A Xanthomonadin-Encoding Gene Cluster for the Identification of Pathovars of *Xanthomonas campestris*

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A gene cluster for xanthomonadin production was obtained from *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot of crucifers. Eighteen mutants affected in pigment production were identified. Seventeen of these mutants were fully pathogenic, and they could be divided into four classes based on the absorption spectra of pigment extracts. Sequences from a 25.4-kbp genomic clone (pIG102) restored pigment production to all 18 mutants and to a naturally occurring nonpigmented strain of *X. c.* pv. *mangiferaeindicae*. Analysis of pIG102 by subcloning and mutant restoration identified six functional domains for pigment production, and this genomic region conferred the production of a xanthomonadin pigment to *Pseudomonas fluorescens*. Clone pIG102 and subcloned derivatives encoding various functional domains were used as DNA probes in colony blot hybridizations with genomic DNA of 105 strains of *Xanthomonas*, *Pseudomonas*, *Erwinia*, and *Clavibacter*. Clone pIG102 hybridized strongly to all strains of 18 pathovars of *X. campestris* and showed little or no hybridization to the other strains. In Southern hybridizations, there was no strict correlation between pathovar designation and the restriction fragment length polymorphism pattern of the pigment-encoding region. A pIG102 subclone, pIG233, hybridized to all of the *X. campestris* strains but showed no hybridization to the non-xanthomonads. These probes should be useful in distinguishing *X. campestris* pathovars from other genera of bacteria associated with plants.

Additional keywords: Brassica, DNA probe, genetics, yellow pigments.

Xanthomonads are the causal agents of disease of many economically important plant hosts (Neergaard 1977). The

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most important species of *Xanthomonas*, *X. campestris*, is divided into over 120 different pathovars, each causing a different disease, usually on different hosts (Bradbury 1984).

Most xanthomonads produce yellow, membrane-bound, brominated, aryl-polyene pigments called xanthomonadins (Starr et al. 1977; Starr 1981), which are used as chemotaxonomic markers (Starr and Stephens 1963; Starr et al. 1977; Bradbury 1984) and diagnostic markers (Moffet and Croft 1983; Schaad and Stall 1988) because they are specific for the genus (Starr 1981). However, on culture medium yellow-pigmented xanthomonads can be very difficult to distinguish from other yellow-pigmented bacteria, and naturally occurring nonpigmented *Xanthomonas* variants can be isolated from plant tissue (Moffet and Croft 1983; Poplawsky and Chun 1992). Thus, a DNA probe specific for xanthomonads (both pigmented and nonpigmented) would simplify the diagnosis and identification of these bacteria.

The purpose of this work was to identify and characterize genomic regions encoding xanthomonadin production in *X. c.* pv. *campestris* and to test these regions for their utility in the identification of xanthomonads.

RESULTS

X. c. pv. *campestris* pigment mutants.

Fourteen mutants (Pm-1 through Pm-9 and Pm-11 through Pm-15) altered in pigment production were obtained by ethyl methanesulfonate (EMS) mutagenesis, and four naturally occurring pigment mutants (B-122, B-1500, B-1501, and B-1502) were previously isolated from commercial crucifer seed (Poplawsky and Chun 1992). All 18 isolates were positive for starch hydrolysis and EPS production.

The 18 mutant strains of *X. c.* pv. *campestris* and a single nonpigmented strain (JF27.1) of *X. c.* pv. *mangiferaeindicae* could be divided into four classes based on spectral absorption characteristics of crude methanolic extracts (Table 1; Fig. 1). Parent strain B-24 showed a single absorption maximum of 0.47 absorbance units at 441 nm with a shoulder at 415–425 nm. Class I mutants showed no distinct absorption maxima, and class II mutants showed a small absorption maximum at 390 nm. The absorption spectra of class III mutants were variable and altered in

shape, although each had a prominent absorption maximum at 415 nm in addition to a maximum of 0.3–0.4 absorbance units at 441 nm. The class IV mutants showed absorption spectra very similar to that of B-24, but with a maximum of 0.2–0.25 absorbance units at 441 nm. In addition to being affected in pigmentation, six of the mutants were also auxotrophic, and one of these was nonpathogenic (Table 1).

Identification of pigment-restoring clones.

Twenty randomly selected clones from the B-24 genomic library were screened for cosmid content. All had different inserts, with an average size of 22 kbp. The B-24 cosmid clone bank was mated en masse to mutants Pm-3 and Pm-4, and two cosmid clones (pIG101 and pIG102) were obtained from pigment-restored mutants. Restriction endonuclease analysis (Fig. 2) and Southern hybridization (data not shown) indicated that cosmids pIG101 and pIG102 had approximately 17.9 kbp of DNA in common, and that these sequences were colinear with the B-24 chromosome.

Tri-parental conjugal transfer of pLAFR3 clones and subclones was used to perform a complementation analysis with all 18 *X. c.* pv. *campestris* pigment mutants and three nonpigmented strains each of *X. c.* pv. *manihotis* and *X. c.* pv. *mangiferaeindicae*. All 18 *X. c.* pv. *campestris* strains and *X. c.* pv. *mangiferaeindicae* strain JF27.1 yielded transconjugants at a frequency of approximately 10^{-4}–10^{-5}. We were unable to obtain transconjugants from

the *X. c.* pv. *manihotis* strains and the remaining *X. c.* pv. *mangiferaeindicae* strains. Although cosmid pIG101 restored pigment production to only 18 of these 19 strains, all were restored by at least one of the three *Eco*RI-*Hind*III fragments of pIG102 (pIG201, pIG202, and pIG203; Fig. 3). Further subclone pigment restoration analyses divided the pigment mutants into six functional domains, distributed throughout the cloned genomic sequences of pIG102 (Fig. 3). In all cases, subclones that restored pigment production to the six auxotrophic pigment mutants (Table 1) did not restore prototrophic growth.

Xanthomonadin production in *Pseudomonas*.

