Xanthomonas campestris pv. *campestris* Requires a Functional *pigB* for Epiphytic Survival and Host Infection

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When cauliflower plants (*Brassica oleraceae*) were misted with bacterial suspensions of *Xanthomonas campestris* pv. *campestris* (causal agent of black rot of cruciferous plants), two separate populations of the pathogen were associated with the leaves. Initially, bacteria removable by sonication and sensitive to sodium hypochlorite treatment predominated (easily removable epiphytic bacteria, EREB). However, after 2 weeks, bacteria not removable by sonication and insensitive to sodium hypochlorite treatment were dominant. Although the exact location of this second population of the pathogen was not determined, evidence is presented to support its location in protected sites on the leaf surface. *pigB* of this pathogen is required for production of extracellular polysaccharide (EPS), xanthomonadin pigments, and the diffusible signal molecule, DF (diffusible factor). DF can extracellularly restore EPS and xanthomonadin production to *pigB* mutant strains. Parent strain B-24 and *pigB* mutant strain B24-B2 were identical for in planta growth and symptomatology after artificial infection by injection in leaf mid-veins. Subsequently, *X. campestris* pv. *campestris* parent strain B-24, Tn3HoHo1 *pigB* insertion mutation strain B24-B2, chromosomally restored *pigB* mutation strain B24-B2R, and strain B24-79 with a Tn3HoHo1 insertion in an unrelated part of the genome were compared for epiphytic survival on, and natural infection of, cauliflower. After application, strains B-24, B24-B2R, and B24-79 all maintained leaf EREB populations of between approximately 3 and 6 (log [1 + CFU per g of fresh weight]) over a 3-week period, whereas B24-B2 populations fell to nearly undetectable levels. Plants sprayed with strains B-24, B24-B2R, and B24-79 averaged between 1.0 and 1.2 lesions, whereas those sprayed with B24-B2 averaged only 0.03 lesions per plant after 3 weeks. Differences in EREB population levels did not explain the observed differences in host infection frequencies, and the results indicated that strain B24-B2 was reduced in its ability to infect the host via the hydathodes, but unaffected in infection via wounds. When strains B-24 and B24-B2 were mixed in equal numbers and sprayed on plants to-

gether, B24-B2 epiphytic populations were intermediate between those of B-24 applied alone and B24-B2 applied alone. These results indicate that a functional *pigB* is required for epiphytic survival and natural host infection under the experimental conditions tested, and suggest that DF, xanthomonadins, and EPS could all be important for survival of this pathogen on the leaf surface, and/or for host infection.

Xanthomonas bacteria are the causal agents of disease on at least 124 monocot and 268 dicot plant hosts (Leyns et al. 1984). These bacteria are known for their mucoid, yellow-pigmented appearance, and many of them can survive and multiply as epiphytes (Starr 1981; Hirano and Upper 1983). Xanthomonadins are yellow, membrane-bound, brominated aryl-polyene pigments of uncertain function and unique to the genus *Xanthomonas* (Starr 1981). In one study, production of these pigments and protection against photobiological damage were associated (Jenkins and Starr 1982). The production of extracellular polysaccharide (EPS; responsible for mucoidy) is related to virulence in other phytopathogenic bacteria (Coplin and Cook 1990; Cook and Sequeira 1991; Kao et al. 1992), and may also be important for tolerance to desiccation (Wilkinson 1958; Roberson and Firestone 1992). An association between EPS production and virulence has been widely reported in *X. campestris* (Corey and Starr 1957; Sutton and Williams 1970a, 1970b; Daniels et al. 1984; Shaw et al. 1988).

In previous work, we identified an 18.6-kbp genomic region from *X. campestris* pv. *campestris* (*pig*) that contained seven transcriptional units involved in xanthomonadin production (Poplawsky et al. 1993; Poplawsky and Chun 1997a). Insertional inactivation of one of these transcriptional units, *pigB*, resulted in 82 and 73% reductions in xanthomonadin and EPS production levels, respectively (Poplawsky and Chun 1997a). Most of the strains of *Xanthomonas* tested produced a diffusible factor (DF) that could extracellularly restore *pigB* mutant strains to normal xanthomonadin and EPS production. DF had the properties of a pheromone (Chun et al. 1997), and a functional *pigB* was required for its production (Poplawsky and Chun 1997a). With artificial infection (syringe and needle inoculations into leaf mid-veins) *pigB* mutant strains were identical to the parent strain in plant symptomatology (Poplawsky

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and Chun 1997a). *pigB* is approximately 2.3 kb in size and probably encodes multiple products (Poplawsky and Chun 1997a). Partial DNA sequencing suggests that the product of the open reading frame ORF 1 has a regulatory role (Poplawsky and Chun 1997b).

