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The Role in Pathogenicity of Some Related Genes in *Xanthomonas campestris* Pathovars *campestris* and *translucens*: A Shuttle Strategy for Cloning Genes Required for Pathogenicity

Maria K. Sawczyc, Christine E. Barber, and Michael J. Daniels

John Innes Institute, Norwich NR4 7UH, U.K.
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To determine whether *Xanthomonas campestris* pathovars possess related pathogenicity genes, a genomic library of DNA of *X. campestris* pv. *translucens* (constructed in the cosmid pLAFR1) was mobilized into nonpathogenic mutants of *X. campestris* pv. *campestris*, 8237 and 8288. Two unrelated *X. c.* pv. *translucens* clones, pIJ3021 and pIJ3022, restored pathogenicity of the mutants to turnips and production of protease by mutant 8237. pIJ3021 was homologous to pIJ3020, a cosmid containing *X. c.* pv. *campestris* DNA known to complement 8237. A Tn*5* insertion in pIJ3022 that abolished the ability to restore function to 8237 was transferred by marker exchange into the genome of *X. c.* pv. *translucens*, yielding a strain that had lost pathogenicity to wheat. Pathogenicity of mutant 8288 was restored by the *X. c.* pv. *translucens* clone pIJ3003. A Tn*5* insertion in pIJ3003 that abolished the restoration of pathogenicity to 8288 was also transferred to the *X. c.* pv. *translucens* genome, but the resulting mutant retained pathogenicity to wheat. In addition, transconjugants arising from mass reciprocal transfer of *X. c.* pv. *campestris* and *X. c.* pv. *translucens* libraries into the heterologous pathovars were tested for modification of pathogenicity to the homologous and heterologous plant hosts (that is, extension or restriction of host range), but no clones showing reproducible effects were found.

Genetic methods for investigating the interaction of pathogens with plants have attracted increasing interest in recent years (Panopoulos and Peet 1985; Daniels *et al.* 1988). The ability to clone genes encoding factors important for parasitism and pathogenicity should greatly broaden the scope for understanding these complex phenomena. The gram-negative bacterial pathogens are particularly suitable subjects because the necessary molecular genetic procedures have been perfected and numerous general- and specific-purpose gene cloning vectors have been developed.

In our laboratory we have studied primarily *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson, which causes black rot of crucifers (Williams 1980) and is one of more than 120 pathovars of the species *X. campestris*. Each pathovar typically infects a small number of related plant species, but taken together the species *X. campestris* includes major pathogens of many wild and cultivated plants.

One strategy that has been used successfully to clone genes required for pathogenicity is to isolate mutants that are defective in pathogenicity and then to screen a genomic library of wild-type DNA for recombinant plasmids that are able to restore pathogenicity to the mutants (Daniels *et al.* 1984a,b). Because this approach often requires the repetitive testing of many hundreds or thousands of single colonies for pathogenicity, it is practicable only for host-pathogen systems for which a simple, rapid, and reliable pathogenicity test is available.

In this paper we describe an approach for studying pathogenicity genes that should be applicable to many pathovars. Because *X. campestris* pathovars are believed to be closely related, it is likely that many pathogenicity genes are conserved (either structurally or functionally) among pathovars. If so, it may be possible to complement mutants of one pathovar with DNA from another.

To test this concept, we compared *X. c.* pv. *campestris* and *X. c.* pv. *translucens* (Jones *et al.*) Dye, the agent of black chaff of wheat (Smith *et al.* 1919). Genes were identified in a library of *X. c.* pv. *translucens* DNA that restored the pathogenicity of two mutants of *X. c.* pv. *campestris* to turnips. The cloned DNA was mutagenized with Tn*5*, and the mutations were transferred to the genome of *X. c.* pv. *translucens* so that the role of the genes in pathogenicity to wheat could be assessed.

In addition, hybridization studies showed that DNA homologous with fragments containing cloned *X. c.* pv. *campestris* pathogenicity genes was present in a range of other pathovars. We also attempted, without success, to modify the behavior of the two pathovars on both homologous and heterologous hosts by transferring cloned DNA between pathovars.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists strains and plasmids used. Bacteria were cultured as described by Turner *et al.* (1984).