Cosmids pLAFR3, pIG101, and pIG102 were transferred to *Pseudomonas fluorescens* strain NRRL-15135 by conjugation. Colonies of NRRL-15135(pIG101) and NRRL-15135(pIG102) appeared slightly yellow. Boiled methanol extracts from these two strains showed distinct absorption maxima at 415 nm, not present in extracts of NRRL-15135(pLAFR3) (Fig. 4).

DNA homology studies.

Strong signals were observed when colony blots of total DNAs from all *Xanthomonas* strains (except for a single strain of *X. c.* pv. *dieffenbachiae*) were hybridized with pIG102 insert DNA (Fig. 5A, A-1 to H-3). The single *X. c.* pv. *dieffenbachiae* strain (B-400; Fig. 5, F-7) was subsequently shown to lack yellow pigmentation, mucoid growth on yeast extract–dextrose–calcium carbonate medium, and pathogenicity on *Dieffenbachia*. Thus it appears not to be *Xanthomonas*. Little or no signal was detected from strains of *Pseudomonas*, *Erwinia*, and *Clavibacter* (Fig. 5A, H-4 to I-10).

Table 1. Absorption classes and other properties of *Xanthomonas campestris* pv. *campestris* strains B-122, B-1500, B-1501, B-1502, and B-24; B-24 mutants affected in pigment production (Pm); and *X. c.* pv. *mangiferaeindicae* strain JF27.1

Absorption class[a]	Strain	Pigmenta-tion[b]	Proto-trophy[c]	Patho-genicity[d]
...	B-24	+	+	+
I	B-122	–	+	+
	JF27.1	–	+	
	B-1502	–	+	+
	Pm-2	–	–	+
	Pm-3	–	–	+
	Pm-4	–	+	+
	Pm-5	–	+	+
	Pm-7	–	+	+
	Pm-9	–	–	+
	Pm-13	–	+	+
	Pm-14	–	+	+
II	Pm-1	–	+/–	+
	Pm-6	–	+	+
	Pm-8	–	+	+
III	Pm-11	+/–	+	+
	Pm-12	+/–	–	–
	Pm-15	+/–	+/–	+
IV	B-1500	+/–	+	+
	B-1501	+/–	+	+

[a] Spectral absorption pattern; see Figure 1.
[b] + = Yellow pigmentation equal to that of strain B-24. – = No pigmentation. +/– = Intermediate pigmentation.
[c] + = Growth on Lukezic basal (LKB) medium. – = No growth on LKB medium. +/– = Intermediate growth on LKB medium.
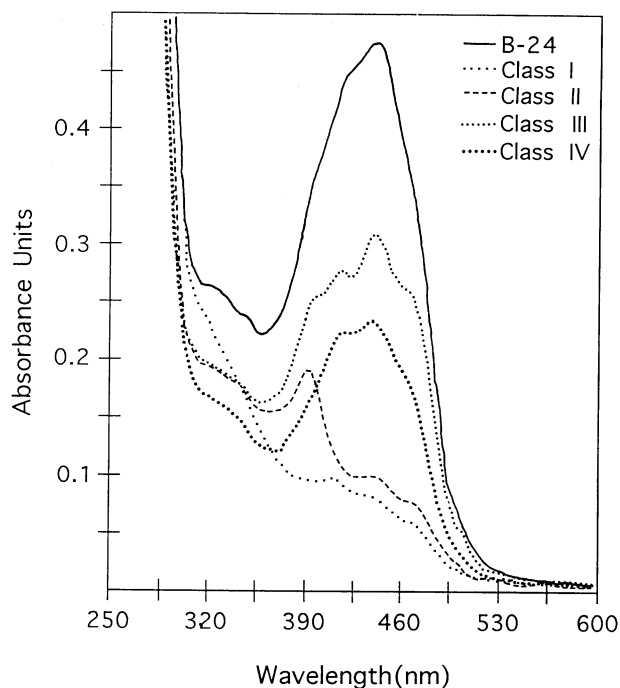[d] + = Typical disease symptoms caused on cauliflower. – = No symptoms observed.

Fig. 1. Absorption spectra of crude methanol extracts of *Xanthomonas campestris* pv. *campestris* strain B-24 and four classes of pigment mutants.

A probe generated from subclone pIG233 (Fig. 3) showed positive signals to all strains of 18 pathovars of *X. campestris* (Fig. 5B, A-1 to H-3, and Fig. 6), including nonpigmented strains (Fig. 5B, H1-H3, and Fig. 6, 2–8). A strain of *X. fragariae* (Fig. 5B, G-3) and all strains of *Pseudomonas*, *Erwinia*, and *Clavibacter* showed no hybridization to this probe.

Southern hybridization was used to determine if DNA from strains of *X. c.* pv. *campestris* and other pathovars had the same or different restriction fragment length polymorphism (RFLP) patterns in the pigment region. As expected, all strains of the seven pathovars tested had DNA sequences homologous to the pigment region (Fig.

7). The hybridization patterns could be divided into two types, with only a few exceptions. The first type had strongly hybridizing *Eco*RI-*Hind*III fragments of 10.3, 8.5, and either 6.4 or 6.2 kbp (or a subset of these fragments), and the second type had strongly hybridizing fragments of 12.0, 9.0, and 4.6 kbp (or a subset thereof). Although patterns of strains within a given pathovar were quite similar, there was not a strict correlation between hybridization pattern and pathovar designation.

