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pigB Determines a Diffusible Factor Needed for Extracellular Polysaccharide Slime and Xanthomonadin Production in *Xanthomonas campestris* pv. campestris†

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Seven xanthomonadin transcriptional units (*pigA* through *pigG*) were identified by transposon saturation mutagenesis within an 18.6-kbp portion of the previously identified 25.4-kbp *pig* region from *Xanthomonas campestris* pv. campestris (strain B-24). Since marker exchange mutant strains with insertions in one 3.7-kbp portion of *pig* could not be obtained, mutations in this region may be lethal to the bacterium. Complementation analyses with different insertion mutations further defined and confirmed the seven transcriptional units. Insertional inactivation of one of the transcriptional units, *pigB*, resulted in greatly reduced levels of both xanthomonadins and extracellular polysaccharide slime, and a *pigB*-encoding plasmid restored both traits to these strains. *pigB* mutant strains could also be restored extracellularly by growth adjacent to strains with insertion mutations in any of the other six xanthomonadin transcriptional units, the parent strain (B-24), or strains of five different species of *Xanthomonas*. Strain B-24 produced a nontransforming diffusible factor (DF), which could be restored to *pigB* mutants by the *pigB*-encoding plasmid. Several lines of evidence indicate that DF is a novel bacterial pheromone, different from the known signal molecules of *Vibrio*, *Agrobacterium*, *Erwinia*, *Pseudomonas*, and *Burkholderia* spp.

Members of the genus *Xanthomonas* are distributed worldwide and are the causal agents for disease for at least 124 monocot and 268 dicot plant hosts (20). Many of the phytopathogenic xanthomonads are disseminated on seed (22), and at least some of these pathogens survive and multiply as epiphytes prior to plant infection (13, 35). Two common traits of *Xanthomonas* spp. are the production of yellow pigments (xanthomonadins) and extracellular polysaccharide slime (EPS) (32). The xanthomonadins are yellow, membrane-bound, brominated aryl-polyene pigments unique to the genus *Xanthomonas* (35). Thus, these pigments are commonly used as chemotaxonomic (2, 36) and diagnostic (32) markers. Although the biological role of xanthomonadins is not well understood, a correlation between these pigments and protection against photobiological damage has been observed (14). A correlation between EPS production and virulence has been widely reported for *Xanthomonas campestris* (7, 9, 33, 38, 39).

In a previous study with *X. campestris* pv. campestris (causal agent of black rot of crucifers), six functional domains for xanthomonadin production were identified within a 25-kbp genomic clone (pIG102) (25). Sequences from this region (*pig*) restored pigment production to all 19 induced and naturally occurring xanthomonadin mutants tested (25). Clone pIG102 also conferred xanthomonadin production on a strain of *Pseudomonas*. Thus, it was concluded that at least some of these functional domains must be structural genes. The original purpose of this work was to perform a transcriptional analysis of *pig* by using transcriptional *lacZ* fusions created with the transposon Tn*3*HoHo1. In the course of this study, we made two unexpected observations. First, in some mutants, both xanthomonadin and EPS production were affected. Second, both traits could be restored in these mutants by growth on agar medium adjacent to other xanthomonadin mutants. Details of these two observations and the transcriptional analysis are reported herein.

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MATERIALS AND METHODS

Description and culture of bacterial strains and plasmids. *Escherichia coli* strains used for genetic manipulations in this study were TB1, HB101 (44), and C2110 (*polA*) (34). *X. campestris* pv. campestris mutant strains were all derived from strain B-24, which is resistant to cephalexin (25). *Erwinia carotovora* A-4, A-8, and A-18 (25) and *Agrobacterium tumefaciens* NT1(*pTiC58ΔaccR*) and NT1(pJM749, pSVB33) (1, 23) were previously described. Three strains of *Burkholderia solanacearum* (K56, S244, and MPS2) of diverse biovar designations and geographical origins were obtained from Caitilyn Allen (Department of Plant Pathology, University of Wisconsin—Madison) and used in this study. Recombinant plasmids were constructed with the cosmid vector pLAFR3, as described earlier (25). Plasmid pIG102 contains the entire *pig* region (kbp 0.0 to 25.4) (Fig. 1), pIG203 contains the region of *pig* encompassing kbp 0.0 to 5.7, and pIG201 contains the region of *pig* encompassing kbp 14.2 to 24.5. *Xanthomonas* strains were grown at 28°C in nutrient yeast glycerol (NYGB) medium (40), nutrient starch agar (NSA) medium (31), or minimal medium (4) supplemented with 1 mg of methionine per liter (MAKC). *A. tumefaciens* strains were grown on AB minimal medium (5), and *E. coli* was grown at 37°C in Luria-Bertani medium (10). Antibiotics were added to the medium when appropriate at the following concentrations: kanamycin, 50 mg/liter; tetracycline, 12 mg/liter; cephalexin, ampicillin, chloramphenicol, and nalidixic acid, each at 100 mg/liter.

