated. This suggests that there are independent positive factors determining host range in this species, rather than an avirulence gene system such as those determining race specificity in other plant pathogens.**

Xanthomonas campestris pv. *translucens* causes bacterial leaf streak of cereal grains and range grasses, a disease rapidly growing in economic importance throughout most cereal-growing areas of the world. Characteristic symptoms of the disease include watersoaking and translucence of infected leaf blades, formation of a sticky substance, gum xanthan, on the plant tissue, and blackening of the chaff and kernels (3). The disease is primarily transmitted through infested seed, and in recent years, it has been estimated to cause as much as 40% yield loss under irrigated conditions (24).

X. campestris pv. *translucens* is one of more than 100 pathovars of *X. campestris*. Each pathovar has a different set of compatible hosts (4). Included in the host range of *X. campestris* pv. *translucens* are barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), oats (*Avena sativa* L.), rye (*Secale cereale* L.), triticale (*Triticosecale* Whittmack), and several range grasses (6). Specificity within this host range is exhibited by individual strains and is characteristic of this pathovar. Each individual strain may affect single hosts or combinations of hosts among these major cereals (2, 3, 10, 13, 15, 16, 22, 26, 27).

Factors determining the host range of plant-pathogenic bacteria have been studied in only a few systems, but already two distinct principles have emerged: (i) host range may be determined by genes that provide positive functions for pathogenicity against specific hosts, or (ii) host range may be controlled by avirulence genes limiting host range. In *Agrobacterium* and *Rhizobium* spp., genes encoding positive host range functions have been identified by mutations causing a reduction in host range and by expansion of host range upon introduction of plasmids containing host range determinants (1, 18, 19, 32). In contrast, studies with *X. campestris* pv. *vesicatoria*, a pathogen of pepper and tomato (8, 30), *X. campestris* pv. *malvacearum* on cotton (11; D. Gabriel, *ASM News* **52**:19–25, 1986), and *Pseudomonas syringae* pv. *glycinea*, a pathogen of soybean (30) have established that avirulence genes function as negative dominant factors determining specificity toward different cultivars of their respective hosts. The avirulent reaction is a hypersensitive response or rapid tissue necrosis at the inoculation point, and it has been shown that mutations in these avirulence genes result in an expansion of host range (31).

Preliminary work with *X. campestris* pv. *translucens* suggested that positive factors played a role in determining host range. In a study by Kim (H. Kim, Ph.D. dissertation, Montana State University, Bozeman, 1982), a wide-host-range strain of *X. campestris* pv. *translucens* was subjected to transposon Tn*5* mutagenesis, and the resulting kanamycin-resistant colonies were screened on the five commercial members of the host range; 1 colony in 1,200 was determined to have lost its pathogenicity on barley but was able to cause disease symptoms on other members of the host range. In addition, a hypersensitive response was lacking when strains of *X. campestris* pv. *translucens* were inoculated to plants that were not within their host range. Work described here showed that coinoculation of a wide- and a narrow-host-range strain resulted in the wide-host-range reaction rather than the avirulent reaction that would be predicted if avirulence genes controlled host specificity. In this study, chemical and transposon mutants of a wide-host-range strain were isolated with specific host range reductions, further supporting a role for positive functions in host range determination.

MATERIALS AND METHODS

Bacterial strains, growing conditions, and media. The bacterial strains used and mutants obtained are listed in Table 1. Nutrient-yeast-glucose broth (NYGB) (23) was used to grow *Xanthomonas* spp. and *Escherichia coli* cultures before mutagenesis and for hypodermic inoculations. Conjugations were conducted on yeast extract-dextrose-$CaCO_3$ agar (YDC) (23) containing glucose at 1 instead of 20 g/liter; this lower level of glucose reduced the production of extracellular polysaccharide (EPS), which is thought to inhibit conjugation (17). A modification of Starr minimal medium (29) was devised for selection of transposon mutants and was designated XMAA. In addition to the basal salts, medium XMAA included glutamic acid (1 mg/ml) and methionine (200 μg/ml) to satisfy the growth requirements of the xanthomonads. Lysine (100 μg/ml) was added for isolation of lysine auxotrophs. Kanamycin was included at 25 μg/ml in agar and 10