DISCUSSION

Although xanthomonadin pigments have been used extensively for the diagnosis and identification of *Xantho-*
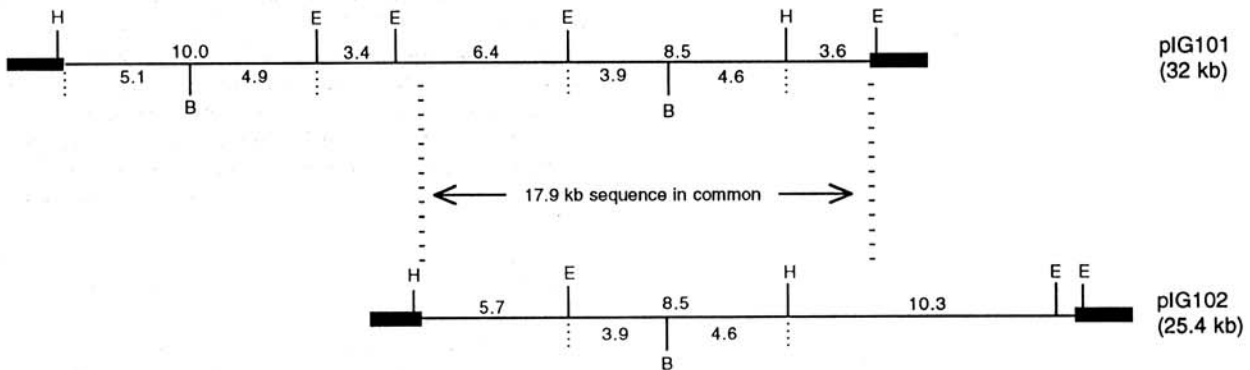

Fig. 2. Restriction endonuclease maps of two *Xanthomonas campestris* pv. *campestris* strain B-24 genomic cosmid clones that restored pigment production to pigment-negative strains. Thick line denotes vector sequences; thin line denotes cloned genomic sequences. Numbers are sizes in kilobases. E = *Eco*RI; H = *Hind*III; B = *Bam*HI.
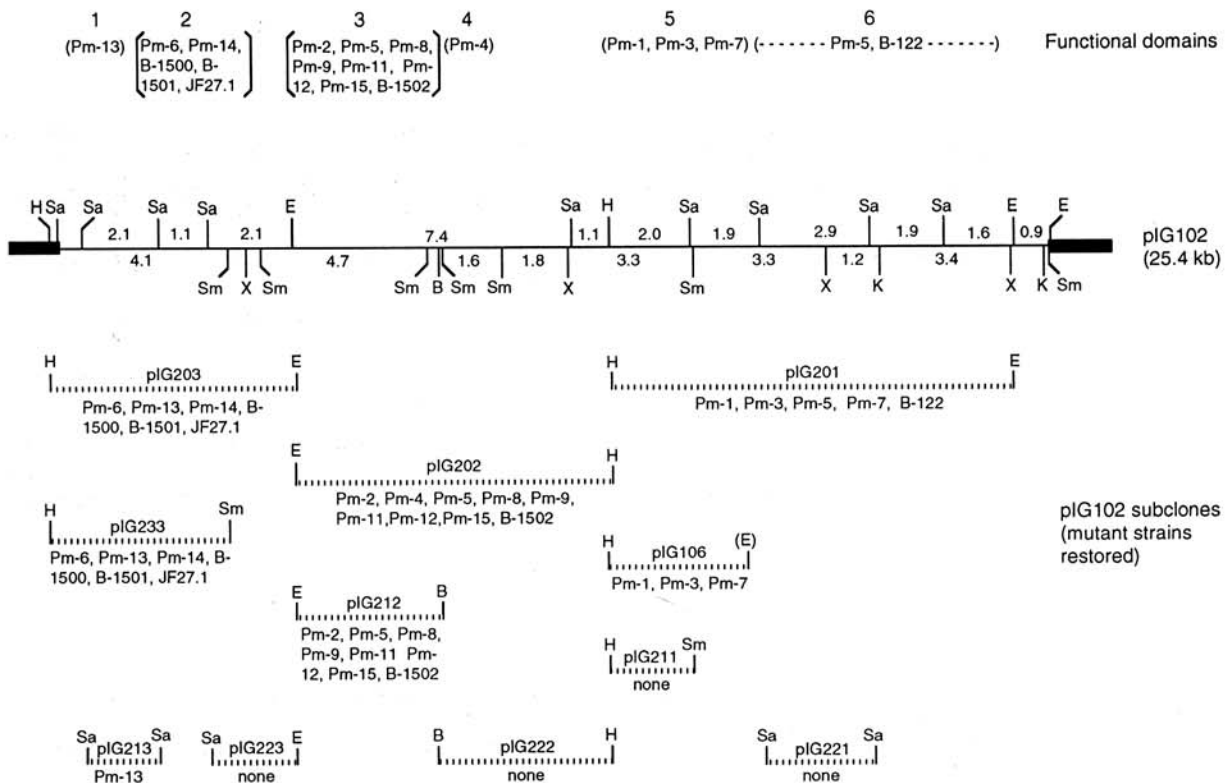

Fig. 3. Six pigment-functional domains, detailed restriction endonuclease map of *Xanthomonas campestris* pv. *campestris* genomic clone pIG102, and pIG102 subclones with the mutant strains that they restored to pigment production. Subclone pIG106 was constructed from pIG101. Numbers are sizes in kilobases. E = *Eco*RI; H = *Hind*III; B = *Bam*HI; Sa = *Sal*I; Sm = *Sma*I; K = *Kpn*I; X = *Xho*I.
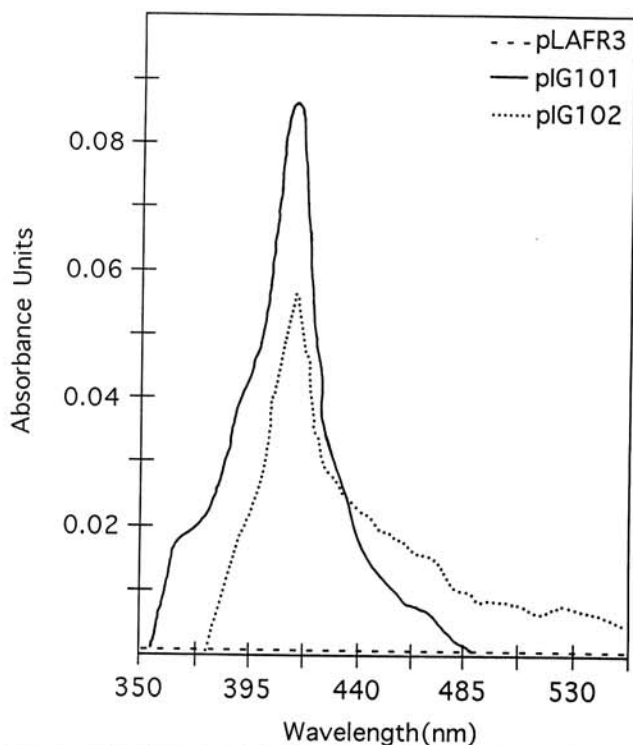
Fig. 4. Absorption spectra of crude methanol extracts of *Pseudomonas fluorescens* strain NRRL-15135 harboring either vector (pLAFR3) or one of the *Xanthomonas campestris* pv. *campestris* genomic clones pIG101 and pIG102. The NRRL-15135(pLAFR3) absorption spectrum was subtracted from each of the three spectra.