Molecular and biochemical techniques. DNA isolations, Southern hybridizations, restriction endonuclease digestions, plasmid transformations, triparental conjugal matings, and xanthomonadin extractions and quantifications were performed as previously described (25). The relationship between the A_{441} readings of methanol extracts from *Xanthomonas* strain B-24 and xanthomonadin levels is linear (28). β-Galactosidase assays were performed with cultures grown to late log phase in NYGB medium, as described by Miller (21). Methods used for the extraction and quantification of EPS were similar to those of Kamoun et al. (16). Strains were grown on MAKC or NSA medium for 5 or 7 days, respectively, resuspended in sterile double-distilled water, and adjusted to an A_{600} of 2.0; equal volumes were then harvested in microcentrifuge tubes. EPS was precipitated from the resulting supernatants, first with 1/3 volume of 1% hexadecyltrimethylammonium bromide, then twice more with 4 volumes of 95% ethanol. Subsequent quantification was with Anthrone reagent (29).

Tn*3*HoHo1 insertion mutagenesis. To generate transposon insertion mutations in the *pig* region of *X. campestris* pv. campestris B-24, we utilized the

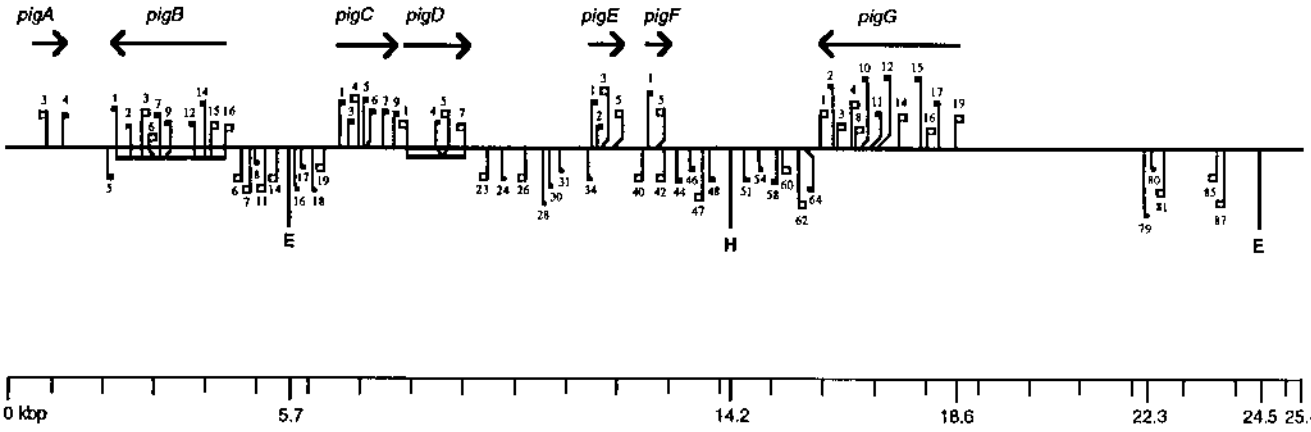

FIG. 1. Tn*3*HoHo1 insertion mutation map of pIG102, showing seven *pig* transcriptional units (*pigA* through *pigG*). Flags above the line represent mutations which caused a complete loss of pigmentation and are numbered sequentially within each transcriptional unit; flags partially below the line represent mutations which caused a reduction in pigmentation; and flags completely below the line represent mutations which did not alter pigmentation and are numbered separately from the *pig* transcriptional units. Mutant strains with full (greater than 30 U), intermediate (9 to 23 U), and no (less than 3 U) β-galactosidase production are indicated by solid square flags, solid round flags, and open square flags, respectively. With each insertion, the flag is oriented in the direction of transcription of the β-galactosidase gene as determined by restriction endonuclease analysis. Distances are in kilobase pairs. Letters indicate the *Eco*RI (E) and *Hin*dIII (H) restriction endonuclease digestion sites.