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TABLE 1. Bacterial strains

Strain	Host range[a]				Relevant characteristics	Source
	B	W	R	T		
X. campestris pv. *translucens*						
Xct4	+	+	+	+	Wild type; Glu, Met; kasugamycin resistant	L. Moore
Xct4s	+	+	+	+	Streptomycin-resistant derivative of Xct4	This work
Xct67	+	−	+	+	Wild type; Glu, Met; kasugamycin resistant	D. C. Sands
Xct4.1	+	+	−	+	Tn*5* insertion mutant of Xct4, kanamycin resistant	This work
Xct4.2	+	+	−	+	EMS mutant of Xct4s	This work
Xct4.3	+	+	−	+	EMS mutant of Xct4s	This work
Xct4.4	+	+	−	+	EMS mutant of Xct4s, reduced EPS	This work
Xct4.5	+	−	+	+	Tn*5* insertion mutant of Xct4, kanamycin resistant	This work
Xct4.6	+	−	+	+	EMS mutant of Xct4s	This work
Xct4.7	−	+	+	+	EMS mutant of Xct4s	This work
Xct4.8	+	+	+	−	EMS mutant of Xct4s	This work
Xct4.9	−	+	−	+	EMS mutant of Xct4s	This work
Xct4.10	+	−	+	−	EMS mutant of Xct4s	This work
Xct4.11	−	−	−	−	EMS mutant of Xct4s	This work
E. coli						
HB101(pUW964)::Tn*5*, Tn*7*					Tn*5* donor; streptomycin, kanamycin, trimethoprim resistant	A. Weiss
S17(pSUP2021)::Tn*5*					Tn*5* donor; kanamycin resistant	Simon (25)

[a] B, Barley; W, wheat; R, rye; T, triticale.

μg/ml in broth. Since the xanthomonads have high levels of natural resistance to kasugamycin, this antibiotic was added (25 μg/ml) to select against *E. coli* donors. Medium XMAB included all components of XMAA except lysine and was used to screen the transposon mutants for lysine auxotrophy. Chemical mutants were selected on medium XMAC, equivalent to XMAA but without kanamycin and including streptomycin (200 μg/ml). Medium XMAD, containing all components of XMAA except lysine and kanamycin and including streptomycin (200 μg/ml), was used to screen the chemical mutants for lysine auxotrophy.

Coinoculation experiments. Wild-type strain Xct4, pathogenic on all hosts tested, was coinoculated with Xct67, a wild-type strain avirulent on wheat but virulent on barley, rye, and triticale (Table 2). Both strains were grown in NYGB to a density of 5.0×10^7 CFU/ml and were mixed in ratios of 1:10, 1:1, and 10:1. These suspensions were inoculated to seedling grain plants of each member of the host range by injecting the mixed suspensions into the stems of the plants by the hypodermic injection technique described below. The plants were assessed after 1 week for the watersoaking reaction characteristic of the disease.

Transposon mutagenesis. The *E. coli* Tn*5* donor strains HB101(pSUP2021) and S17(pUW964) and the recipient Xct4 were grown in NYGB at 28°C without shaking for 24 and 48 h, respectively. The bacteria were gently mixed in a ratio of 9 parts recipient to 1 part donor and spread on plates of YDC agar. After a 24-h incubation at 32°C, the cultures were placed at 4°C for 6 h to enhance the recovery of transposon mutants. The resulting bacterial growth was suspended in 10 ml of sterile distilled water and placed on XMAA medium. Seven to 10 days of incubation were required to obtain kanamycin-resistant derivatives of Xct4 on this medium.