monas bacteria, there are at least two major limitations to this approach. First, *X. campestris* strains that produce little or no xanthomonadin pigments do occur. In a recent 2-year study, 3.6% of commercial crucifer seed samples judged positive for black rot yielded pathogenic isolates reduced or negative for xanthomonadin production (Poplawsky and Chun 1992). Also, most naturally occurring strains of some pathovars of *X. campestris* (for example *X. c.* pv. *manihotis* and *X. c.* pv. *mangiferaeindicae*) are nonpigmented (Moffet and Croft 1983). Second, since the yellow pigments of other plant-associated bacteria such as *Erwinia* or *Clavibacter* can be confused with xanthomonadins, tedious chromatographic techniques must be utilized to verify the presence of these pigments (Irey and Stall 1981). We reasoned that DNA probes based on xanthomonadin genes would have the reliability of the xanthomonadin pigments but might overcome these two limitations. Thus, the purpose of this study was to first identify and clone genes specifically involved in xanthomonadin production and then test these genes for their utility in the identification of *Xanthomonas* bacteria.

There are four lines of evidence indicating that the gene cluster identified on pIG102 (*pig*) is the major genomic region that specifies xanthomonadin production.

First, the major xanthomonadin-encoding region should restore pigment production to most or all of the pigment mutants tested. *pig* clones restored pigment production to all five naturally occurring mutants and all 14 induced mutants. Thus, all of the naturally occurring and induced
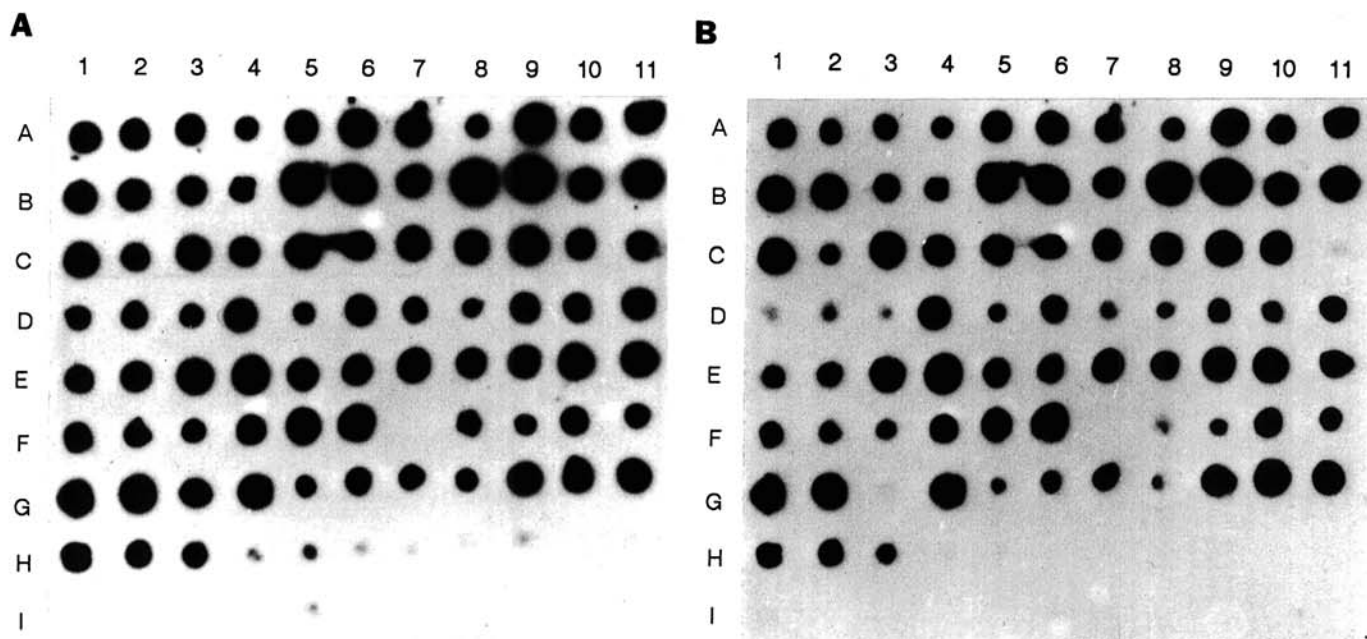

Fig. 5. Colony blots of bacterial genomic DNA from 98 strains probed with ^{32}P-labeled pIG102 insert sequences (**A**) and ^{32}P-labeled pIG233 insert sequences (**B**): A-1 to C-9, *Xanthomonas campestris* pv. *campestris*; C-10 to D-3, *X. c.* pv. *vesicatoria*; D-4 to D-8, *X. c.* pv. *phaseoli*; D-9 to E-3, *X. c.* pv. *carotae*; E-4 to E-6, *X. c.* pv. *malvacearum*; E-7 to E-11, *X. c.* pv. *translucens*; F-1 to F-3, *X. c.* pv. *pelargonii*; F-4 to F-5, *X. c.* pv. *pruni*; F-6, *X. c.* pv. *juglandis*; F-7 to F-9, *X. c.* pv. *dieffenbachiae*; F-10 to F-11, *X. c.* pv. *nigromaculans*; G-1, *X. c.* pv. *corylina*; G-2, *X. c.* pv. *alfalfae*; G-3 to G-4, *X. fragariae*; G-5 to G-7, *X. c.* pv. *citri*; G-8, *X. c.* pv. *glycines*; G-9 to G-10, *X. c.* pv. *oryzae*; G-11 to H-3, *X. c.* pv. *manihotis*; H-4 to H-5, *Pseudomonas syringae* pv. *syringae*; H-6 to H-7, *P. s.* pv. *phaseolicola*; H-8 to H-9, *P. s.* pv. *pisi*; H-10 to I-1, *Erwinia carotovora*; I-2 to I-5, *E. chrysanthemi*; I-6 to I-7, *E. herbicola*; I-8 to I-10, *Clavibacter michiganensis* subsp. *michiganensis*. See Table 2 for sources and origins of strains.

variability in pigment production occurred in the *pig* region.