mutagenesis scheme of Stachel et al. (34) with the following modifications. *pig* DNA fragments to be mutagenized were cloned in pLAFR3 (Tc^r), and these target plasmids (pIG102 and pIG201) were used for transformation of HB101 (pHoHo1, pSShe). The plasmid pHoHo1 contains the Ap^r, β-galactosidase-encoding, transposase-disarmed Tn*3*HoHo1. Plasmid pSShe encodes a functional transposase and chloramphenicol resistance. Transformants were selected with tetracycline, ampicillin, and chloramphenicol and were stored at 4°C for 4 to 8 weeks before use. Storage at 4°C greatly increased the frequency of Tn*3*HoHo1 insertion mutations isolated in subsequent manipulations. Strain C2110 (Nal^r *polA*), which allows replication of pLAFR3 clones but not of the other plasmids from the above transformants, was subsequently used as the recipient in triparental matings with the transformants and HB101(pRK2013). Transconjugants with putative Tn*3*HoHo1 transpositions to target plasmids were selected with nalidixic acid, ampicillin, and tetracycline. Since strain C2110 is unsuitable for plasmid DNA analysis, plasmid DNA was isolated from each transconjugant and used in the transformation of HB101. Plasmid DNA was subsequently isolated from each Ap^r Tc^r HB101 transformant, and the sites and orientations of the Tn*3*HoHo1 insertions were determined by restriction endonuclease digestion by using the previously constructed *pig* restriction endonuclease map (25).

In order to analyze the effect(s) of insertion mutations, each mutated plasmid was transferred from *E. coli* HB101 to *X. campestris* pv. campestris B-24 by triparental matings with HB101(pRK2013). Then, recombinational exchange events between the mutated, plasmid-borne *pig* region and the functional, B-24 chromosomally borne *pig* region were selected with each transconjugant in the following manner. Ap^r Tc^r Ce^r transconjugants were purified and then grown for several cycles on medium containing ampicillin, tetracycline, and cephalexin to allow for recombinational exchange. Each transconjugant was then grown in NYGB (with no antibiotics) for several cycles to allow for loss of the plasmid, and dilutions were plated on NYGB medium with ampicillin. Ap^r Tc^s isolates were considered presumptive B-24, Tn*3*HoHo1 insertion mutant strains. In all cases, recombinational exchange of insertion mutations was verified by Southern hybridization, with the *pig* region used as a probe.

Insertion mutation strains were tested for pathogenicity by artificial infection and methods which were previously described (25).

Extracellular complementation. *Xanthomonas*, *Erwinia*, and *Burkholderia* strains were tested for extracellular complementation by streaking fresh bacterial resuspensions at right angles to each other on NSA and MAKC agar media. When the portion of a streak of a nonpigmented strain adjacent to the streak of a second (pigmented or nonpigmented) strain turned yellow and mucoid within 3 to 5 days, this was interpreted as extracellular complementation. Methods for quantification of extracellular complementation were as follows. Potential donor strains were grown to late log phase in NYGB. Bacterial cells were harvested and discarded, and the supernatant was sterilized with a 0.2-μm-pore-size filter. Sterilized culture supernatants were mixed in a 1:1 ratio with either fresh (2×) NYGB or fresh (2×) molten NSA. The supplemented NYGB was then used for growth of a *pigB* mutant and quantification of xanthomonadin production (25), whereas solidified, supplemented NSA medium was used for quantification of EPS.

The *X. campestris* pv. campestris parent strain, B-24, was tested for extracellular complementation of the *A. tumefaciens tra* system on AB minimal medium with the strains and methods of Piper et al. (23). NT1(*pTiC58ΔaccR*) is the positive-control strain, which constitutively synthesizes the *Agrobacterium* autoinducer, and NT1(pJM749, pSVB33) is the autoinducer indicator strain, which is negative for autoinducer production and has a *lacZ* fusion to a *tra* gene.