Genetic and molecular genetic techniques. Plasmid transfer and other genetic techniques were described by Turner *et al.* (1984). pLAFR1 libraries of *X. c.* pv. *campestris* and *X. c.* pv. *translucens* were constructed as described by Daniels *et al.* (1984b), and Tn*5* mutagenesis, marker exchange, and DNA hybridization procedures followed the protocols of Turner *et al.* (1985), except that the plasmid used to displace pLAFR1 derivatives for marker exchange was pIJ3011, a spontaneous Sp^s

Address correspondence to M. J. Daniels.
Present address of M. J. Daniels: The Sainsbury Laboratory, John Innes Institute, Colney Lane, Norwich NR4 7UH, U.K.

derivative of pPH1JI (Beringer *et al.* 1978), isolated in our laboratory.

Pathogenicity tests. Aseptic turnip seedlings and mature plants were inoculated with bacterial colonies or with suspensions containing a range of bacterial concentrations (Daniels *et al.* 1984a). Essentially identical techniques were used to prepare aseptic wheat (cv. Maris Huntsman) seedlings, which were inoculated by clipping the tips of leaves with sterile scissors and applying inoculum with a toothpick. Compost-grown wheat seedlings were also inoculated by infiltrating bacterial suspensions of a range of concentrations into leaf tissue using a hypodermic syringe with a fine needle (26 standard wire gauge). Plants were maintained at 25° C under high humidity after inoculation.

Enzyme assays. Polygalacturonate lyase and protease in culture fluids or cell extracts were assayed as described by Dow *et al.* (1987).

RESULTS

Properties of the DNA library from *X. c.* pv. *translucens*. The library of *X. c.* pv. *translucens* DNA consisted of 2,900 recombinant plasmids maintained in *Escherichia coli* ED8767. Plasmids were isolated by the method of Holmes and Quigley (1981) from 14 randomly chosen colonies and digested with *Eco*RI, and the products were separated by electrophoresis. Each plasmid gave a 21.6-kilobase (kb) fragment (the pLAFR1 vector) together with two to seven (average 5.1) additional fragments of *X. c.* pv. *translucens* DNA. The mean total insert size per plasmid was 23 kb (range 14–33 kb).

The pooled library was transferred into appropriate *E. coli*, *X. c.* pv. *campestris*, or *X. c.* pv. *translucens* strains by conjugation using a triparental mating (Daniels *et al.* 1984b), selecting for transfer of the tetracycline-resistance (Tc^r) marker of pLAFR1. The transfer frequency was in the range of 10^{-5} to 10^{-3} per recipient.

By testing the phenotype of a sample of the resulting transconjugants, we found that *X. c.* pv. *translucens* DNA was able to "complement" or restore function to some auxotrophic and unpigmented mutants of *X. c.* pv. *campestris* and *X. c.* pv. *translucens* and some *E. coli* auxotrophs. These experiments showed that the library has characteristics similar to those of the *X. c.* pv. *campestris* library described by Daniels *et al.* (1984b).

Effect of *X. c.* pv. *campestris* and *X. c.* pv. *translucens* on turnip and wheat. The symptoms produced by wild-type and mutant *X. c.* pv. *campestris* in seedlings and mature leaves of turnip have been described previously (Daniels *et al.* 1984a). Turnip seedlings whose stems were stabbed with a needle loaded with *X. c.* pv. *translucens* inoculum showed no visible symptoms, and leaves infiltrated with broth cultures containing up to 10^9 colony-forming units per milliliter developed only faint chlorosis restricted to the infiltrated area, a response similar to that produced by sterile, uninoculated broth.

In wheat seedlings, *X. c.* pv. *translucens* produced a translucent, chlorotic lesion, which later became necrotic and which spread from the inoculation point to reach a length of 2–5 cm after 7 days (Fig. 1), whereas *X. c.* pv. *campestris* caused no visible symptoms.

Although the inoculation techniques do not involve natural routes of infection of plants by the two pathovars, having been devised to permit rapid screening of large numbers of strains in genetic experiments, the responses of the two types of plants to the homologous and heterologous pathovars are clearly distinguishable. Counts of viable bacteria in homogenized plant tissue indicated that both incompatible interactions (that is, turnip-*X. c.* pv. *translucens* and wheat-*X. c.* pv. *campestris*) were also characterized by inability of the bacteria to multiply significantly, in contrast to the compatible situation (data not shown).

Complementation of *X. c.* pv. *campestris* pathogenicity mutants with DNA from *X. c.* pv. *translucens*. The *X. c.* pv. *translucens* DNA library was transferred into *X. c.* pv.

