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Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1

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A genomic library was prepared in *Escherichia coli* from DNA of wild-type *Xanthomonas campestris* pv. *campestris* (aetiological agent of crucifer black rot), partially digested with endonuclease *Eco*RI, using the mobilisable broad host range cosmid vector pLAFR1. Recombinant plasmids contained inserts ranging in size from 19.1 to 32.3 kb (mean 26.6). Certain of the clones complemented *E. coli* auxotrophic markers. Using the narrow host range plasmid pRK2013 as a helper the pooled recombinant plasmids were transferred conjugally to *X. c. campestris* mutants, and clones were identified which restored yellow pigmentation to white mutants, prototrophy to amino acid auxotrophs and pathogenicity towards turnip plants to two non-pathogenic mutants. The lesion in one mutant (8288, complemented by the plasmid pIJ3000) is unknown. However mutant 8237 is defective in production of extracellular protease and polygalacturonate lyase and restoration of pathogenicity by complementation with the plasmid pIJ3020 concomitantly restored both enzyme levels to wild-type values.

Key words: cloning/cosmid/pathogenicity/*Xanthomonas*

Introduction

The molecular biology of bacterial pathogenicity to plants has received little attention until recently and no clear picture has emerged of the mechanisms by which disease is produced. The roles of a few factors which are suspected *a priori* to contribute to disease symptoms (e.g., toxins, polysaccharides, pectolytic enzymes and auxin) have been investigated by isolating mutants specifically altered in their production (reviewed by Chatterjee and Vidaver, 1984), but there are many aspects of disease, notably the specificity of pathogens for different tissues, organs and plant varieties or taxa, which cannot be explained by the action of these factors and which therefore cannot easily be analysed by this approach.

Our strategy for investigating unknown pathogenicity factors has been to inoculate plants with large numbers of single colonies of mutagenised bacteria to isolate mutants which, while being unimpaired in growth *ex planta*, show alterations in the patterns of interaction with the plant host. This approach does not pre-judge the biochemical nature of the mutant gene product. Recombinant DNA techniques are then used to clone the genes defined by the mutations, as a prelude to characterising the gene products and investigating their action on plants (Daniels, 1984). In this way it should be possible to compile a 'catalogue' of pathogenicity determinants and considerably expand our knowledge of plant disease.

Most plant pathogens do not lend themselves to this approach because reliable plant inoculation requires procedures which are unsuitable for use in the large-scale screens necessary to identify mutants. Empirically we found that *Xanthomonas campestris* pv. *campestris* (hereafter called *X. c. campestris*), causing black rot of crucifers (Williams, 1980), is a suitable model pathogen. We have previously described a procedure for pathogenicity screening and its use to isolate a range of non-pathogenic mutants (Daniels *et al.*, 1983, 1984).

Molecular cloning of *Xanthomonas* genes has not previously been reported. Based on the ability of members of the genus to accept and maintain broad host-range IncP plasmids (Lai *et al.*, 1977; Turner *et al.*, 1984), we anticipated that cloning vectors derived from these plasmids, such as pRK290 (Ditta *et al.*, 1980) and its derivative, the mobilisable cosmid pLAFR1 (Friedman *et al.*, 1982), would be suitable. Here we describe the use of pLAFR1 to prepare a genomic library of wild-type *X. c. campestris* DNA. By complementation of mutants, clones were isolated containing segments of *Xanthomonas* DNA; two clones were found each of which restored virulence to a separate non-pathogenic mutant. In addition DNA involved in the biosynthesis of several amino acids and the pigment xanthomonadin (Starr *et al.*, 1977) was identified.