RESULTS

Transcriptional analysis of *pig*. Tn*3*HoHo1 mutagenesis of pIG102 and pIG201 (Fig. 1, kbp 0.0 to 25.4 and 14.2 to 24.5, respectively) in *E. coli* yielded 153 insertion mutations that mapped within the 25.4 kbp of target sequences. Of these, 125 were chosen for marker exchange mutagenesis with *X. campestris* pv. campestris B-24. From these experiments, 76 separate marker exchange mutants were generated (Fig. 1) and subsequently verified by Southern hybridization with pIG102 and pigment restoration with the appropriate *pig* subclone (data not shown). This resulted in the saturation of *pig* with insertion mutations, except for a 3.7-kbp region near the right end (Fig. 1, kbp 18.6 to 22.3). Although 13 insertion mutations were generated in this region in *E. coli*, and these plasmids were transferred to and stably maintained in strain B-24, we were unable to obtain any B-24 recombinational-exchange mutants in this region despite numerous attempts. When exchange mutants were characterized with respect to pigment and β-galactosidase production, the presence of seven transcriptional units (*pigA* through *pigG*) involved in pigment production was revealed (Fig. 1). Although insertions in most of the transcriptional units caused a complete loss of visible pigmentation, mutants with insertions in *pigB* and *pigD* appeared slightly pigmented after prolonged growth. Except for one instance (*pigC* and *pigD*), transcriptional units were separated by insertion mutations which did not affect pigmentation (Fig. 1). All 125 mutations (including those which could not be transferred to *X. campestris* B-24 by recombinational exchange and are not shown in Fig. 1) were designated numerically within each pigment transcriptional unit. Thus, the first mutation shown in Fig. 1 is *pigA3*, and the B-24 exchange mutant strain containing this mutation is designated B24-A3.

Two insertion mutation strains from each of the seven transcriptional units (with the exception of *pigB*) were compared to the parent strain for pathogenicity. *pigB* insertion mutation strains B24-B2, B24-B9, B24-B12, and B24-B14 were also tested. All of these insertion mutation strains caused typical

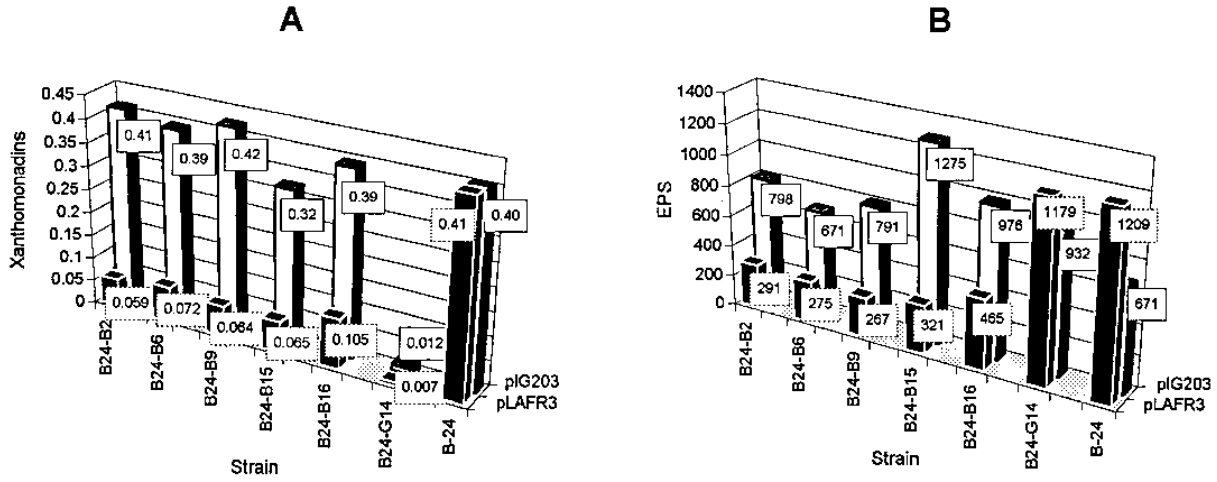

FIG. 2. Levels of xanthomonadin, measured as A_{441} (A), and EPS, measured in micrograms per milliliter (B), expressed by mutants (front bars), complemented mutants (back bars), and the parent strain (B-24). See Fig. 1 for locations of insertion mutations. Mutant strains harbored the cloning vector (pLAFR3) or the *pigB* clone (pIG203). Values are averages of two replicate experiments. Least significant difference was 0.018 ($P \leq 0.05$) for xanthomonadins and 195.3 ($P \leq 0.05$) for EPS.

black rot symptoms which were indistinguishable, with respect to timing and severity, from those caused by the parent strain.