Chemical mutagenesis. Strain Xct4s, a spontaneous streptomycin-resistant derivative of Xct4 selected by the gradient plate method of Carlton and Brown (5), showed wild-type levels of virulence in greenhouse tests and was useful for chemical mutagenesis. Before exposure to ethyl methanesulfonate (EMS), Xct4s was grown in shake culture at 28°C for 24 h in NYGB. The culture was diluted 1:1 with a 4% solution of EMS in NYGB and shaken for an additional 6 h at 28°C. The culture was pelleted, washed twice with TE (pH 8.0) buffer (20), suspended in 10 ml of XMAD broth, and incubated for 6 h in a 28°C shaker. Dilutions were plated on XMAC agar.

Plant assays of putative mutants. The seed used in the plant assays of mutants was obtained from the California Crop Improvement Program, University of California, Davis. Varieties of the four grains used in host range testing included "Anza" wheat, "CM67" barley, "Merced" rye, and "Juan" triticale. Selection of the varieties of the four grains used in host range testing was based on the least amount of time needed for symptoms to appear and the greatest severity of symptoms. Additional varieties used in the cultivar specificity experiment are listed in Table 3 and were chosen on the basis of availability. All plants were inoculated in the three-leaf stage, approximately 10 days after planting.

Two inoculation techniques were used in host range testing: spray and hypodermic injection (10). Inoculum was prepared for the spray method by placing an agar block

TABLE 2. Results of coinoculation of wide- and narrow-host-range strains of *X. campestris* pv. *translucens*

Strain	Host range reaction[a]			
	B	W	R	T
Controls				
Xct4	+	+	+	+
Xct67	+	−	+	+
Coinoculation (Xct4-Xct67 ratio)				
1:1	+	+	+	+
1:10	+	+	+	+
10:1	+	+	+	+

[a] See Table 1, footnote *a*.

TABLE 3. Results of inoculating additional varieties of grains with mutant and wild-type *X. campestris* pv. *translucens* isolates

Plant and variety	Virulence				Plant source
	Xct4s	Xct4.4	Xct4.6	Xct67	
Barley					
CM67[a]	+	+	+	+	CCIP[b]
Kimberly	+	+	−	+	D. Mills
Hector	−	−	−	−	D. Sands
3066 Piroline	+	+	+	+	D. Sands
Wheat					
Anza[a]	+	+	−	−	CCIP
Newana	+	−	−	−	D. Sands
Lew	+	+	+	−	D. Sands
Glenman	+	−	−	−	D. Sands
Oats					
Cayuse	−	−	−	−	D. Sands
Border	−	−	−	−	D. Mills
Oatana	−	−	−	−	D. Sands
Glenman	−	−	−	−	D. Sands
Rye					
Merced[a]	+	−	+	+	CCIP
Butte	+	−	+	+	D. Sands
Triticale					
Juan[a]	+	+	+	+	CCIP
McIntosh	+	+	−	−	D. Sands

[a] Varieties used in mutant screening.
[b] CCIP, California Crop Improvement Program.

containing a single bacterial colony in 10 ml of sterile distilled water and mixing well. The resulting suspension was sprayed onto the leaves of the plants with a sprayer (Sigma Chemical Co., St. Louis, Mo.). This technique was used for preliminary screening of transposon mutants. Inoculum for hypodermic injection was grown in sterilized microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) containing 150 μl of NYGB amended with appropriate antibiotics in each well. The plates were covered with sealing tape (Dynatech Laboratories) and incubated for 4 days at 28°C without shaking. Bacterial suspensions from each well were drawn into a 1-ml syringe (Becton Dickinson Co., Rutherford, N.J.) through a 27-gauge needle (American Pharmaseal Corp., Glendale, Calif.) punched through the sealing tape and were inoculated individually into the stems of two seedlings of each cereal in the host range. The plants were kept at 22 to 25°C for 1 week, at which time they were examined for the watersoaking and translucent streaking characteristic of the pathogenic reaction. Mutants showing any deviation from the wild-type host range were inoculated a total of three times for verification.