Results

Behaviour of pLAFR1 in X. c. campestris

pLAFR1 was derived, by a series of steps, from the R factor RK2 (Ditta *et al.*, 1980; Friedman *et al.*, 1982), which is itself

Table I. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
X. c. campestris		
8000	Wild-type	NCPBB 1145
8002	As 8000 but *rif*-1 *pro*-1	This work
8004	As 8000 but *rif*-14	Turner *et al.* (1984)
8010	As 8000 but *str*-1 *leu*-1	This work
8027	As 8000 but *spc*-1 *thr*-1	This work
8194	As 8000 but *spc*-1 *met*-1	This work
8251	As 8004 but non-pigmented	This work
8220	As 8004 but non-pathogenic	Daniels *et al.* (1984)
8237	As 8004 but non-pathogenic	Daniels *et al.* (1984)
8255	As 8004 but non-pathogenic	Daniels *et al.* (1984)
8288	As 8004 but non-pathogenic	Daniels *et al.* (1984)
8305	As 8004 but non-pathogenic	This work
E. coli		
CBK 001	*phe*A::Tn5	Shaw and Berg (1979)
CSH 50	*pro*	Miller (1972)
ED 8767	*rec*A *met*	Murray *et al.* (1977)
HB 101	*pro*A2 *leu*B6	Boyer and Roulland-Dussoix (1969)
UNF 510	*his trp*	Merrick *et al.* (1978)
Plasmids		
pLAFR1	Tc^R Tra^- Mob^+, RK2 replicon	Friedman *et al.* (1982)
pRK2013	Km^R Tra^+ Mob^+, Col E1 replicon	Figurski and Helinski (1979)

considered to be identical to RP1 and RP4 (Burkhardt *et al.*, 1979). Previous studies had shown that IncP plasmids can be transferred conjugally from *Escherichia coli* donors to *X. c. campestris* at frequencies of $\sim 10^{-4}$ per recipient, all three antibiotic resistance functions [to ampicillin, kanamycin/neomycin and tetracycline (Tc)] being expressed in *X. c. campestris* (Turner *et al.*, 1984). pLAFR1 retains only the Tc^R function which can be used for selection in *X. c. campestris*. The plasmid is not self-transmissible, but it can be mobilised by *trans*-acting functions on the narrow host range plasmid pRK2013, which cannot establish replication in *X. c. campestris* (Turner *et al.*, 1984). In tripartite matings between *X. c. campestris* recipients carrying chromosomal mutations for resistance to rifampicin (Rif), spectinomycin (Sp) or streptomycin (Sm) and *E. coli*/pLAFR1 (donor) and *E. coli*/pRK2013 (helper), pLAFR1 was found to be transferred to *X. c. campestris* at frequencies around 10^{-4} per recipient. Sm^R recipients consistently gave 10- to 100-fold higher frequency of transconjugants than Rif^R strains, with Sp^R strains being intermediate. A similar variation in ability to receive R68.45 was also noticed (Turner *et al.*, 1984). The presence of pLAFR1 in Tc^R transconjugants was verified by agarose gel electrophoresis of lysates. The plasmid could also be transferred conjugally, but with variable efficiency, from *X. c. campestris* to *E. coli* in the presence of an *E. coli*/pRK2013 helper. Presumably pRK2013 can enjoy a transient non-replicating existence in *X. c. campestris* and express its mobilising function to permit transfer of pLAFR1.

Properties of the DNA library

When partially-digested, size-fractionated *X. c. campestris* DNA was ligated with linear, alkaline phosphatase-treated pLAFR1, packaged into λ pseudovirions and introduced into *E. coli* ED 8767 the yield of Tc^R colonies was ~6000 per μg of *Xanthomonas* chromosomal DNA, a value comparable with that obtained by Friedman *et al.* (1982) for *Rhizobium meliloti*. A random sample of 16 colonies was purified and subcultured; plasmid-containing lysates (Holmes and Quigley, 1981) were digested to completion with *Eco*RI and the products analysed electrophoretically. All the samples contained a band in the position expected for a linear double-stranded DNA molecule of 21.6 kb (the pLAFR1 vector), and also 1–7 additional bands (average number 4.4), assumed to be *X. c. campestris* DNA inserts. The sum of lengths of the inserts in each clone was in the range 19.1–32.3 kb, mean 26.6 kb, a value consistent with the size-selection imposed by the λ packaging system and similar to results with *R. meliloti* (Friedman *et al.*, 1982) and *Pseudomonas* sp. (Moores *et al.*, 1984). Strains containing recombinant plasmids were usually grown in the presence of Tc. Stability under non-selective conditions varied. Two randomly chosen clones were picked and grown to saturation in NYGB and then plated out to give single colonies. 97% and 67% respectively of the colonies were Tc^R. For pLAFR1 the corresponding figure was 95%.