Xanthomonadin and EPS production of *pigB* mutants. Strain B-24 *pigB* exchange mutants appeared to have reduced EPS production on agar medium. To verify this, pIG203 (Fig. 1, kbp 0.0 to 5.7) was transferred to five mutants distributed throughout the *pigB* region, and the xanthomonadin and EPS production of each mutant and complemented mutant was quantified (Fig. 2). Xanthomonadin production levels of *pigB* mutants averaged 18% of the levels of the parent strain (B-24), whereas a *pigG* mutant strain which appeared completely nonpigmented (B24-G14) had levels which were only 2% of the B-24 levels. Subclone pIG203 restored the xanthomonadin levels of the *pigB* mutants to 98% or greater of the parent strain levels, except for mutant B24-B15, which was restored to only 80% of the B-24 levels. As expected, pIG203 had no effect on the xanthomonadin levels of B24-G14. EPS production of *pigB* mutants averaged 27% of production by the parent strain, and that of B24-G14 was the same as that of strain B-24. The subclone pIG203 restored the *pigB* mutants to EPS levels ranging from 56 to 106% of those of the parent strain. Although pIG203 had no effect on xanthomonadin production in either B24-G14 or B-24, it inhibited EPS production in both of these strains (Fig. 2).

Extracellular complementation of *pigB* mutants. During the short-term storage of multiple B-24 gene exchange mutants on the same petri dish, we noticed that production of both xanthomonadin and EPS appeared to be restored in *pigB* mutants in the presence of other mutants (extracellular complementation). Thus, two mutants from each *pig* transcriptional unit were tested in pairwise combinations with each of two mutants from every other transcriptional unit. Only strains with mutations in *pigB* could be restored by extracellular complementation. Mutants from all of the other transcriptional units, as well as the parent strain, B-24, were capable of extracellular complementation of *pigB* mutants. Extracellular complementation was observed on both NSA and MAKC minimal medium. These results suggested that *pigB* determines the production of a diffusible, extracellular complementation factor (DF).

The diffusible, extracellular, nontransforming nature of the factor was confirmed in the following manner. The parent strain, B-24, was grown on agar medium, and an agar block was aseptically excised from the area adjacent to the streak. The agar block was transferred to fresh medium, and strain B24-B2 was streaked adjacent to it. The portion of this streak adjacent to the agar block became pigmented and mucoid after 3 days of growth, whereas the portion of the streak furthest from the agar block remained nonpigmented and nonmucoid. When B24-B2 was complemented extracellularly on agar medium by the parent strain, B-24, and cells from the complemented (pigmented, mucoid) portion of the streak were recultured on fresh agar medium, growth of only nonpigmented, nonmucoid colonies was observed.

To determine the relationship between *pigB* and DF, a *pigB* mutant harboring pLAFR3 was streaked at right angles to the same mutant harboring pIG203 (pLAFR3 with the cloned, functional *pigB* sequences). With all of the *pigB* mutations tested (*pigB1*, *pigB2*, *pigB3*, *pigB6*, and *pigB9*), the portion of the pLAFR3-harboring *pigB* mutant streak adjacent to the pIG203-harboring mutant became pigmented and mucoid within 5 days. To quantify this effect, B24-B9 was grown in filter-sterilized medium conditioned by prior growth of either B24-B9 harboring the cloning vector pLAFR3 or B24-B9 harboring pIG203. Growth in medium previously conditioned by B24-B9(pIG203) allowed B24-B9 to produce xanthomonadins and EPS at levels comparable to those of the parent strain (Table 1 and Fig. 2). However, growth of B24-B9 in medium conditioned by B24-B9(pLAFR3) resulted in xanthomonadin and EPS levels more typical of B24-B9 growth in nonconditioned medium. Uninoculated conditioned media were always tested for possible contamination due to ineffective sterilization, and contamination was never observed.