Table 1. Bacteria and plasmids

Strain or plasmid	Characteristics[a]	Source or reference
Bacteria		
Xanthomonas campestris pv. *campestris*		
8004	Rif^r	Turner *et al.* 1984
8237	Nonpathogenic derivative of 8004	Daniels *et al.* 1984a
8288	Nonpathogenic derivative of 8004	Daniels *et al.* 1984a
Xanthomonas campestris pv. *translucens*		
XT02	Sp^r	Atkins *et al.* 1987
XT022	::Tn*5* derivative of XT02	This study
XT023	::Tn*5* derivative of XT02	This study
Escherichia coli		
ED8767	*rec*A	Murray *et al.* 1977
PCT800	::Tn*5* derivative of ED8767	Turner *et al.* 1985
HB101	*rec*A Str^r	Boyer and Roulland-Dussoix 1969
Plasmids		
pLAFR1	Tc^r	Friedman *et al.* 1982
pIJ3000	pLAFR1 containing *X. c. campestris* DNA	Daniels *et al.* 1984b
pIJ3020	pLAFR1 containing *X. c. campestris* DNA	Daniels *et al.* 1984b
pIJ3003	pLAFR1 containing *X. c.* pv. *translucens* DNA	This study
pIJ3021	pLAFR1 containing *X. c.* pv. *translucens* DNA	This study
pIJ3022	pLAFR1 containing *X. c.* pv. *translucens* DNA	This study
pRK2013	Km^r, Tra^+, Mob^+, ColE1 replicon	Figurski and Helinski 1979
pRK2073	Sp^r, Km^s derivative of pRK2013	Leong *et al.* 1982
pIJ3011	Sp^s, Gm^r, Cm^r, Tra^+, Mob^+ IncPi replicon	This study

[a]Rif = rifampicin, Sp = spectinomycin, Str = streptomycin, Tc = tetracycline, Km = kanamycin, Gm = gentamycin, and Cm = chloramphenicol; and ^{r} = resistant and ^{s} = sensitive.

campestris mutants 8237 and 8288. These mutants were originally identified by screening prototrophic survivors of mutagenesis of the wild-type strain 8004 on turnip seedlings to detect altered pathogenicity (Daniels *et al.* 1984a). Subsequently it was found that 8237 is defective in the production of protease, polygalacturonate lyase, endoglucanase, amylase, and extracellular polysaccharide, which can be readily detected on indicator plates (Daniels *et al.* 1984b; Tang 1989). A recombinant plasmid, pIJ3020, from the *X. c.* pv. *campestris* library restores pathogenicity and the ability to produce all enzymes and polysaccharide. Because of these pleiotropic effects of the genes cloned in pIJ3020 and because detection of protease is simpler and less laborious than pathogenicity testing, we first screened the *X. c.* pv. *translucens* library for clones that restored protease production and then tested candidates for other phenotypic characters.

Of the 360 8237 transconjugant colonies containing *X. c.* pv. *translucens* DNA that were inoculated onto skimmed milk plates, seven colonies showed enhanced protease activity. The plasmids were isolated from these strains and were found by *Eco*RI digestion to be of two types, designated pIJ3021 and pIJ3022. pIJ3021 was also able to restore polygalacturonate lyase production to near-normal levels in 8237, but pIJ3022 gave only slight activity (Table 2). However, both plasmids restored pathogenicity of the mutants to turnip seedlings (Fig. 2) and mature leaves. Symptoms developed at the same rate as, and were indistinguishable from those incited by the wild-type 8004. The insert DNA of pIJ3021 did not hybridize to pIJ3022; however, pIJ3021 showed homology with the *X. c.* pv. *campestris* DNA in pIJ3020.

The possible presence of a homologue of the *X. c.* pv. *translucens* sequences cloned in pIJ3022 in the *X. c.* pv. *campestris* genome was studied by probing Southern blots of electrophoretically separated *Eco*RI- and *Bam*HI-digested DNA. Two *Eco*RI and three *Bam*HI fragments hybridized with pIJ3022. However, these sequences were missing from the *X. c.* pv. *campestris* DNA library (a pool of about 3,000 clones) and were not, therefore, investigated further. The *Eco*RI bands were larger than 23 and 5 kb, and it is possible that the size constraints on DNA fragments that can be cloned in pLAFR1 are partly responsible for their absence from the library.