Second, in a previous study five fractions of xanthomonadin pigments from a single strain of *X. c.* pv. *campestris* were differentiated on the basis of absorption spectra, molecular weights, degrees of bromination, and chromatographic properties (Starr et al. 1977). Thus, different types of genes, such as structural, regulatory, and modification/processing genes, could be involved in xanthomonadin production. One would expect mutations in the different types of genes to result in different pigment absorption spectra. We observed four different absorption spectrum classes among the 19 pigment mutants. Thus, the *pig* region may encode a number of different types of genes involved in pigment production.

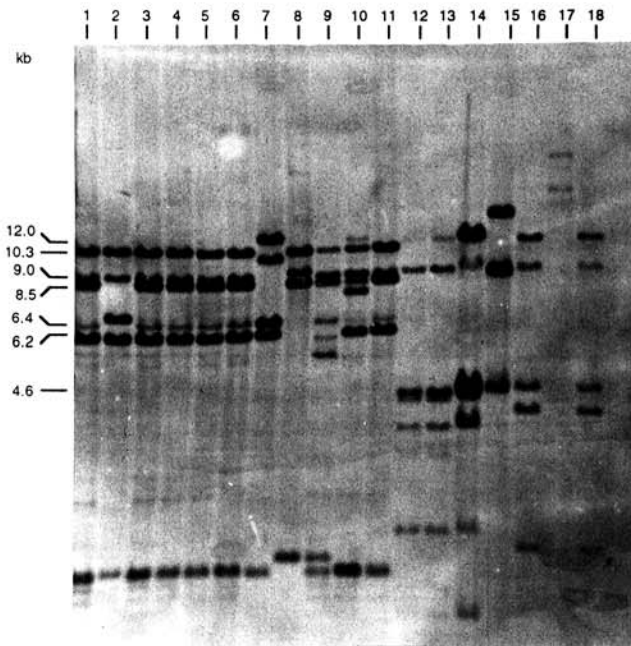Third, the structural complexity of xanthomonadins in-

dicates that several genes should be involved in their production. We identified six different functional domains for pigment production within the *pig* region. Since three of these domains (2, 3, and 5) included more than one class of pigment mutant, they probably are comprised of multiple genes for xanthomonadin production. Thus, the *pig* region encodes six or more genes for xanthomonadin production.

Fourth, if the complete information for xanthomonadin production is present in a clone, then its transfer to another genus of bacterium might confer the production of the pigment. One can place the five previously identified xanthomonadin fractions (Starr et al. 1977) into two absorption spectrum groups based on their absorption spectra maxima in acetone: group I, with maxima at 441–445 nm, and group II, with maxima at 424 nm. In our study, crude methanol extracts from strain B-24 showed a broad peak with a maximum at 441 nm and a poorly defined shoulder at 415–425 nm. Methanol extracts of pigment class III mutants showed two well-defined maxima at 415 and 441 nm. Thus, it is likely that the spectra of these crude preparations represented absorption spectra of both group I pigments (maxima at 441–445 nm) and group II pigments (maxima at 415–425 nm). Transfer of pIG101 or pIG102 to a *P. fluorescens* strain conferred the production of a methanol-soluble yellow pigment with an absorption maximum at 415 nm. This pigment was extracted by the same methods used for xanthomonadin extraction from *Xanthomonas*. The 415-nm wavelength corresponds to a group II
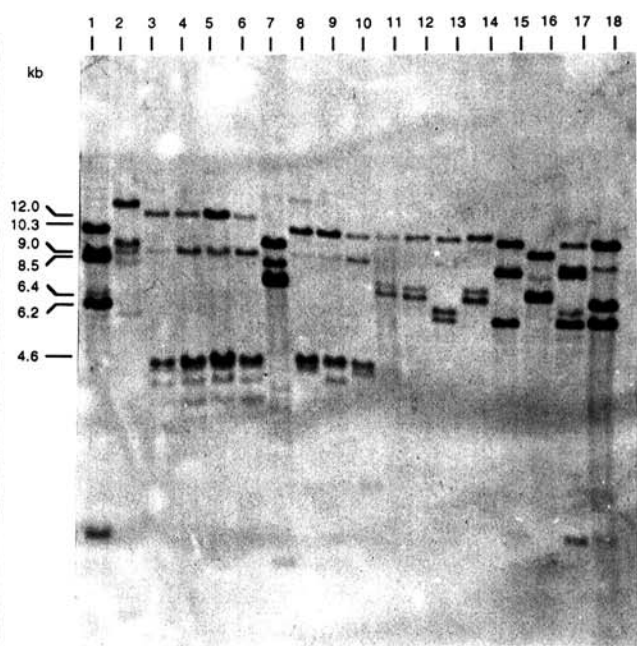

Fig. 6. Colony blots of bacterial genomic DNA from nine strains probed with digoxigenin-labeled pIG233 insert sequences: 1, *Clavibacter michiganense* H-108 (negative control); 2–5, naturally occurring *Xanthomonas campestris* pv. *campestris* strains reduced or negative for pigmentation; 6–8, naturally occurring nonpigmented strains of *X. c.* pv. *mangiferaeindicae*; 9, pigmented strain of *X. c.* pv. *mangiferaeindicae*. See Table 2 for sources and origins of strains.