The extent of DF production in *Xanthomonas* was tested

TABLE 1. Response of *pigB* mutant B24-B9(pLAFR3) to two conditioned media[a]

Medium	EPS[b]	Xanthomonadin[c]
B24-B9(pLAFR3)	497.2	0.06
B24-B9(pIG203)	1,071.3	0.35

[a] Values are averages of two replicate experiments.
[b] In micrograms per milliliter.
[c] Absorbance at 441 nm of methanol extracts.

TABLE 2. Pigmentation and production of DF by strains of *Xanthomonas*

Strain[a]	Pigmentation[b]	DF[c]
X. arboricola pv. pruni B-1800	+4	+
X. axonopodis pv. manihotis		
B-468	+4	+
B-472	−	+
B-901	−	+
B-902 (CIAT 1067)	−	+
X. axonopodis pv. phaseoli		
B-494	+4	+
B-703 (ATCC 43276)	+4	+/−
B-707	+4	+
B-957	+4	−
X. axonopodis pv. vesicatoria		
B-272	+4	+/−
B-274	+4	+/−
B-277	+4	+/−
B-280	+4	+/−
X. campestris pv. campestris		
B-1	+4	+
B-4	+4	+
B-18	+4	+
B-24	+4	+
B-293 (ATCC 33913)	+4	+
B-32 (PDDCC-8)	+4	+/−
B-122	−	+
B-1502	−	+
B-1500	+2	−
B-1501	+2	−
X. campestris pv. carotae		
B-801	+4	+/−
B-802 (NCPPB 425)	+4	+
B-804	+4	+
B-817	+4	+
B-810 (PDDCC-1407)	+4	−
X. campestris pv. mangiferaeindicae		
B-1120 (PDDCC 4087)	−	+
B-1121 (NCPPB 2438)	−	+
B-1122	+4	+
X. translucens pv. translucens		
B-430	+4	+
B-433	+4	+
B-451 (ATCC 19319)	+4	+
B-452 (NCPPB 1836)	+2	+
B-454 (NCPPB 1943)	+4	+
X. vesicatoria B-218	+4	+

[a] According to the reclassification proposed by Vauterin et al. (41).
[b] Visual evaluation of each strain on NSA medium after 4 days of growth. −, no pigmentation; +2, intermediate pigmentation; +4, maximum pigmentation, similar to that of *X. campestris* pv. campestris B-24.
[c] Determined by testing for extracellular complementation of mutant B24-B2. The strain to be tested and B24-B2 were streaked at right angles adjacent to each other on the same NSA plate. Changes in pigmentation of B24-B2 were visually evaluated on days 4 and 9. +, noticeable pigmentation of the portion of the B24-B2 streak adjacent to the test strain after 4 days; +/−, noticeable pigmentation of the portion of the B24-B2 streak adjacent to the test strain after 9 days; −, no noticeable pigmentation changes in B24-B2 on day 4 or 9. Although EPS production was more difficult to determine visually than pigmentation, increased pigmentation was generally accompanied by increased mucoid.

with 37 strains of five different species and eight different pathovars (Table 2). Production was detected in 33 strains representing all five species and eight pathovars. In order to determine whether DF was identical to some known bacterial autoinducers, *Erwinia*, *Agrobacterium*, and *Burkholderia* strains were tested. The three strains of *E. carotovora* and three strains of *B. solanacearum* tested were unable to extracellularly complement the *pigB* mutant for either xanthomonadin or EPS production. *A. tumefaciens* NT1(*pTiC58ΔaccR*) extracellularly complemented *A. tumefaciens* NT1(pJM749, pSVB33), as determined by color change on AB minimal medium with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates. However, *X. campestris* pv. campestris B-24 showed no extracellular complementation activity of the *A. tumefaciens* indicator strain on this medium. In separate tests with AB minimal medium, strain B-24 clearly complemented B24-B2 for both xanthomonadin and EPS production.