Restoration of pathogenicity was not observed for any of the 1,000 transconjugant colonies of mutant 8288

Table 2. Extracellular protease and polygalacturonate lyase production by *Xanthomonas campestris* strains[a]

	Protease		Polygalacturonate lyase	
Strain	**Plate test**	**Assay**	**Plate test**	**Assay**
X. c. pv. *campestris*				
8004	+++	1.3	+++	1.9
8237	±	0.6	±	0.1
8237/pIJ3020	+++	1.2	+++	1.5
8237/pIJ3021	+++	1.0	++	1.3
8237/pIJ3022	++	1.1	+	0.3
8237/pIJ3022::Tn*5*	+	0.7		
8288	+	0.7	++	
8288/pIJ3000	++	2.2	+++	
8288/pIJ3003	++	1.1	++	
8288/pIJ3033::Tn*5*	+/++	0.9		
X. c. pv. *translucens*				
XT02	++++	4.4	−	
XT022	+	0.7		
XT023	++	0.8		

[a]Plate test assessments are based on the diameter of zones of substrate degradation surrounding colonies of comparable size incubated for 48 hr on plates containing skimmed milk or sodium polygalacturonate. Enzyme assays were performed on supernatant fluids of 24-hr cultures, adjusted to $A_{600} = 0.5$ (protease assay, A_{340} per hour per 10^9 colony-forming units (cfu); polygalacturonate lyase assay, micromole of product per minute per 10^9 cfu).

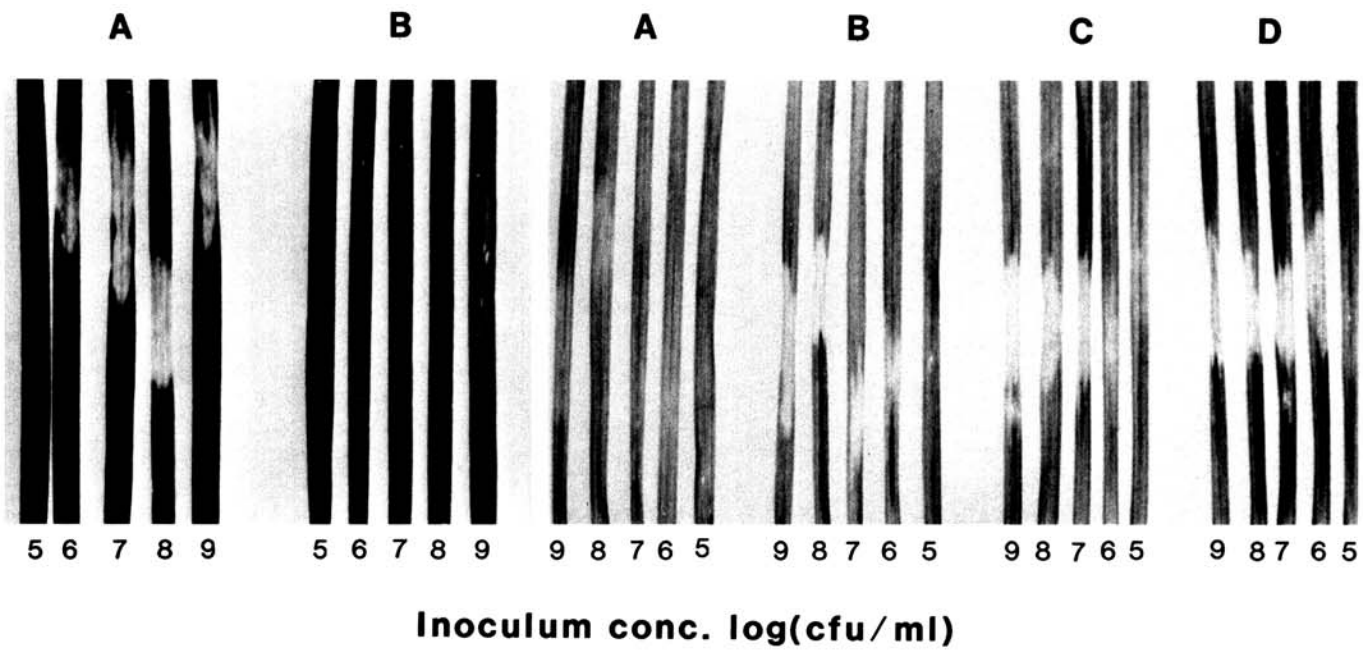

Fig. 1. Leaves from wheat seedlings infiltrated by *Xanthomonas campestris* pv. *translucens* strains. Compost-grown seedlings were infiltrated with suspensions adjusted to a range of bacterial concentrations. Seedlings were photographed 7 days after inoculation. Left panel: **A,** XT02; **B,** XT022. Right panel: **A,** XT022; **B,** XT022/pIJ3020; **C,** XT022/pIJ3021; **D,** XT022/pIJ3022.