Fig. 7. Southern hybridizations of *Eco*RI-*Hind*III–digested bacterial genomic DNA probed with pIG102 insert sequences. **A,** Lanes 1–10, *Xanthomonas campestris* pv. *campestris* strains B-24, B-1, B-4, B-18, B-33, B-32, B-122, B-1500, B-1501, and B-1502, respectively; lanes 11–14, *X. c.* pv. *manihotis* strains B-468, B-472, B-901, and B-902, respectively; lanes 15–18, *X. c.* pv. *mangiferaeindicae* strains JF27.1, JF26.1, A34, and JA162.1, respectively. **B,** Lane 1, *X. c.* pv. *campestris* strain B-24; lanes 2–6, *X. c.* pv. *vesicatoria* strains B-218, B-272, B-274, B-277, and B-280, respectively; lanes 7–10, *X. c.* pv. *phaseoli* strains B-494, B-703, B-707, and B-957, respectively; lanes 11–14, *X. c.* pv. *carotae* strains B-801, B-802, B-804, and B-817, respectively; lanes 15–18, *X. c.* pv. *translucens* strains B-430, B-451, B-452, and B-454, respectively.

xanthomonadin pigment. Pseudomonads are known to produce yellow-pigmented antibiotics (phenazines), but these pigments are not methanol-soluble and do not exhibit absorption maxima near 415 nm (Chang and Blackwood 1969). Thus, these *Pseudomonas* transconjugants apparently produced xanthomonadin pigments of absorption spectrum group II. Xanthomonadin production has never previously been observed in *Pseudomonas*, and methanol extracts of *Pseudomonas* transconjugants containing only vector sequences showed no absorption maxima typical of xanthomonadins. Thus, assuming that *Pseudomonas* does not encode any genes specifically involved in xanthomonadin production, the *pig* region does encode complete information for the production of at least some of the xanthomonadin pigments.

DNA probes based on xanthomonadin genes could have two applications in the diagnosis and identification of *Xanthomonas*. First, probes could be used to distinguish xanthomonads from non-xanthomonads in a colony hybridization assay, and this could replace the procedures for the identification of xanthomonadin pigments. Second, if each *X. campestris* pathovar had a unique RFLP pattern in the *pig* region, this could be used to distinguish strains of different pathovars.

We have shown that DNA probes based on xanthomonadin genes are useful for the first application. Probes from pIG102 and pIG233 showed differential hybridization between strains of *X. campestris* and non-xanthomonads. With pIG102, a strong hybridization signal (Fig. 5A, A-1 to H-3, excluding F-7) indicated that the strain was *Xanthomonas*, and a weak signal (Fig. 5A, H-4 to H-9) or a negative signal indicated that the strain was a non-xanthomonad. With pIG233, a weak to strong hybridization signal was positive and a negative signal was negative for designation as *Xanthomonas* (Fig. 5B).

DNA probes based on the *pig* region probably will not be useful for the differentiation of *X. campestris* pathovars. When the hybridization patterns of four to 10 strains of seven different pathovars of *X. campestris* were compared to each other using the pIG102 probe, they could be divided into two RFLP types. Since each of the two types was typical of a number of different pathovars, this technique cannot be used to determine pathovar designation.

pig-based DNA probes solve both of the problems inherent in the use of xanthomonadin pigments for the identification of xanthomonads. These probes provide a relatively simple and fast method to distinguish between *X. campestris* and non-xanthomonads. This method is more reliable and objective than the visual determination of xanthomonadins, and it is simpler and faster than the chromatographic separation of the pigments. The probes also showed strong hybridization signals to DNA from all 10 of the naturally occurring *X. campestris* strains tested that were reduced or negative for pigmentation. Thus, these probes identified strains as *Xanthomonas* that could not be identified by the conventional pigment extraction methods. All of the non-xanthomonads tested in this study were from plant habitats, thus these probes should be useful for distinguishing pathovars of *X. campestris* from other genera of bacteria associated with plants.

MATERIALS AND METHODS

Bacterial plasmids, strains, and culture conditions.

Escherichia coli strains used for genetic manipulations were JM109 and TB1 (Yanisch-Perron *et al.* 1985). All other strains used in this study are listed in Table 2. *X. c.* pv. *campestris* strains B-1500 and B-1501 were reduced in pigmentation. *X. c.* pv. *campestris* strains B-122 and B-1502, all *X. c.* pv. *manihotis* strains (except B-468), and all *X. c.* pv. *mangiferaeindicae* strains (except A-34) were negative for pigment production. *X. c.* pv. *campestris* strains and *X. c.* pv. *mangiferaeindicae* strain JF27.1 were naturally resistant to cephalexin, and this trait was used in conjugal matings. The cosmid cloning vector pLAFR3 (Staskawicz *et al.* 1987) was used for genomic library construction, and this cosmid as well as plasmid pUC18 (Yanisch-Perron *et al.* 1985) was used in subcloning. *Xanthomonas* strains were grown at 28° C on nutrient yeast glycerol (NYGB) medium (Turner *et al.* 1984), nutrient starch agar (NSA) medium (Schaad and Kendrick 1975), Lukezic basal (LKB) medium (Lukezic 1979), and 523 medium (Kado and Heskett 1970). NYGB medium was used unless otherwise specified. *E. coli* was grown at 37° C in Luria Bertoni (LB) medium (Davis *et al.* 1980). Antibiotics and other supplements were added to media when appropriate, at the following concentrations: kanamycin, 50 µg/ml; cephalexin, 100 µg/ml; tetracycline, 12 µg/ml; X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 25 µg/ml; and IPTG (isopropyl-β-D-thiogalactopyranoside), 20 µg/ml.

Selection and characterization of mutants.

EMS treatments were used to obtain pigment-negative mutants of *X. c.* pv. *campestris* strain B-24. The methods of Thorne *et al.* (1987) were used, except 523 medium was substituted for YT medium. EMS treatments of 2.5 µl/ml for 1 hr and 0.5 µl/ml for 2 hr resulted in approximately 99% lethality. These treatments yielded pigment-negative mutants at a frequency of approximately 2.5×10^{-3}. Naturally occurring mutants reduced or negative for pigment production were previously isolated from commercial crucifer seed (Poplawsky and Chun 1992).