TABLE 3. Intracellular complementation analyses with Tn*3*HoHo1 insertion mutations

Strain	Plasmid[a]	DF[b]	Pigmentation[c]
B-24		+	+4
B24-A1[d]		Nt	Nt
B24-A3		Nt	−
B24-A3	pIG102-*pigA1*	Nt	+4
B24-B15		−	+1
B24-B2		−	+1
B24-B2	pIG102-*pigB15*	−	+2
B24-B15	pIG102-*pigB2*	−	+2
B24-D7		Nt	+1
B24-C3		Nt	−
B24-C3	pIG102-*pigD7*	Nt	+4
B24-E2		Nt	−
B24-F5		Nt	−
B24-F5	pIG102-*pigE2*	Nt	+4
B24-G1		Nt	−
B24-G19		Nt	−
B24-G19	pIG201-*pigG1*	Nt	−

[a] Plasmid pIG102 or pIG201 with Tn*3*HoHo1 insertions as shown in Fig. 1.
[b] Only strains with mutations in *pigB* were tested for DF production in this experiment. Nt, not tested.
[c] Relative pigmentation. −, no pigmentation; +1, very little pigmentation; +2, intermediate pigmentation; +4, maximum pigmentation, similar to that of the parent strain, B-24.
[d] Mutation *pigA1* is at map position 0.25 kbp (see Fig. 1). This mutation could not be transferred to strain B-24; thus, pigment production was not tested.

Intracellular complementation analyses with Tn*3*HoHo1 insertion mutations. The polar nature of insertional inactivation was used in complementation analyses with different Tn*3*HoHo1 insertion mutations to further confirm and define the *pig* transcriptional units (Table 3). In all cases, pigmentation of multiple transconjugant isolates was visually determined, and each was checked for the presence of an unaltered plasmid by restriction endonuclease analysis. Mutations *pigA1* and *pigA3* were able to complement each other for xanthomonadin production, indicating that *pigA* transcription is initiated between kbp 0.25 and 0.9 within *pig*. Mutations *pigB2* and *pigB15* were unable to complement each other for DF production, confirming that *pigB* is a single transcriptional unit. Mutations *pigC3* and *pigD7* were able to complement each other for xanthomonadin production, indicating the separate natures of *pigC* and *pigD*. Likewise, mutations *pigE2* and *pigF5* were able to complement each other for xanthomonadin production, confirming the separate natures of *pigE* and *pigF*. Finally, mutations *pigG1* and *pigG19* were unable to comple-

ment each other for xanthomonadin production, confirming that *pigG* is a single transcriptional unit.

DISCUSSION

Transposon saturation mutagenesis of the previously identified 25.4-kbp *pig* region revealed seven distinct xanthomonadin transcriptional units within the region from kbp 0 to 18.6. Although we obtained transposon insertions in *E. coli* in the cloned *pig* region from kbp 18.6 to 22.3, we could not transfer these mutations to *X. campestris* pv. campestris B-24 by recombinational exchange. Since mutations on either side of this region were transferred to strain B-24, sufficient homologous DNA for recombinational exchange was not the limiting factor in these experiments. Thus, mutation in this region may be lethal to the bacterium.

The locations of the transcriptional units correlate with the approximate location of six xanthomonadin functional domains previously identified by restoration analysis of chemically induced mutants (25). *pigA* and *pigB* correspond to functional domains 1 and 2, respectively; *pigC* corresponds to nonpigmented mutants of functional domain 3; and *pigD* corresponds to partially pigmented mutants of functional domain 3. The existence of *pigE* and *pigF* was previously suggested by functional domain 4, and the previous identification of functional domains 5 and 6 indicates that *pigG* encodes at least two separate products. Thus, the xanthomonadin-encoding region of *X. campestris* pv. campestris (*pig*) is 18.4 kbp in size, consists of seven transcriptional units, and encodes at least eight different products needed for full xanthomonadin expression.

In addition to intracellular complementation, *pigB* mutants also had pigment and EPS production restored extracellularly, by a DF. Since insertional inactivation of *pigB* resulted in the loss of the production of xanthomonadins, EPS, and DF, and a plasmid encoding a functional *pigB* restored their production, it is clear that *pigB* is needed for the production of all three products. There are two possibilities concerning the nature of *pigB* and DF. First, *pigB* may encode a structural gene(s), and DF may be a biosynthetic intermediate which is lost from the cell and taken up by adjacent bacteria (cross-feeding). Alternatively, *pigB* may have a regulatory function, and DF may be a signal molecule (pheromone) needed for full xanthomonadin and EPS production.