The vast majority of research concerning the interaction between phytopathogenic xanthomonads and their hosts has focused on the pathogenic interaction that occurs after entry of the pathogen into the host. These studies have identified several pathogenicity, virulence, and host-specificity genes (for review see Keen et al. 1993). However, other stages of phytopathogenic bacterial development are important to the establishment of disease. In particular, epiphytic development prior to the entry of a bacterial pathogen into the host may be a prerequisite to plant infection and disease development (for reviews see Leben 1981; Hirano and Upper 1983). Natural infection of cruciferous hosts by *X. campestris* pv. *campestris* occurs through wounds or the hydathodes at the margins of host plant leaves (Russell 1898; Cook et al. 1952). This vascular pathogen then forms characteristic, V-shaped lesions emanating from the margins of the leaves. We reasoned that since xanthomonadins may be important for protection against photo-biological damage, and EPS may have a role in protection against desiccation, these two bacterial products could be important for survival of the pathogen on the leaf surface. Since a functional *pigB* is required for the production of EPS and xanthomonadins, we reasoned that *pigB* might be crucial for epiphytic survival and host plant infection. The following study tests this hypothesis.

RESULTS

Strain development and characterization.

Twelve pigmented, mucoid, ampicillin-sensitive, tetracycline-sensitive strains were isolated from continuous culture of B24-B2(pIG102) under conditions that were nonselective for the retention of pIG102. No pigmented strains were obtained from the simultaneous experiment conducted with strain B24-B2. When subjected to Southern hybridization with pIG102 as a probe, these 12 strains all showed the DNA rearrangement expected for replacement of the genomic, mutant *pigB* region with the plasmid-borne, unaltered *pigB* region (data not shown). One of these isolates was selected and designated B24-B2R.

Parent strain B-24, mutant strain B24-79 (a strain with a Tn3HoHo1 insertion in an unrelated part of the genome), and restored *pigB* mutant strain B24-B2R all had similar levels of EPS and pigment production, whereas the *pigB* mutant strain B24-B2 had xanthomonadin levels that were approximately 10% and EPS levels that were approximately 7% of those observed with the other strains (Table 1). Similar levels of DF production were detected from the first three strains, and no DF production was detected from B24-B2.

Recovery of strains B-24 and B24-B2 on a rich medium (NSA) was compared with recovery on the semi-selective medium, FS(M). Recovery of both strains on FS(M) medium was greater than 94% of that observed on NSA medium.

Epiphytic survival and disease development.

In preliminary experiments with strains B-24 and B24-B2, treatment of leaves with sodium hypochlorite was shown to

have no effect on internal numbers of the pathogen after syringe inoculations in leaf mid-veins (data not shown). Sodium hypochlorite treatment of leaves at 1 day or 1 week after misting with bacterial suspensions eliminated approximately 99.5 or 75%, respectively, of the bacteria that could be detected by sonication of the leaves.

Initially, it was of interest to determine the sizes and locations of different pathogen populations after spraying cauliflower plants with bacterial suspensions, and incubation of plants at 28°C and 40 to 50% relative humidity (RH). We found that strain B-24 bacteria removable by sonication (easily removable epiphytic bacteria, EREB) predominated at week 0 (Fig. 1A). However, a second population of the pathogen that was not removable by sonication steadily gained prominence during the 3-week period thereafter. After 3 weeks it represented 99% or greater of the total pathogen numbers, and was unaffected by treatment of the leaves with sodium hypochlorite. In the same experiment, strain B24-B2 also developed two similar pathogen populations (Fig. 1B), although at weeks 2 and 3 the numbers of both were, respectively, approximately 10- to 50-fold and 60- to 500-fold lower than those of strain B-24. Occasional, small, black rot lesions were first noticed at the margins of leaves after 2 weeks, and fully developed, chlorotic lesions with blackened veins became apparent after 3 weeks of incubation. At this time, plants sprayed with strain B-24 averaged 2.82 lesions, whereas those sprayed with A-7 averaged 0.08. This experiment was repeated with similar results (data not shown).