containing *X. c.* pv. *translucens* DNA that were tested for pathogenicity to turnip seedlings. A further 800 transconjugants were inoculated onto skimmed milk plates, and one colony, harboring the plasmid pIJ3003, showed enhanced protease activity. pIJ3003 restored pathogenicity to 8288, tested on both seedlings and mature leaves. The mutation in strain 8288 lies in a gene that is part of a cluster spanning about 11 kb concerned with the export of a number of enzymes, including protease and polygalacturonate lyase, from the periplasm to the extracellular medium. Mutations in this region result in accumulation of the enzymes in the periplasm (Turner *et al.* 1985; Dow *et al.* 1987). pIJ3003 caused enhanced secretion of protease, but the distribution of polygalacturonate lyase was not changed (Table 2, and data not shown).

There was no homology between the insert DNA of pIJ3003 and pIJ3000. The latter plasmid contains *X. c.* pv. *campestris* DNA and complements 8288 (Daniels *et al.* 1984b).

Tn*5* mutagenesis. pIJ3021, pIJ3022, and pIJ3003 were introduced by transformation into *E. coli* PCT800, and the resulting strains were mated with *E. coli* HB101, using *E. coli* ED8767/pRK2073 as helper; selection for resistance to kanamycin (Km), streptomycin (Str), and Tc gave a collection of Tn*5* insertions in the plasmids harbored by HB101 (Turner *et al.* 1985). In each case the colonies were pooled and the plasmids were transferred *en masse* by conjugation into *X. c.* pv. *campestris* 8237 and 8288. The transconjugants were tested for pathogenicity to turnips and for production of extracellular protease on skimmed milk plates.

Examination of 800 colonies of *X. c.* pv. *campestris* 8237/pIJ3021::Tn*5* failed to give any that were nonpathogenic or protease-deficient. We do not know why no negative mutants were obtained, because examination by restriction analysis of Tn*5*-containing plasmids from a random sample of 20 colonies indicated that transposition had occurred into all fragments of the *X. c.* pv. *translucens* DNA in pIJ3021.

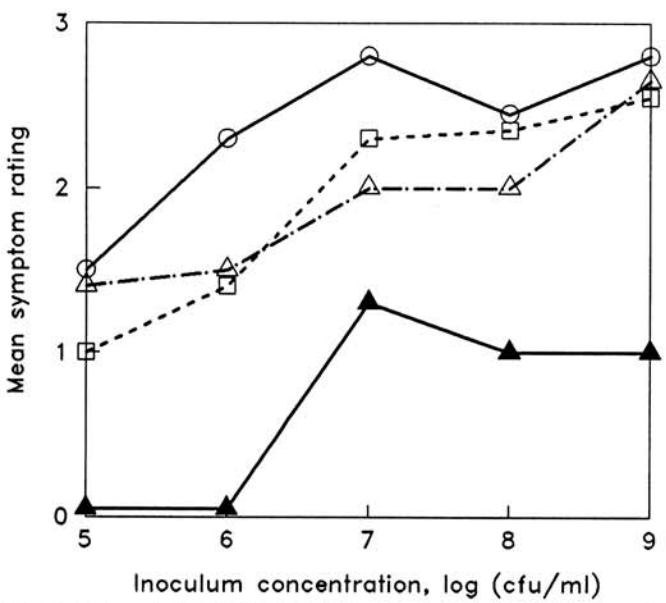

Fig. 2. Pathogenicity of *Xanthomonas campestris* pv. *campestris* strains to turnip seedlings. Groups of five seedlings were inoculated with approximately 0.1 μl of suspensions of the indicated concentrations of strains 8004 (○), 8237 (▲), 8237/pIJ3021 (□), and 8237/pIJ3022 (△). After 4 days at 25° C, the symptoms were rated as 0 (no visible symptoms), 1 (darkening around the inoculation site), 2 (dark, translucent lesion spreading from the inoculation site), and 3 (complete collapse and rotting of seedling). The mean rating for each group is plotted.

Two hundred 8237/pIJ3022::Tn*5* transconjugants were subcultured onto skimmed milk plates, and four were found with low protease levels; they had also lost pathogenicity to turnip seedlings. Restriction analysis of the four plasmids showed that Tn*5* had in all cases inserted into a 6-kb *Eco*RI fragment within the *X. c.* pv. *translucens* DNA.