For a number of reasons, DF is probably not a biosynthetic intermediate. First, with cross-feeding a number of intermediates in a biosynthetic pathway are usually freely diffusible and available for use by adjacent colonies (18). In this case, only one of a possible eight intermediates would have had these properties. Second, xanthomonadins are membrane bound (35); thus, one would not expect biosynthetic intermediates to be freely diffusible. Third, cross-feeding is usually observed due to overproduction (by a donor mutant strain) of a biosynthetic intermediate because of a mutation later in the pathway than the mutation of the recipient (*pigB* mutant) (18). However, in addition to strains with insertion mutations in each of the other six *pig* transcriptional units, the parent strain and 33 other *X. campestris* strains also produced DF. All of these strains would not be expected to have a suitable mutation causing a block in the xanthomonadin biosynthetic pathway, especially since most of them are pigmented. Fourth, DF restored both xanthomonadins and EPS to a *pigB* mutant. Since xanthomonadins and EPS are structurally dissimilar molecules, it is very unlikely that they would have a common biosynthetic intermediate. Finally, *pigB* mutants still produced a low level of xanthomonadins and EPS. This low-level production is more typical of inactivation of a regulatory gene than of inactivation of a gene in a biosynthetic pathway.

Two additional observations are consistent with the interpretation of *pigB* as having a regulatory function. First, only one of five *pigB* mutants had EPS production completely restored by pIG203, and pIG203 actually reduced EPS production in the parent strain, B-24, and the mutant strain B24-G14. Overproduction of a gene regulator, such as would be expected on a multicopy cloning vector such as pLAFR3, could explain these observations in the case of a regulatory component with both positive- and negative-acting functions (17, 19). This idea is supported by the recent observation of complete restoration of EPS production to the mutant strain B24-B2 by chromosomal gene exchange (28). Since pIG203 did not have similar inhibitory effects on xanthomonadin production, DF may operate in different regulatory circuits for these two bacterial products. Second, although proximal and distal mutations in *pigB* did not complement each other for production of DF, they did allow partial restoration of xanthomonadin production (see Table 3). This could be explained if pIG102 encoded all of the structural genes for xanthomonadin production and *pigB* had a positive-acting regulatory function in the case of xanthomonadin production. All of the xanthomonadin structural genes on multiple copies of pIG102 might partially replace the loss of the *pigB* regulatory function, through a gene dosage effect. Prior evidence indicates that pIG102 does encode all of the structural genes for xanthomonadin production (25).

Pheromones regulate key processes in a wide range of prokaryotes (12, 30, 37). Homoserine lactone (HSL) derivatives act as pheromones in several of these systems, including bioluminescence in the fish symbiont *Vibrio* spp. (3, 11), conjugal transfer of the Ti plasmid in the plant pathogen *A. tumefaciens* (23, 45), cell wall-degrading enzyme production in the plant pathogen *E. carotovora* (15, 24), and phenazine antibiotic production in the plant disease, biocontrol strain *Pseudomonas aureofaciens* (43). Our results indicate that the HSL autoinducers identified from *A. tumefaciens* and *E. carotovora*, as well as the volatile extracellular factor produced by *B. solanacearum* (6), are not functionally equivalent to the *X. campestris* DF. In addition, *X. campestris* pv. campestris B-24 culture supernatants did not activate phenazine antibiotic production in *P. aureofaciens* (42). We observed production of DF in 33 of 37 *Xanthomonas* strains tested, including naturally occurring nonpigmented strains of *X. campestris* pv. campestris, *X. axonopodis* pv. manihotis, and *X. campestris* pv. *mangiferaeindicae*. Two of the four strains in which production was not observed (*X. campestris* pv. campestris B-1500 and B-1501) have reduced xanthomonadin production and appear to have naturally occurring insertion mutations in the *pigB* region (27). These results suggest that the role of DF in xanthomonadin and EPS production may extend to most of the xanthomonads.

Recent results in our laboratory indicate that DF is active at very low concentrations, may have a lactone ring as part of its structure (8), and is important for the epiphytic survival of this phytopathogenic bacterium (26). Thus, DF is a novel signal molecule that may be related to the HSLs and may play a role in the regulation of bacterial metabolism important for *Xanthomonas* epiphytic survival.

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