Complementation of mutants

The general procedure was to transfer the DNA library in 3000 pooled *E. coli* colonies into *X. c. campestris* mutants by conjugation and then to screen Tc^R transconjugants for restoration of wild-type phenotype. In the case of a non-pigmented (white) mutant (8251) this was accomplished by inspection: ~2% of colonies had acquired the normal yellow colour of the wild-type. The reversion frequency of this mutation was $< 10^{-4}$. Plasmids were extracted from a sample of six of these yellow colonies, introduced into *E. coli* ED 8767 by transformation (with selection for Tc^R) and again transferred by mating into 8251. One isolate failed to complement the white mutation; of the others one gave 77% and the other four yielded 97–100% yellow colonies, indicating that restoration of pigmentation was caused by complementation of the mutation by a wild-type plasmid-borne gene. *E. coli* colonies containing the plasmid were not pigmented, but when the plasmid was introduced into normally-pigmented *X. c. campestris* strains they assumed a darker tone of yellow. In fact, introduction of the DNA library directly from *E. coli* into wild-type strains gave ~1% 'super yellow' colonies. In subsequent work this was a useful preliminary indicator for the detection of complementation of other mutations.

Table II. Complementation of mutations by wild-type *X. c. campestris* DNA library

Strain	Complemented phenotype	Frequency (no. of complemented colonies/no. of Tc^R transconjugants)
X. c. campestris		
8002	Pro^+	4/498 (0.8%)
8010	Leu^+	6/361 (1.6%)
8027	Thr^+	0/250
8194	Met^+	2/439 (0.5%)
8220	Pathogenic	0/500
8237	Pathogenic	1/1700 (0.06%)
8255	Pathogenic	0/700
8288	Pathogenic	3/500 (0.6%)
8251	Pigmented	15/800 (1.9%)
E. coli		
CBK 001	Phe^+	8.7×10^{-4}
CSH 50	Pro^+	$< 10^{-6}$
ED 8767	Met^+	7×10^{-4}
HB 101	Pro^+	$< 10^{-6}$
	Leu^+	$< 10^{-6}$
UNF 510	Trp^+	$< 10^{-6}$
	His^+	+ (frequency not determined)

The library was introduced from *E. coli* into auxotrophic *X. c. campestris* strains 8002 (Pro^-), 8010 (Leu^-), 8027 (Thr^-) and 8194 (Met^-) and prototrophic Tc^R transconjugants were isolated either by direct plating on minimal medium + Tc or by replica plating colonies grown on NYGA + Tc to minimal medium. The frequency of prototrophic derivatives is given in Table II. No Thr^+ colonies were found among 250 8027 transconjugants tested.

The DNA library was also able to complement certain *E. coli* auxotrophic mutations (Table II). ED8767 containing the library gave Met^+ colonies at a frequency of 7×10^{-4} (but the cloned DNA complementing this mutation did not complement *X. c. campestris* 8194). CBK 001 (*phe*::Tn5) was complemented and also the *his* marker of UNF510. The *trp* marker of UNF510, *pro* of CSH 50 and *pro* and *leu* of HB 101 were not complemented (i.e., frequency $< 10^{-6}$). The observed complementation frequencies were all several orders of magnitude above the reversion frequencies of the markers.

Complementation of pathogenicity mutants

The DNA library was transferred from *E. coli* into the non-pathogenic *X. c. campestris* mutants 8220, 8237, 8255 and 8288, chosen because they show no leakiness and give an unambiguous result in pathogenicity tests on individual turnip seedlings (Daniels *et al.*, 1984). In initial tests no complementation was obtained with 8220 (500 colonies tested), 8237 (700 tested) and 8255 (700 tested). However, three out of

500 transconjugants with 8288 gave wild-type symptoms, initially screened on single seedlings and subsequently using groups of 10 for each colony. The three plasmids were isolated and introduced into *E. coli* ED 8767 by transformation. Electrophoresis of *Eco*RI-digested lysates showed that the three isolates were identical, having (in addition to the 21.6-kb pLAFR1 band) two bands of 16 and 9 kb respectively. When one of the plasmids, designated pIJ3000, was again introduced into 8288 by mating from *E. coli* all the 25 Tc^R colonies tested gave wild-type symptoms on seedlngs. The virulence of the complemented mutant was compared with 8288 and 8004 (wild-type) by inoculating groups of seedlings with standard doses from serially-diluted suspensions (Daniels *et al.*, 1984). The results shown in Figures 1 and 2 confirm that pIJ3000 restored full virulence to 8288.