One of the four plasmids was transferred by transformation to *E. coli* ED8767 and thence by conjugation to *X. c.* pv. *translucens* XT02. The Tn*5* mutation was transferred into the chromosome by introducing the incompatible plasmid pIJ3011 into the *X. c.* pv. *translucens* XT02/pIJ3022::Tn*5* strain, selecting for resistance to gentamycin, Km, and spectinomycin (Sp). Occurrence of marker exchange was verified as described by Turner *et al.* (1985) by Tc sensitivity and Southern blotting. The *X. c.* pv. *translucens* Tn*5* mutant generated in this way was designated XT022.

Five hundred *X. c.* pv. *campestris* 8288/pIJ3003::Tn*5* strains were similarly screened, and one strain carrying Tn*5* in a 7-kb *Eco*RI fragment was found to be low in extracellular protease and nonpathogenic to turnip seedlings. The plasmid was introduced into XT02, and the mutation was transferred into the genome by marker exchange as described above to yield the mutant XT023.

Both XT022 and XT023 showed reduced protease activity compared with XT02 (Table 2). When serially diluted suspensions were infiltrated into wheat leaves, XT023 retained pathogenicity, whereas XT022 was nonpathogenic. The pathogenicity could be restored by introducing pIJ3020, pIJ3021, or pIJ3022 (Fig. 1). XT022 grew more slowly in wheat leaves than XT02, and the final concentration of bacteria attained in the plant tissue was one or two orders of magnitude lower.

Homologous DNA in other *X. campestris* pathovars. Because our experiments revealed *X. c.* pv. *translucens* DNA sequences homologous to and probably functionally equivalent to regions of the *X. c.* pv. *campestris* genome previously cloned in pIJ3000 and pIJ3020, we extended our studies by probing *Eco*RI-digested DNA extracted from other *X. c.* pv. *campestris* wild strains (five isolates), from two strains of *X. c.* pv. *oryzae* (Ishiyama) Dye, and from single strains of *X. c.* pv. *glycines* (Nakano) Dye, *X. c.* pv. *graminis* (Egli, Goto & Schmidt) Dye, *X. c.* pv. *holcicola* (Elliott) Dye, *X. c.* pv. *malvacearum* (Smith) Dye, *X. c.* pv. *phaseoli* (Smith) Dye, *X. c.* pv. *pisi* (Goto and Okabe) Dye, *X. c.* pv. *vesicatoria* (Doidge) Dye, *X. c.* pv. *vitians* (Brown) Dye, and *X. c.* pv. *zinniae* (Hopkins & Dowson) Dye with ^{32}P-labeled pIJ3000 and pIJ3020. Homologous bands were detected in all cases (data not shown).

Effect of heterologous DNA on the pathogenicity of *X. c.* pv. *campestris* and *X. c.* pv. *translucens*. The *X. c.* pv. *translucens* DNA library was transferred *en masse* by conjugation into *X. c.* pv. *campestris* 8004, and similarly an *X. c.* pv. *campestris* library (Daniels *et al.* 1984b) was transferred into *X. c.* pv. *translucens* XT02. Approximately 1,000 randomly chosen colonies from each transfer were screened for pathogenicity, initially by inoculating each

colony into single wheat and turnip seedlings. Any colonies that seemed to induce an atypical response on either plant were retested on populations of at least 10 seedlings, using a range of inoculum concentrations, and suspensions were also infiltrated into mature leaves.

No recombinant plasmids were detected in either library that had detectable, reproducible effects on the behavior of the heterologous pathovar in either plant host; that is, no *X. c.* pv. *campestris* transconjugants induced visible symptoms on wheat or reduced virulence on turnips, and conversely, no *X. c.* pv. *translucens* transconjugants induced symptoms on turnip or reduced virulence on wheat.

DISCUSSION

The experiments described in this paper had two objectives. Our first objective was to investigate whether the two pathovars possessed pathogenicity genes (in the general sense defined by Daniels *et al.* [1984a]) that were either homologous or functionally equivalent. The strategy was to isolate *X. c.* pv. *translucens* clones that could restore function to two previously characterized *X. c.* pv. *campestris* mutants. The *X. c.* pv. *translucens* clones were then used as vehicles for localized mutagenesis of the *X. c.* pv. *translucens* genome, and the resulting strains were tested for pathogenicity to wheat. In addition to providing comparative data on the two pathovars, this approach represents a useful, general, indirect method for studying diverse *X. campestris* pathovars, which may be difficult to study directly for technical or other reasons.