Mutants altered in pigment production were evaluated for prototrophic growth after 3 days on LKB agar medium. For pathogenicity tests, cauliflower or cabbage plants (at the four-leaf stage) were inoculated with a syringe and 26-gauge needle as described by Schaad and Stall (1988). Plants were incubated for 10 days in a temperature-controlled (25° C) dew chamber (Model E-54-DL, Percival Mfg. Co., Boone, IA). All tests were repeated at least once.

For xanthomonadin analysis, equal cell numbers of each mutant strain were extracted with boiling methanol as previously described (Starr and Stephens 1963; Schaad and Stall 1988). Absorption spectra were obtained from a Beckman DU50 analytical spectrophotometer with the PeakPick feature (Beckman Instruments Inc., Fullerton, CA). Restoration of pigment production in mutants was determined by visual inspection and absorbance measurements (at 441 nm) of methanol extracts.

Table 2. Bacterial strains used in this study[a]

Genus, species, and pathovar	Strain	Genus, species, and pathovar	Strain
Xanthomonas campestris pv. *campestris*	B-1 (BBS, 1, CA, A-1)	*X. c.* pv. *translucens*	B-430 (—, 16, GA, E-7)
	B-4 (—, 2, GA, A-2)		B-433 (—, 16, GA, E-8)
	B-18 (Y-3, 2, FL, A-3)		B-451 (ATCC 19319, 16, WI, E-9)
	B-24 (—, 3, OR, A-4)		B-452 (NCPPB 1836, 16, USA, E-10)
	B-30 (K-2, 4, DEU, A-5)		B-454 (NCPPB 1943, 16, JPN, E-11)
	B-31 (K-2V, 4, DEU, A-6)	*X. c.* pv. *pelargonii*	B-937 (XP-39, 17, NY, F-1)
	B-32 (PDDCC-8, 5, NZL, A-7)		B-975 (XPEL-1370, 11, FL, F-2)
	B-33 (ATCC 33913, 5, GBR, A-8)		B-983 (INRA 1718, 18, FRA, F-3)
	B-36 (—, —, JPN, A-9)	*X. c.* pv. *pruni*	B-977 (XPRU-G14, 11, FL, F-4)
	B-38 (—, 2, JPN, A-10)		B-985 (ATCC 10016, —, IL, F-5)
	B-65 (UBL-25, 2, BRA, A-11)	*X. c.* pv. *juglandis*	B-981 (INRA 1024, 19, FRA, F-6)
	B-70 (—, 2, JPN, B-1)	*X. c.* pv. *dieffenbachiae*	B-400 (—, 20, FL, F-7)
	B-75 (—, 2, WA, B-2)		B-969 (XDIF 068-1163, 11, FL, F-8)
	B-82 (—, 6, AUS, B-3)		B-970 (XDIF 067-1260, 11, FL, F-9)
	B-85 (—, 2, GA, B-4)	*X. c.* pv. *nigromaculans*	B-967 (XNIG 069-1200, 11, FL, F-10)
	B-89 (—, 2, CA, B-5)		B-968 (XNIG 069-1238, 11, FL, F-11)
	B-94 (—, 2, CA, B-6)	*X. c.* pv. *corylina*	B-980 (ATCC 19313, 21, USA, G-1)
	B-107 (—, 2, CA, B-7)	*X. c.* pv. *alfalfae*	B-974 (XALF-G22, 11, FL, G-2)
	B-110 (—, 2, CA, B-8)	*X. fragariae*	B-467 (070-1866, —, FL, G-3)
	B-111 (—, 2, WA, B-9)		B-978 (XFRA-G12, 11, FL, G-4)
	B-112 (—, 2, WA, B-10)	*X. c.* pv. *citri*	B-778 (NCPPB 1472, 22, HKG, G-5)
	B-117 (#1, 7, WA, B-11)		B-729 (021, 13, BRA, G-6)
	B-122 (#11-3, 7, AZ, C-1 and 6:2)		B-769 (380, 23, BRA, G-7)
	B-123 (#12, 7, AZ, C-2)	*X. c.* pv. *glycines*	B-965 (XGLY-G56, 11, BRA, G-8)
	B-127 (#31, 7, CA, C-3)	*X. c.* pv. *oryzae*	B-442 (PXO-71, 24, PHL, G-9)
	B-161 (—, 2, —, C-4)		B-719 (—, 25, CHN, G-10)
	B-165 (—, 2, —, C-5)	*X. c.* pv. *manihotis*	B-468 (ENA-2647, 26, AFR, G-11)
	B-186 (—, 2, CA, C-6)		B-472 (ENA-2648, 26, BRA, H-1)
	B-301 (AB, 8, HI, C-7)		B-901 (XM-12, 27, —, H-2)
	B-305 (G-3-27, 8, HI, C-8)		B-902 (CIAT 1067, 27, VEN, H-3)
	BT-1 (—, 9, THA, C-9)	*X. c.* pv. *mangiferae-indicae*	JF26.1 (NCPPB 2438, 38, AFR, 6:7)
	B-1500 (—, 37, USA, 6:3)		JF27.1 (PDDCC 4087, 38, BRA, 6:6)
	B-1501 (—, 37, USA, 6:4)		A-34 (CFPB 29188, 38, AFR, 6:9)
	B-1502 (—, 37, USA, 6:5)		JA162.1 (CFPB 2933, 38, SAF, 6:8)
X. c. pv. *vesicatoria*	B-218 (549, 10, DE, C-10)	*Pseudomonas syringae* pv. *syringae*	C-203 (Nag-29, 288, ID, H-4)
	B-272 (XVES 81-19, 11, VEN, C-11)		C-227 (C-1, 29, MI, H-5)
	B-274 (XVES 81-23, 11, FL, D-1)	*P. s.* pv. *phaseolicola*	C-233 (NCPPB 1104, —, HUN, H-6)
	B-277 (XVES 71-21, 11, FL, D-2)		C-237 (NCPPB 380, —, SWE, H-7)
	B-280 (XVES 75-3, 11, FL, D-3)	*P. s.* pv. *pisi*	C-243 (ATCC 1105, —, —, H-8)
X. c. pv. *phaseoli*	B-494 (XP-11, 12, MI, D-4)		C-255 (NCPPB 1358, —, CAN, H-9)
	B-703 (ATCC 43276, 13, BRA, D-5)	*P. fluorescens*	NRRL-15135 (—, 37, WA, —)
	B-707 (—, 2, ID, D-6)	*Erwinia carotovora*	A-4 (—, 30, MO, H-10)
	B-957 (XP6061, 13, BRA, D-7)		A-8 (—, —, FL, H-11)
X. c. pv. *phaseoli* var. *fuscans*	B-959 (XPF-5825, 13, BRA, D-8)		A-13 (—, 31, GA, I-1)
X. c. pv. *carotae*	B-801 (XCAR-9, 11, CA, D-9)	*E. chrysanthemi*	A-33 (—, 32, GA, I-2)
	B-802 (NCPPB 425, 14, CAN, D-10)		A-308 (—, 33, WI, I-3)
	B-804 (UMB-46, 2, BRA, D-11)		A-310 (—, 34, FL, I-4)
	B-809 (ATCC 10547, —, —, E-1)		A-311 (—, 34, FL, I-5)
	B-810 (PDDCC-1407, —, USA, —)	*E. herbicola*	A-705 (—, 35, —, I-6)
	B-817 (—, 2, ID, E-2)		A-707 (—, 35, —, I-7)
	B-822 (—, 2, WA, E-3)	*Clavibacter michiganensis* subsp. *michiganensis*	H-108 (—, 36, NE, I-8 and 6:1)
X. c. pv. *malvacearum*	B-412 (XM-5, 15, CA, E-4)		H-119 (—, —, IA, I-9)
	B-941 (XM-3174, 13, BRA, E-5)		H-157 (—, —, CAN, I-10)
	B-945 (XM-4003, 13, BRA, E-6)		