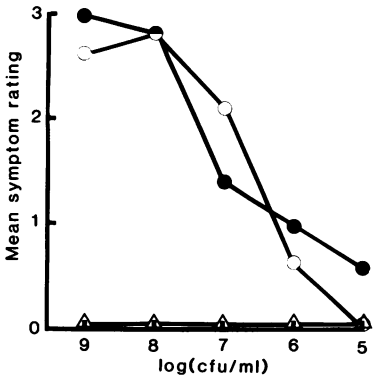

Fig. 1. Virulence of *X. c. campestris* strains. Groups of five turnip seedlings were inoculated from serially-diluted suspension of strains 8004 (wild-type, ●), 8288 (non-pathogenic mutant, △) and 8288/pIJ3000 (○). After 3 days the symptoms were scored on the scale 0 (no visible symptoms), 1 (darkening around the inoculation site), 2 (dark, translucent lesion spreading from inoculation site) and 3 (complete collapse and rotting of seedling). The mean rating for each group is plotted.

Identical results were found with all three isolates of the plasmid. We verified that the 16- and 9-kb inserts were of *Xanthomonas* origin by digesting 8004 DNA with *Eco*RI, separating fragments by electrophoresis and transferring them to hybridisation membranes for probing with radioactive plasmids. pIJ3000 hybridised to only two of the large number of bands in the digest, at positions corresponding to 16 and 9 kb, whereas pLAFR1 gave no hybridisation. Similar experiments using 8004 DNA digested with *Bam*HI and *Hind*III (pIJ300 insert DNA has five sites for *Bam*HI and none for *Hin*dIII) indicated that the 16- and 9-kb *Eco*RI fragments are contiguous in the *X. c. campestris* genome (data not shown).

pIJ3000 did not restore pathogenicity to other mutants in the collection described by Daniels *et al.* (1984), but complemented 8305, a recently isolated mutant which has not yet been characterised in detail, although the phenotype is similar to 8288.

While examining the growth of *X. c. campestris* on various indicator media we discovered that 8237 produces less extracellular protease than the wild-type, judged by the size of clear zones around colonies on agar containing skimmed milk. The DNA library was again transferred into 8237 and Tc^R colonies were subcultured on to skimmed milk plates. Although the nature of the test and the leakiness of the mutant made objective judgement difficult, 14 colonies out of 1000 appeared to produce larger clear zones and each of these was inoculated into five seedlings. One was found to have regained full pathogenicity; the recombinant plasmid (designated pIJ3020) was introduced into *E. coli* by transformation, characterised physically [the insert DNA gave eight *Eco*RI fragments of size 6.4, 4.7, 3.9 (doublet), 3.4, 2.2, 1.8 and 1.1 kb] and transferred again to 8237. Twenty four out of twenty five transconjugant colonies tested gave full symptoms, and the one which remained non-pathogenic had also