The mutation in strain 8237 lies in one of at least seven linked "global" regulatory genes that are required for synthesis of protease, polygalacturonate lyase, cellulase, and amylase (Tang 1989). *X. c.* pv. *campestris* DNA cloned in pIJ3020 restores both pathogenicity and the ability to synthesize all these enzymes (Daniels *et al.* 1984b), and because marker exchange Tn*5* mutants produced using this DNA have phenotypes identical to 8237, pIJ3020 probably restores function by complementation (Tang 1989).

All four *Eco*RI fragments of *X. c.* pv. *translucens* DNA in pIJ3021 hybridized with pIJ3020, suggesting that the respective genes are homologues. In addition to restoring pathogenicity, pIJ3021 restored production of polygalacturonate lyase and protease. Protease assessment was based on the semiquantitative plate assay because the limited, specific cleavage of proteins by the *X. c.* pv. *campestris* protease (Tang *et al.* 1987) makes a more precise assay impracticable for routine screening of strains.

Pathogenicity and protease production were also restored by pIJ3022, which contained *X. c.* pv. *translucens* DNA with no sequence homology to pIJ3020. Surprisingly, pIJ3022 gave no significant increase in polygalacturonate lyase levels. In the pathogenicity tests used in this work, protease is relatively unimportant for pathogenicity (Tang *et al.* 1987), but polygalacturonate lyase is believed to be essential for rotting tissues (Dow *et al.* 1987). One possible explanation is that pIJ3022 cannot restore polygalacturonate lyase production *in vitro* in response to polygalacturonate or plant cell walls as inducers but can do so when the bacteria are in plants. A further possibility is that pIJ3022 contains the *X. c.* pv. *translucens* protease structural gene allowing protease expression in 8237 independently of the regulatory functions. This is, however, unlikely because the *X. c.* pv. *campestris* protease gene cloned in pIJ3070 cannot restore pathogenicity to 8237, although protease production is restored (Tang *et al.* 1987), and because the homologue of pIJ3022 revealed by probing Southern blots of *Eco*RI- and *Bam*HI-digested *X. c.* pv. *campestris* DNA had a different pattern of restriction enzyme sites than the relevant region of pIJ3070 (data not shown).

We suggest that pIJ3022 encodes a regulatory gene product that can partially substitute for the factor missing in 8237, but a definitive investigation will require extensive molecular genetic and biochemical characterization. The *X. c.* pv. *campestris* genome contains sequences homologous to pIJ3022, but we were unable to investigate the function and role in pathogenicity of this DNA because it was not represented in the *X. c.* pv. *campestris* DNA library.

We could not determine whether the pIJ3021 genes are required for pathogenicity of *X. c.* pv. *translucens* because of our inexplicable failure to isolate Tn*5* insertions that abolished the ability to restore pathogenicity to 8237. However, the mutational approach was successfully used to demonstrate that certain genes in the DNA cloned in pIJ3022 are essential for pathogenicity to wheat. The only detectable extracellular enzyme activity produced by *X. c.* pv. *translucens* XT02 is protease, and the nonpathogenic mutants generated by marker exchange produced much less of this enzyme. However, it is not known whether the protease deficiency alone accounts for the reduced pathogenicity (see below).

X. c. pv. *campestris* 8288 is unable to export extracellular enzymes from the periplasm to the medium; enzyme export and pathogenicity are restored by pIJ3000, which contains a cluster of genes spanning 10 kb of DNA (Turner *et al.* 1985; Dow *et al.* 1987). *X. c.* pv. *translucens* DNA in pIJ3003 was able to restore pathogenicity and protease export but had relatively little effect on the distribution of polygalacturonate lyase. It was not possible to determine whether enzyme export was restored more efficiently when the bacteria were growing *in planta*.

The insert DNA of pIJ3003 and pIJ3000 showed no homology, although studies of genomic digests of *X. c.* pv. *translucens* suggested the presence of a homologue of the export gene cluster in this pathovar (which could not be studied in the present experiments because of the incompleteness of the library). It is likely that pIJ3003 suppresses the mutation in 8288 sufficiently to restore pathogenicity. It is possible that alternative protein export pathways exist and that components of one can partially substitute for the other.

The genes in pIJ3003 were not required for pathogenicity of *X. c.* pv. *translucens* to wheat, as judged by the symptoms produced following the application of bacteria mutant in the genes of pIJ3003 to clipped wheat leaf tips or their infiltration into wheat leaf spaces. However, alternative pathogenicity assays may reveal a role for the products of these genes.