[a] Information in parentheses refers to prior strain designation, source, geographical origin, and position in Figure 5 or 6 (designated 6:), respectively. A dash indicates that the information is not available. The numerical designations of sources are as follows: 1, R. G. Grogan; 2, N. W. Schaad; 3, L. Moore; 4, K. Rudolph; 5, D. Dye; 6, D. S. Trimboli; 7, R. H. Morrison; 8, A. Alvarez; 9, N. Thaveechai; 10, M. Sasser; 11, R. Stall; 12, A. Saettler; 13, K. Mohan; 14, B. Paine; 15, W. Schnathorst; 16, B. Cunfer; 17, R. S. Dickey; 18, B. Digat; 19, J. Luisetti; 20, Knass Apoka; 21, P. W. Miller; 22, A. C. Hayward; 23, J. R. Neto; 24, T. W. Mew; 25, H. Azad; 26, O. Kimura; 27, Ikotun; 28, D. Webster; 29, D. Fulbright; 30, R. Goodman; 31, D. Summer; 32, Scarborough; 33, A. Kelman; 34, J. Miller; 35, Sowell; 36, A. Vidaver; 37, A. Poplawsky; 38, O. Pruvost. For geographical origin, two-letter postal codes are used for U.S. states. Abbreviations for countries (ANSI three-letter standard codes) are as follows: AFR, Africa; AUS, Australia; BRA, Brazil; CAN, Canada; CHN, People's Republic of China; DEU, Federal Republic of Germany; FRA, France; GBR, United Kingdom; HKG, Hong Kong; HUN, Hungary; JPN, Japan; NZL, New Zealand; PHL, Phillipines; SWE, Sweden; THA, Thailand; USA, United States; VEN, Venezuela.

Genetic and molecular techniques.

Tri-parental conjugal transfer of pLAFR3 clones with the helper plasmid pRK2013 was performed as described by Ditta *et al.* (1980). Cosmid and plasmid DNA were isolated by alkaline lysis techniques and *Xanthomonas* genomic DNA was isolated by standard procedures (Ausubel *et al.* 1987). Standard methods were used for other DNA manipulations (Maniatas *et al.* 1982). The B-24 clone bank was constructed by partial digestion of genomic DNA with *Sau*3A, collection of the 20- to 30-kbp size fraction, ligation to *Bam*HI-digested pLAFR3 DNA, packaging into λ particles (Promega Corporation, Madison, WI), and transduction of *E. coli* JM109.

DNA probes were obtained by digestion of cosmid clones with appropriate restriction enzymes, electrophoresis in SeaKem GTG agarose (FMC BioProducts, Rockland, ME), and electroelution of excised bands in dialysis membranes (Ausubel *et al.* 1987). For most colony blots, isolated DNA fragments were then labeled with [^{32}P]dATP using random oligonucleotide primer extension (Ausubel *et al.* 1987). For Southern blots and one colony blot, DNA was labeled with digoxigenin using a commercially available kit (Genius, Boehringer Mannheim Corporation, Indianapolis, IN).

Standard methods were used for the preparation of colony and Southern blots (Maniatas *et al.* 1982) and DNA hybridization (Ausubel *et al.* 1987) using Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, CA). With colony blots, approximately equal volumes of bacteria from each strain were transferred to the membranes. Most colony hybridizations were performed with 50% formamide at 42° C, and subsequent washes were in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS (sodium dodecyl sulfate) at 65° C. Probe DNA was removed for reuse of the same blot by washing in 0.4 N NaOH at 42° C for 30 min and subsequently washing in 0.1× SSC, 0.5% SDS, and 0.2 M Tris (pH 7.5). Between hybridizations, blots were checked by autoradiography for residual signals. Southern hybridizations, one colony hybridization, and subsequent washes were performed as recommended by the manufacturer (no formamide and 68° C for hybridizations and, subsequently, two 15-min washes in 0.1× SSC and 0.1% SDS at 68° C). Detection of hybrids was according to the manufacturer's instructions (Genius, Boehringer Mannheim).

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