DNA isolation and hybridization. DNA was isolated for hybridization experiments from kanamycin-resistant strains grown in XMAA broth. A modified Currier and Nester (7) method was used; lysis time was increased to 2 h at 37°C and NaOH was omitted to allow isolation of total DNA. Two phenol and two chloroform extractions were performed, and the DNA was purified on cesium chloride-ethidium bromide gradients before digestion with *Eco*RI for hybridizations. Southern blotting and hybridizations were conducted by the method of Maniatis et al. (20). An internal fragment of Tn*5* for use as a probe was excised from plasmid ColE1::Tn*5* by digestion with *Hin*dIII and gel purified. Nick translation of the Tn*5* probe, labeling with ^{32}P or biotin, and detection of the blot followed the procedures described by Maniatis et al. (20) and in the nick translation reagent kit (Bethesda Research Laboratories, Gaithersburg, Md.) and DNA detection system (Bethesda Research Laboratories).

Comparison of colonization of an avirulent mutant and the wild type in plant tissue. Cultures of wild-type Xct4s and EMS mutant Xct4.4, nonpathogenic on rye, were grown in NYGB for 48 h to a density of 6.0×10^7 CFU/ml. Rye plants were inoculated by hypodermic injection as described above, and samples of the plants were taken daily for 10 days beginning immediately after inoculation. The leaves and stems were severed from the plant, and segments of the leaf blade 2 cm in length, with the inoculation point as the center, were excised. Five random segments were placed in a test tube containing 10 ml of sterile distilled water and mixed so that all leaf segments were immersed; these were allowed to soak at room temperature for 4 h. Dilutions were made of the resulting suspensions, and 100 μl of each dilution was plated on YDC agar amended with cycloheximide (50 μg/ml) and streptomycin (400 μg/ml). There were four replications of this isolation procedure daily for plants inoculated with the wild-type and with the mutant.

RESULTS

Coinoculation experiments. Coinoculation of wild-type strain Xct4, virulent on barley, wheat, rye, and triticale, and Xct67, virulent on barley, rye, and triticale, resulted in the virulent reaction on all hosts regardless of the relative concentration of each strain (Table 2).

Transposon mutagenesis and screening of mutants. Transposon mutagenesis of the wide-host-range strain Xct4 produced approximately 7,000 kanamycin-resistant colonies from several matings. As an indicator of the frequency of mutation, the Tn*5* mutants were screened for lysine auxotrophy, and 0.1% required lysine.

Approximately 5,300 Tn*5* mutants were screened in plant assays by spraying or injecting a suspension of each isolate into each of the major hosts. Seventeen mutants were detected as having an aberrant host range. Five of the 17 mutants were used in Southern hybridization experiments, and the presence of a single copy of Tn*5* was detected in each. Two mutants, Xct4.1, nonpathogenic on rye, and Xct4.5, nonpathogenic on wheat, retained their normal levels of virulence on the other hosts. Several additional mutants exhibited reduced virulence but still produced the characteristic watersoaking.

Chemical mutagenesis and screening of mutants. A 33% survival rate was determined after exposure of the streptomycin-resistant strain Xct4s to EMS. A total of 1,500 survivors were selected for host range assessment, and the frequency of lysine auxotrophy among these putative mutants was 0.26%. Plant assays disclosed a variety of mutations involving host range (Table 1). Mutations for pathogenicity toward individual members of the host range included single mutants nonpathogenic on barley, wheat, or triticale. Three strains producing no watersoaking or characteristic disease symptoms on rye were detected (Fig. 1), one with a reduced level of EPS. The reduction in EPS in this mutant may be unrelated to host range determination, since two additional mutants with reduced amounts of EPS were virulent on all hosts. Two of the mutants gave double host range variations, one nonpathogenic on barley and triticale and the other nonpathogenic on wheat and rye. All reduced-host-range mutants produced normal levels of virulence and watersoaking on the other members of the host range. One strain gave no pathogenic reaction on any host. In no case did a host range mutant cause a hypersensitive response (Fig. 1).