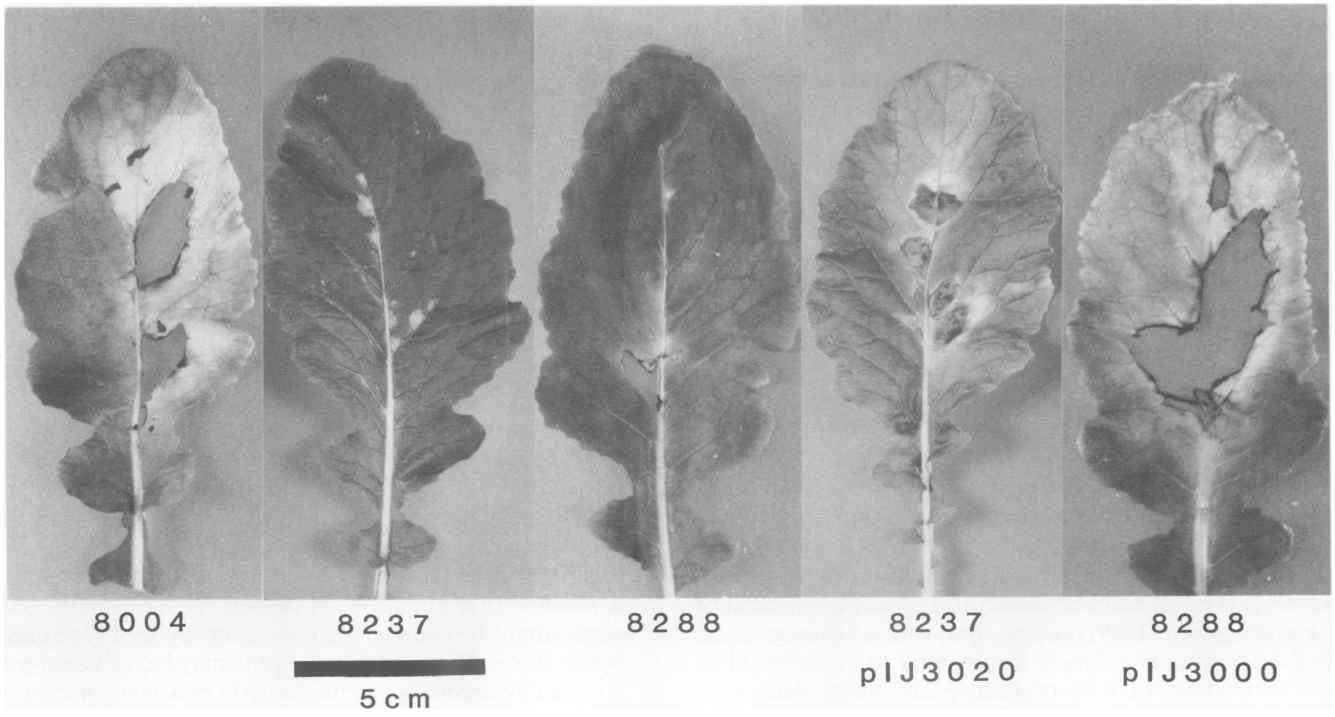

Fig. 2. Symptoms produced in turnip leaves by infiltration with suspensions of *X. c. campestris* strains (wild-type 8004, non-pathogenic mutants 8237 and 8288, and complemented mutants restored to pathogenicity). Leaves were photographed 2 days after inoculation.

Table III. Polygalacturonate lyase and protease production by *X. c. campestris* strains

Strain	Polygalacturonate lyase (A_{235} min^{-1})			Relative protease activity		
	Expt 1.	Expt 2.	Expt 3.	Expt 1.	Expt 2.	Expt 3.
8004	0.07, 0.045	0.09, 0.05	0.03, 0.03	1	1	1
8237	0, 0.01	0, 0	0, 0.005	0.1	0.06	0.25
8237/pIJ3020	0.17, 0.27	0.05, 0.06	0.05, 0.055	1.22	0.78	0.83
8288	0.105, 0.115	0.07, 0.09	(not done)	0.78	0.56	0.69

lost the capacity to produce normal protease levels, possibly because of a deletion in the plasmid.

Table III shows relative protease levels of strains 8004, 8288, 8237 and 8237 complemented with pIJ3020. Attempts to assay protease activity in culture supernatant fluids by a casein digestion method (Laskowski, 1955) gave variable and unsatisfactory results, probably because of the low concentration of enzyme present. However we found that the diameter of the zone of clearing on skimmed milk agar was proportional to the diameter of the colony for a given strain and incubation period, and this relationship was used to derive the relative values in Table III. Parallel studies revealed that mutant 8237 is also defective in production of polygalacturonate lyase (PGL), although pectinesterase levels were normal. Introduction of pIJ3020 restored PGL levels to wild-type values (Table III). Mutant 8288 produced wild-type levels of PGL.