The development of EREB populations of four strains of *X. campestris* pv. *campestris* was compared after cauliflower plants were sprayed, and incubation at approximately 28°C and 40 to 50% RH. Over a 3-week period, EREB populations of the parent strain B-24 and restored mutant strain B24-B2R stayed close to the starting level (5.0 to 6.0, log [1 + CFU/g]), whereas the populations of *pigB* mutant strain B24-B2 decreased from this level to approximately 1.0 (log [1 + CFU/g]) when detectable (Fig. 2A). This experiment was repeated with similar results (data not shown). No populations of the pathogen were ever observed on plants sprayed only with buffer. When B24-79 (a Tn3HoHo1 mutant strain with an insertion in an unrelated part of the genome) was tested, EREB population levels of this strain and B-24 fluctuated between the starting level (approximately 6.0, log [1 + CFU/g]) and approximately 3.0 (log [1 + CFU/g]), (Fig. 2B). EREB population levels of strain B24-B2 were essentially undetectable. This experiment was also repeated with similar results (data not shown).

Table 1. Characterization of four strains of *Xanthomonas campestris* pv. *campestris*

Strain	Xanthomonadins ^w	EPS (mg/ml) ^x	DF ^y
B-24 (parent)	0.476 a ^z	3.06 a	+
B24-B2 (<i>pigB</i> mutant)	0.048 b	0.21 b	–
B24-B2R (restored mutant)	0.461 a	2.63 a	+
B24-79 (unrelated mutant)	0.462 a	2.91 a	+

^w Absorbance at 441 nm of boiled methanol extracts.

^x Extracellular polysaccharide, quantified with Anthrone reagent.

^y Diffusible factor.

^z Values are averages of two replicates. Values in each column followed by different letters were statistically different: LSD(0.05) = 0.03 for xanthomonadins, and 0.50 for EPS.

In the above experiments, typical black rot lesions were observed originating at the margins of leaves after 2 to 3 weeks. Treatment of plants with strains B-24, B24-B2R, and B24-79 resulted in approximately 1.0 to 1.2 black rot lesions per plant after 3 weeks (Table 2). However, only one, small, black rot lesion was observed in all of the plants sprayed with strain B24-B2 (0.03 lesions per plant). When this diseased tissue was macerated and dilutions were plated on FS(M) medium, only *Xanthomonas* bacteria typical of strain B24-B2 (nonmucoid, nonpigmented, and Ap^r) were recovered.

Incubation of plants at 95% RH instead of 40 to 50% RH allowed for increased EREB population levels of *X. campestris* pv. *campestris* (Fig. 3). Strain B-24 EREB population levels increased approximately 10- to 100-fold, whereas B24-B2 levels decreased 10- to 100-fold during the 3-week period. After 3 weeks, plants sprayed with strain B-24 averaged 1.00 lesions, and those sprayed with strain B24-B2 averaged 0.04 lesions (Table 2). This experiment was repeated with similar results (data not shown).

Since lesion numbers were normally distributed (data not shown) and bacterial epiphytic populations are lognormally distributed (Hirano et al. 1982), a Pearson's one-tailed correlation analysis for normally distributed variables was performed on the number of black rot lesions observed at 3 weeks, and EREB population levels ($\log [1 + \text{CFU/g}]$) of all of the strains in the above described experiments. Correlations at 16 h and at 1, 2, and 3 weeks were 0.07, 0.45, 0.47, and 0.56, respectively, and none of these was significant.

When parent strain B-24 and *pigB* mutant strain B24-B2 were mixed in equal numbers and sprayed on cauliflower plants that were then incubated at 28°C and 40 to 50% RH, B24-B2 population levels were intermediate between the levels of B24-B2 inoculated alone and B-24 inoculated alone at weeks 1 and 2, but then fell to levels close to those of B24-B2 inoculated alone at week 3 (Fig. 4). Strain B-24 population levels from the mixed applications were nearly identical to those of B24-B2 from the mixed applications until week 3, when they climbed to a level similar to that of B-24 inoculated alone. Similar results were obtained when this experiment was repeated (data not shown). Lesion numbers could not be assessed, since lesions caused by strains B-24 and those caused by strain B24-B2 were not distinguishable.

In planta growth and symptomatology.