Cultivar specificity experiments. The results of inoculation of rye mutant Xct4.4 and wheat mutant Xct4.6 to additional

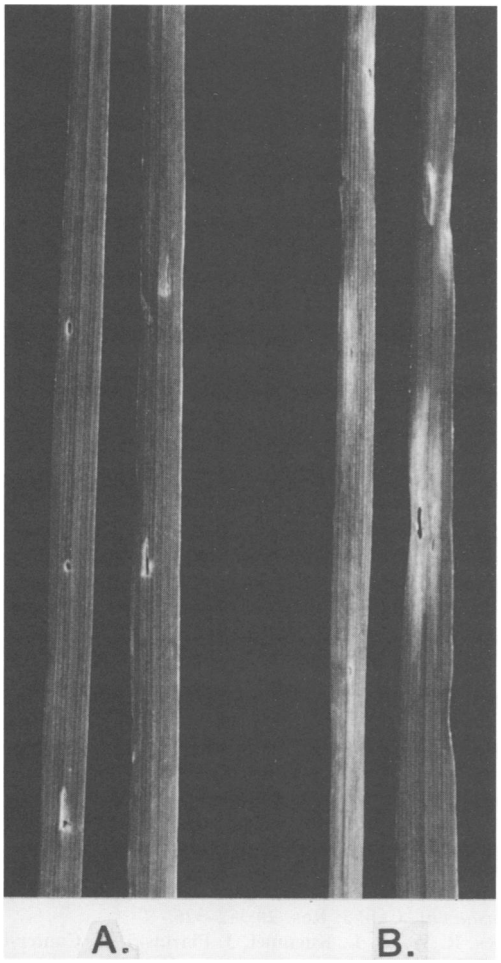

FIG. 1. Reaction on rye 7 days after inoculation with (A) EMS mutant Xct4.4, avirulent on rye (*Secale cereale*), and (B) wild-type Xct4S.

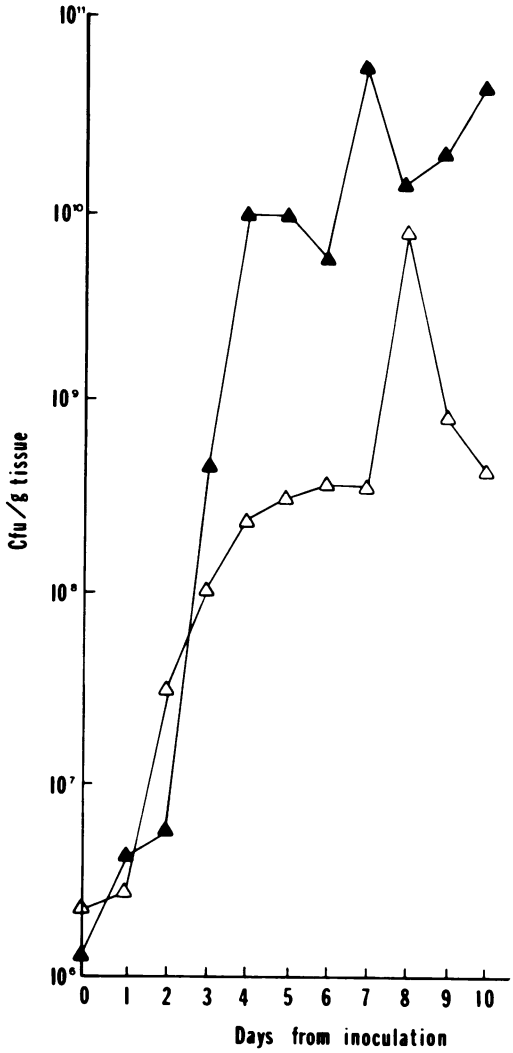

FIG. 2. Colonization of EMS mutant Xct4.4 (△), avirulent on rye, and wild-type Xct4s (▲) in rye (*S. cereale*) tissue over a 10-day period.

varieties are listed in Table 3. Some cultivar specificity was indicated in these mutants. Xct4.4, the rye mutant, remained negative on "Butte," the additional rye variety tested. However, it was also nonpathogenic on two additional varieties of wheat, "Newana" and "Glenman." The wheat mutant Xct4.6 was nonpathogenic on three of the four varieties of wheat tested, and also on "Kimberly" barley and "McIntosh" triticale. In no case did the mutants gain pathogenicity toward hosts that the wild type did not affect.