As an alternative to inoculating individual seedlings with single *X. c. campestris* colonies we introduced the transconjugant pool from ~1000 colonies resulting from transfer of the DNA library into strain 8255 into mature turnip leaves by syringe infiltration of suspensions containing 10^6, 10^7, 10^8 or 10^9 c.f.u./ml (Daniels *et al.*, 1984). The plants were maintained at 25°C for 8 days, but there was no indication that any bacteria in the mixtures inoculated had initiated wild-type pathogenic responses. The infiltrated leaf portions were macerated and dilutions were plated on NYGA containing tetracycline (5 μg/ml). Two hundred and fifty single *X. c. campestris* colonies were then tested individually on seedlings. None developed symptoms, suggesting that the plant could not act as a selective agent to enrich for pathogenic cells in the mixture, at least in the case of mutant 8255.

Discussion

We have shown that the broad host-range cosmid pLAFR1 can be used as a gene cloning vector in *X. c. campestris*. An advantage of broad host-range vectors is that genes may be cloned in a convenient host such as *E. coli* and therefore be accessible to all the molecular genetical techniques developed for that organism, but at the same time the cloned genes may be transferred into the original host species for functional tests. The genomic library was prepared from partially digested DNA to permit the recovery of genes spanning restriction sites, and size fractionation of the DNA was employed to minimise the possibility of inserts containing non-contiguous fragments (Grosveld *et al.*, 1981). The genome size of *X. c. campestris* has not been determined; if, however, it is assumed to be similar to *E. coli* the equation of Clark and Carbon (1979) predicts that, with an average insert size of 26.6 kb, 708 colonies would need to be screened to give a probability of 0.99 of finding any given unique sequence. Since the pathogenicity test for *X. c. campestris* (Daniels *et al.*, 1984) permits one person to screen at least 500 colonies per day we felt confident that clones complementing pathogenicity mutants should be obtainable by direct screening on plants.

The behaviour of the library revealed in complementation experiments with auxotrophic and pigment mutants closely parallels previous work with a pLAFR1 library of *R. meliloti* (Friedman *et al.*, 1982) and clones were recovered in practice at frequencies up to 1%. *X. c. campestris* genes could complement some *E. coli* auxotrophic mutations but others could not be complemented. Several explanations are possible for this, e.g., (i) the genes may not be expressed in *E. coli*; (ii) the gene products may not be sufficiently active in *E. coli*, or be produced in sufficient amounts to generate the required intermediates; (iii) the biosynthetic pathways in the two organisms may differ, or (iv) the intact genes may be missing from the library.

The yellow pigments of *Xanthomonas* (xanthomonadins) are brominated arylpolyenes (Starr *et al.*, 1977), a class of compound found only in the genus and one of the few bromine-containing compounds known to occur in living organisms. The biosynthesis of xanthomonadins has not yet been studied, but the ease with which clones complementing mutations in the pathway could be isolated indicates that the genes involved could be readily studied.

Our primary aim was to develop means for analysing pathogenicity determinants, which are at present defined only by mutations producing an alteration in the symptoms shown by infected plants (Daniels *et al.*, 1984). Clones complementing symbiotic mutants of *Rhizobium* can be isolated by inoculating the whole transconjugant mixture resulting from pLAFR1 library transfer on to plants which act as a 'selective medium' for the minority of nodulation-proficient bacteria (Brewin *et al.*, 1980; Long *et al.*, 1982). An analogous approach with *X. c. campestris* 8255 transconjugant mixtures did not work, probably because the majority of non-pathogenic bacteria in the mixture induced plant defence reactions which inhibited both mutants and cells of wild-type phenotype produced by complementation. Mutant 8255 gives a hypersensitive response on turnips whereas certain other mutants do not (Daniels *et al.*, 1984). It is possible that the mixed inoculation strategy may work with other mutant classes. We opted to test colonies individually and have isolated two unrelated recombinant plasmids which restore full pathogenicity to two of the four mutants (8237 and 8288) we have studied in detail. Our failure to complement strains 8220 and 8255 may indicate that the corresponding intact wild-type genes are missing from the library or that insufficient colonies were tested to detect the clones. Alternatively it is possible that mutations in certain classes of pathogenicity gene cannot be complemented in *trans* by wild-type plasmid-borne alleles. For example, the gene may be, or be subject to, a *cis*-acting regulator, or the bacteria producing a mixture of mutant and wild-type gene product may have reduced pathogenicity (i.e., the mutant allele is dominant). Genes of this type could be cloned if the original defining mutation was

a transposon insertion which could be used as a molecular probe for sequences flanking the insertion, in turn permitting the isolation of a clone from the DNA library containing the gene. This approach cannot yet be applied because of the lack of a suitable system for large-scale transposon mutagenesis in *X. c. campestris* (Turner *et al.*, 1984).