After artificial inoculation into leaf mid-veins by injection, bacterial numbers in planta increased from approximately 3 to approximately 10 ($\log [1 + \text{CFU/g}]$) over a 6-day period (Fig. 5). The in planta growth characteristics of parent strain B-24 and mutant strain B24-B2 were nearly identical. The symptomatology of these two strains was also nearly identical, with approximately 65% of inoculated leaves exhibiting black rot symptoms (chlorosis, vein blackening, and wilting) at 6 days after inoculation, and 90% of the leaves exhibiting symptoms after 8 days. This experiment was repeated with similar results. Alternatively, each of these two strains was spray-inoculated onto 36 plants, each with one leaf wounded at the margin. After 2 weeks, lesions had developed at 25 and 17%

Fig. 1. A, Bacterial populations of *Xanthomonas campestris* pv. *campestris* parent strain B-24. **B,** *pigB* Tn3HoHo1 insertion mutation strain B24-B2. Both associated with cauliflower leaves after spraying with bacterial suspensions and incubation at 40 to 50% relative humidity. Suffix "S" refers to populations removed by sonication; suffix "T" refers to populations remaining after sonication; suffix "B" refers to populations remaining after sonication and treatment with sodium hypochlorite. Each data point is the mean of eight replicates. Bars at each point represent the standard error of the mean.

of the wound sites on plants sprayed with B-24 and B24-B2 suspensions, respectively, and these infection frequencies were not significantly different based on an F test ($P = 0.10$). However, in this same experiment, infection frequencies of 0.917 and 0.028 lesions per plant were observed at leaf margin, nonwound sites for plants sprayed with strain B-24 and B24-B2 suspensions, respectively, and these infection frequencies were significantly different based on an F test ($P = 0.005$). This experiment was repeated with similar results. In general, the lesions observed at wound sites were much larger and more highly developed than those at nonwound sites. After 3 weeks, many of the wounded, black-rot-infected leaves were completely senescent; thus, the numbers of lesions could not be enumerated reliably.

DISCUSSION

Although the phytopathogenic xanthomonads are not well characterized with respect to epiphytic survival, the few existing studies support the idea that the epiphytic phase is important in the disease cycle of these pathogens (Leben 1963; Cafati and Saettler 1980a, 1980b; Kuan et al. 1986; Timmer et al. 1987; Azad and Schaad 1988). In all of these studies, leaves were either washed or sonicated, or symptomless leaves were homogenized, and the resulting numbers were considered the surface or epiphytic populations of the pathogens.

In this study, we present evidence for two populations of the pathogen after spraying leaves with bacterial suspensions. Initially, bacteria removable by sonication predominated. However, by week 2 bacteria not removable by sonication, and not affected by treatment with sodium hypochlorite, predominated. These bacteria were detected by homogenization of leaves, thus we cannot be sure whether they were internal, or in "protected sites" on the leaf surface. Previous reports have suggested the presence of bacteria in protected sites on the leaf surface (Haeefe and Lindow 1987; Beattie and Lindow 1994), and for two reasons it is likely that at least some of the bacteria in our study were in similar sites. First, they were detected at only 16 h after leaves were sprayed. It is unlikely that infection and significant bacterial growth in planta (an increase to 3 to 5, $\log [1 + \text{CFU/g}]$) would have occurred in this short period of time. If this were the case, one would expect to have seen symptom development after 1 week, instead of the observed 2 to 3 weeks (see Figure 5). Second, these bacterial populations were detected with strain B24-B2 also, and since this mutant strain infected the host plant only very rarely (0.08 lesions per plant = 1.0 lesion per 25 leaves sampled), internal bacteria could not have accounted for the population levels observed. The levels of both bacterial populations were reduced with mutant strain B24-B2, compared with the parent strain B-24. In preliminary experiments, treatment with sodium hypochlorite had no effect on the pathogen in planta; thus, this treatment only affected epiphytic

Fig. 2. Epiphytic (EREB) populations of (A) *Xanthomonas campestris* pv. *campestris* parent strain B-24, *pigB* Tn3HoHo1 insertion mutation strain B24-B2, and chromosomally restored *pigB* mutant strain B24-B2R; (B) parent strain B-24, *pigB* Tn3HoHo1 insertion mutation strain B24-B2, and unrelated Tn3HoHo1 insertion mutation strain B24-79 detected on cauliflower plants incubated at 40 to 50% relative humidity. Each data point is the mean of five replicates. Bars at each point represent the standard error of the mean.

bacteria. Also, since most of the bacteria removable by sonication could not be recovered by sonication after sodium hypochlorite treatment, we conclude that the bacteria removable by sonication were epiphytic. Since we could be sure of the location of only those bacteria removable by sonication, all further experiments were conducted with sonication, and the bacterial populations detected were referred to as easily removable epiphytic bacteria (EREB).