Comparison of colonization in plant tissue. The relative growth patterns for wild-type Xct4s and mutant Xct4.4 inoculated to rye plants are shown in Fig. 2. Symptoms appeared on day 4 on plants inoculated with the wild-type strain, and the number of wild-type bacteria in plant tissue continued to increase throughout the experiment. After the second day, the mutant strain grew more slowly than the wild type, and there was a significant difference in the numbers achieved by each strain. By day 10, the watersoaking and leaf streaking had spread the length of the leaf blade in most plants inoculated with the wild type. The avirulent mutant failed to produce watersoaking or streaking throughout the experiment.

DISCUSSION

Work by Kim (Ph.D. dissertation) provided preliminary indications that factors determining host range in *X. campestris* pv. *translucens* were positive and could be inactivated by mutagenesis. Our results for coinoculations of wide- and narrow-host-range strains also suggest that virulence factors, not avirulence genes, are the probable determinants of host range. Had the plant reaction been determined by dominant avirulence genes in the bacterium, the narrow-host-range reaction would have been expected when the strains were coinoculated. Therefore, we attempted to inactivate the assumed positive virulence factors through mutagenesis.

Virulence was inactivated for each member of the host range, suggesting that there are individual determinants for each of the economically important hosts. The frequency of mutations for nonpathogenicity or reduced virulence on rye was higher than for any of the other hosts, suggesting the possibility that more than one gene determines the rye-positive phenotype. Results of the cultivar specificity experiments indicated additional changes in host range among the mutants, together with the originally selected mutation. The rye mutant remained negative on both rye varieties used; however, it was also unable to produce watersoaking on two additional varieties of wheat. A similar situation occurred

with the wheat mutant. Although the mutations caused additional changes in cultivar specificity, all changes were effective in reducing host range. In no case was an expansion of host range noted. Cultivar specificity as well as host specificity appears to be determined by positive factors in *X. campestris* pv. *translucens*.

In addition to the mutants that lost virulence on single members of the host range, two mutants were found to have double host range mutations. This may indicate that the genes for pathogenicity on those hosts are in proximity and that a single mutation may inactivate both. The single mutant producing no pathogenic reaction on any host may indicate that a gene for general pathogenicity on all hosts was present in the wild type. Such genes for general pathogenicity could be conserved among the xanthomonads; M. J. Daniels (North Atlantic Treaty Organization Advanced Research Workshop, Dillington College, Somerset, United Kingdom, 1 to 6 September, 1985) was able to restore pathogenicity to a nonpathogenic mutant of *X. campestris* pv. *campestris*, the bacterial agent of black rot of crucifers, by complementation with a genomic library clone of *X. campestris* pv. *translucens*.

After comparison of the wild type and the rye mutant in the colonization study, it became obvious that the wild type was capable of increasing in number more quickly and attaining higher final numbers than the rye mutant. However, the mutant was able to colonize the plant tissue and was not lacking in overall fitness, as shown by its ability to cause disease on the other members of the host range.

In the *Agrobacterium tumefaciens* system, the failure to produce cytokinins has been established as a factor in limiting host range (33). Little is known about the pathogenicity of *X. campestris* pv. *translucens*; however, uncharacterized pathogenicity factors may have a role in determining host range. It is not likely that binding of the nonpathogenic mutants of *X. campestris* pv. *translucens* to plant cells functions to reduce host range, as the rye-negative mutant was able to increase in number and colonize the tissue over the 10-day period. It is reasonable to assume that the mutants are lacking in some factor necessary to begin the pathogenic watersoaking response in the plant. The factor may be a structural or chemical signal produced by the bacterium and recognized by the plant, or bacterial recognition of a plant product specific to each host. In either case, a mutation in the bacterium may disrupt the interaction and cause a nonpathogenic response.

It is apparent that in this system, host range may be determined by factors having a positive rather than a negative effect. Experimentation is in progress to reestablish pathogenicity in reduced-host-range mutants by complementation with genomic cosmid clones and to begin studies of the structure and function of the genes involved in host range determination in *X. campestris* pv. *translucens*.

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