Xanthomonas products which may contribute to pathogenicity include the polysaccharide xanthan (Jeanes *et al.*, 1961), pectolytic, proteolytic and cellulolytic enzymes (Starr and Nasuno, 1967; Knoesel and Garber, 1967; Reddy *et al.*, 1971) and toxic acidic metabolites (Noda *et al.*, 1980; Perreaux *et al.*, 1982). We have shown that mutant 8237 is deficient in protease and polygalacturonate lyase; studies are in progress to determine if any other non-pathogenic mutants show altered levels of the possible pathogenicity factors. If they do, the availability of complementing clones will permit an analysis of their function in disease. Alternatively, if the mutants show normal levels of the factors, it may be possible to deduce some properties of the pathogenicity determinants from studies of the cloned genes.

It is not yet clear whether the phenotype of mutant 8237 results from a single mutation affecting both protease and polygalacturonate lyase production, or whether there are two linked mutations each affecting one of the enzymes. Mutants of the former type affecting a range of extracellular enzymes have been described in *Pseudomonas aeruginosa* (Wretlind and Pavlovskis, 1984). Transposon mutagenesis of cloned DNA followed by introduction of the mutation into the wild-type chromosome by marker exchange should enable us to resolve this question (P.C. Turner, C.E. Barber and M.J. Daniels, in preparation).

As we have shown, it is possible to isolate cloned genes involved in pathogenicity of *X. c. campestris* by complementation of non-pathogenic mutants. Two approaches have been used. The nature of the lesion in mutant 8288 is unknown and the complementing recombinant plasmid pIJ3000 was identified by testing for restoration of pathogenicity directly on plants. In the case of 8237 we supposed that the protease defect led to reduced pathogenicity, and by screening for restoration of protease activity we recovered a recombinant plasmid pIJ3020 which restored full pathogenicity. Further application of these and other approaches should enable us to assemble a collection of cloned pathogenicity genes, the study of which may be expected to expand greatly our knowledge of the mechanisms of pathogenicity to plants.

Materials and methods

Microbiological methods

Bacterial strains and plasmids are listed in Table I. Media, cultural and mating procedures were described by Turner *et al.* (1984). The complete medium for *E. coli* was usually L broth (Miller, 1972), but NYGB was used for cultures which were to be mated with *X. c. campestris*. For reasons which are not understood, prior growth of *E. coli* strains in L broth inhibited subsequent recovery of *X. c. campestris* transconjugants. Extracellular protease activity was detected using NYGA plates containing 1.5% (w/v) skimmed milk (Difco).

Pathogenicity tests

Pathogenicity to turnip seedlings and mature leaves was tested using the procedure of Daniels *et al.* (1984).