Tang *et al.* (1987) found that protease appeared to be relatively unimportant for pathogenicity of *X. c.* pv. *campestris* following seedling inoculation or leaf infiltration, but more recent experiments have shown that protease may be necessary for earlier penetration stages of the infection process (J. L. Tang and M. J. Daniels,

unpublished data). It is possible that protease is needed for analogous functions in *X. c.* pv. *translucens* and that the deficiency is not apparent in assays that bypass the earlier stages of natural infections. *X. c.* pv. *campestris* causes much greater tissue destruction in turnip than *X. c.* pv. *translucens* causes in wheat, suggesting that extracellular degradative enzymes are more significant pathogenicity determinants for the former pathovar.

Our experiments have shown that it is possible to use DNA transfer and "cross-complementation" between pathovars to study putative related pathogenicity genes. Moreover, the *X. c.* pv. *translucens* genes isolated in this way provided evidence for the existence of previously unidentified genes in *X. c.* pv. *campestris* with a potential role in pathogenicity, indicating that an "interpathovar shuttle" strategy may be an efficient way to widen the range of known genes.

Our approach should prove applicable to many *X. campestris* pathovars. Lazo *et al.* (1987) showed that there is considerable nucleotide sequence homology between random genomic fragments of different pathovars. We have shown that *X. c.* pv. *campestris* DNA in pIJ3000 and pIJ3020 shows homology to DNA of many pathovars, and this has been confirmed by Todd (1987), not only for pIJ3000 and pIJ3020, but also for pIJ3070, which carries an *X. c.* pv. *campestris* protease gene (Tang *et al.* 1987). Further extension of the approach may be possible because *hrp* genes needed for interaction with plants are conserved in *Pseudomonas syringae* pathovars (Lindgren *et al.* 1988) and between *P. solanacearum* and *X. campestris* pathovars (Boucher *et al.* 1987).

Our second objective was to determine whether the nonoverlapping plant host range of two pathovars of *X. campestris* (*X. c.* pv. *campestris* and *X. c.* pv. *translucens*) could be modified by transfer of heterologous DNA fragments. Putative genes determining host range could act either negatively (analogous to avirulence genes) or positively; therefore, we tested the two sets of transconjugants on both their homologous hosts (to detect negatively acting, growth-restricting factors) and the heterologous plant (to detect positive factors that extend the host range).

It is well established that specificity of several *P. syringae* and *X. campestris* pathovars at the race-cultivar level is determined by negatively acting avirulence genes (Staskawicz *et al.* 1984; Gabriel *et al.* 1986; Swanson *et al.* 1988; Hitchin *et al.* 1989), but the determination of specificity at higher levels (such as pathovar-plant species or genus) is poorly understood. Kobayashi *et al.* (1988), Whalen *et al.* (1988), and Roberts *et al.* (1987) reported avirulence-like genes interacting with plants at this level in *P. s.* pv. *tomato, X. c.* pv. *vesicatoria*, and *X. c.* pv. *vitians*, but Ma *et al.* (1988) described positively acting host range gene(s) in *P. solanacearum*. Mellano and Cooksey (1988) also found evidence for positively acting host range determinants in *X. c.* pv. *translucens*.

Our inability to find clones influencing the behavior of *X. c.* pv. *campestris and X. c.* pv. *translucens* may be because any one of several necessary conditions was not satisfied. First, the genomic libraries may have been incomplete, lacking the host range gene(s). Evidence for incompleteness was in fact provided by our inability to find a clone homologous to pIJ3022 in the *X. c.* pv. *campestris* library even though hybridization to genomic DNA digests suggested that homologues should exist. Second, we may not have tested enough transconjugant colonies. Libraries were introduced into recipients by conjugation *en masse*. We know from subsequent work (M. J. Daniels and C. E. Barber, unpublished data) that different clones have different transfer frequencies, so that *en masse* transfer skews the distribution of library clones in the transconjugant population. However, numerous other genes have been isolated from these libraries by the same procedures. Third, the effects would have to be the result of transfer of a single gene (or group of closely linked genes) carried on a single DNA fragment not exceeding 30 kb (the maximum size of DNA insert carried by pLAFR1 clones). Finally, the gene(s) would have to be expressed and the products would have to function normally in the heterologous background. It is known that *X. c.* pv. *campestris* genes encoding amylase, endoglucanase, and polygalacturonate lyase can be expressed in *X. c.* pv. *translucens* (Gough *et al.* 1988; Dow *et al.*, 1989).

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