Preparation and analysis of the DNA library

Chromosomal DNA was prepared from an overnight culture (100 ml) of *X. c. campestris* 8004. After chilling, the cells were recovered by centrifugation, washed twice with ice-cold TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and suspended in 40 ml TE. Protease (Sigma Type XIV, pre-digested for 1 h at 37°C) and SDS were added to final concentrations of 500 μg/ml and 1% (w/v), respectively, and the mixture incubated for 45 min at 0°C before being allowed to warm to 20°C with occasional gentle swirling. The lysate was extracted three times with TE-saturated phenol containing 0.1% 8-hydroxyquinoline. After centrifugation the aqueous phase was made 0.3 M in sodium acetate and an equal volume of isopropanol was added. Precipitated nucleic acids were centrifuged, washed with ethanol and dried *in vacuo*. The pellet was dissolved in 10 ml TE, 0.5 ml of 0.1 M spermine was added and the precipitated DNA recovered by centrifugation. Spermine was extracted by suspending the pellet in 10 ml of 0.1 M sodium acetate, 3 mM $MgCl_2$ in 70% ethanol. After 1 h the DNA was centrifuged and the extraction repeated with 20 ml solution. Finally the DNA was washed with ethanol and dried. Partial digestion with *Eco*RI was achieved by incubating 200 μg DNA samples with 0.1 – 0.2 enzyme units/μg for 15 min at 37°C using the buffer recommended by the manufacturer (BRL). The reaction was terminated by heating the mixture at 70°C for 5 min. A portion was electrophoresed in 0.2% agarose to verify that a significant fraction of digested DNA fell within the size range 20 – 30 kb. The digest was loaded on to a pre-formed sodium chloride gradient (1.25 – 5 M, in TE) and centrifuged for 4 h at 37 000 r.p.m. in a Beckman SW41 rotor. 1 ml samples were taken from the gradient with an automatic pipette, dialysed exhaustively against TE, concentrated with dry Sephadex G-200, made 0.3 M in sodium acetate and precipitated with an equal volume of isopropanol. The pellets were washed with ethanol, dried and dissolved in 30 μl H_2O. A portion (2 μl) of each was electrophoresed with size markers of λ DNA cut with *Hind*III and RP1 cut with *Sal*G. The fraction yielding most material in the size range 20 – 30 kb was used for further work, and the intensity of ethidium bromide fluorescence compared with the standards was used to estimate roughly the concentration of the sample.

pLAFR1 DNA was purified by ethidium bromide-caesium chloride centrifugation of cleared lysates. After digestion with *Eco*RI (BRL) the linear plasmid DNA was treated with calf intestinal alkaline phosphatase (Boehringer, molecular biology grade) using the manufacturer's recommended incubation conditions. The enzyme was inactivated by heating at 70°C for 10 min and the solution extracted with phenol/chloroform (1/1) and then with chloroform. DNA was precipitated from the aqueous phase with isopropanol following addition of sodium acetate to 0.3 M. The precipitate was washed with ethanol redissolved in 0.3 M sodium acetate, reprecipitated with isopropanol and dried *in vacuo*.

The treated vector DNA and the fractionated chromosomal DNA were mixed (in the ratio 3:1 by weight), ligated with T4 DNA ligase, 'packaged' and introduced into *E. coli* ED8767 essentially as described by Grosveld *et al.* (1981). Recombinant clones were selected on L medium containing tetracycline (15 μg/ml). 3000 colonies were subcultured to fresh LTc plates from which the bacteria were collected by flooding the plates with NYGB containing 20% (v/v) glycerol. The pooled suspension (the DNA library) was dispensed in aliquots and stored at −20°C and −70°C.

Recombinant plasmids were extracted from *E. coli* colonies by the method of Holmes and Quigley (1981). Rapid plasmid extraction procedures did not give reliable yields with *X. c. campestris*. The indirect procedure adopted was to wash bacteria with water to remove most of the extracellular polysaccharide and prepare cleared lysates as described by Birnboim and Doly (1979). Plasmids in the lysates were introduced into *E. coli* ED 8767 by transformation (Cohen *et al.*, 1972), selecting for Tc resistance, permitting subsequent extraction and study by the Holmes and Quigley (1981) procedure. Plasmids and restriction enzyme digests thereof were electrophoresed in horizontal slabs of agarose (0.8% in 0.04 M Tris, 0.02 M sodium acetate, 1 mM EDTA, pH 8.3). DNA was visualised by staining with ethidium bromide, and the mol. wt. of fragments was calculated from photographs using the DNAGEL technique (Kieser, 1984). λ DNA digested with *Hind*III was used as a size standard. Hybridisation procedures have been described by Turner *et al.* (1984).

Polygalacturonate lyase assay

Polygalacturonate lyase (transeliminase, EC 4.2.2.2) activity was assayed by the spectrophotometric method of Nasuno and Starr (1967), using supernatant fluids from cultures grown for 48 h in Nasuno and Starr's medium containing 'Firmajel' pectin (0.5%, from H.P. Bulmer, Hereford, UK).

Protease activity

The widths of annular zones of clearing around colonies of a range of sizes on skimmed milk agar were measured after incubation for 24 – 48 h. For each strain a graph of zone size against colony diameter was drawn and used to interpolate a value for a 1 cm diameter colony, which was used as a measure of relative protease activity, taking the value for 8004 (wild-type) as 1.0.

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