We found that *X. campestris* pv. *campestris* parent and chromosomally restored *pigB* mutant strains maintained high EREB populations, and infected plants at about the same frequency. The EREB population levels of the *pigB*, single-site insertion mutation strain fell to undetectable or nearly undetectable levels, and the infection frequency was only about 3% of that of the other strains. Reductions in EREB population levels and host infection were not simply due to the insertion of Tn3HoH1 in the bacterial genome, since strain B24-79 had EREB population levels and host infection comparable to those of the parent strain, B-24. Thus, a functional *pigB* is required for the maintenance of EREB populations of this pathogen, and also for host infection. Since in the initial experiments (Fig. 1), the levels of the second, unidentified population of the pathogen varied proportionally with the EREB population levels, it is likely that a functional *pigB* is required for all epiphytic populations of the pathogen. Reductions in EREB populations of strain B24-B2 were not as great in these experiments as in the experiments in which EREB populations of all four strains of *X. campestris* pv. *campestris* were monitored (Fig. 2). Although we cannot be sure of the reason for this, the higher starting level of strain B24-B2 in the first experiment (approximately 6.7, log [1 + CFU/g]), compared with that in the later experiments (approximately 6.0, log [1 + CFU/g]) may have contributed to this difference.

These results contribute to what little is known about bacterial epiphytic fitness. Lindow (1991) suggested many "hypothetical" epiphytic fitness traits such as EPS production, tolerance to UV irradiation, motility, catabolic properties, and others. However, only in the case of motility is there evidence for a contribution to epiphytic fitness (Haefele and Lindow 1987). Our work shows that a functional *pigB* is specifically required for a high level of epiphytic fitness of *X. campestris* pv. *campestris* under conditions of moderate or low stress. Since *pigB* is needed for the production of DF, EPS, and xanthomonadins, these results suggest that all three of these bacterial products may contribute to epiphytic fitness. The possibility that xanthomonadins may be important for epiphytic survival is not surprising considering that these pigments may be involved in

protection against photobiological damage (Jenkins and Starr 1982), and there is evidence that *Erwinia herbicola* carotenoid pigments can protect *Escherichia coli* from damage due to UV irradiation (Gibbins and Peterson 1978, Tuveson et al. 1988). But the suggestion that EPS is an epiphytic fitness factor in *X. campestris* pv. *campestris* is more controversial, considering the preponderance of evidence for EPS as a virulence factor (Corey and Starr 1957; Sutton and Williams 1970a, 1970b; Daniels et al. 1984; Shaw et al. 1988), and that efforts to tie EPS to epiphytic fitness with *Pseudomonas syringae* have not been successful (Lindow 1991). However, EPS may function in different capacities during both the epiphytic phase and the pathogenic phase, and we may have observed effects due to the loss of multiple epiphytic fitness factors that might not be observed with the loss of just a single factor. Since EPS is thought to confer tolerance to desiccation, our observation of increased epiphytic survival of strain B24-B2 under conducive conditions (95% RH), compared with moderately restrictive conditions (40 to 50% RH), is consistent with the idea that EPS production could contribute to epiphytic fitness. If EPS is both a virulence factor and an epiphytic fitness factor in this pathogen, then one might expect two regulatory circuits, one for the regulation of EPS production as an epiphyte, and one for the regulation of EPS production as a pathogen. Indeed, a regulatory system for pathogenicity factors (including EPS) in *X. campestris* pv. *campestris* is being studied (Tang et al. 1991; Barber et al. 1996), and the diffusible signal component of this system (DSF) is functionally and biochemically different from DF (Poplawsky et al. 1998).

Table 2. Average number of lesions observed on plants 3 weeks after application of *Xanthomonas campestris* pv. *campestris*

Strain	Lesions per plant ^y	
	40% RH	95% RH
B-24 (parent)	1.16 a	1.00 a
B24-B2 (<i>pigB</i> mutant)	0.03 b	0.04 b
B24-B2R (restored mutant)	1.03 a	NT ^z
B24-79 (unrelated mutant)	0.97 a	NT

^y At two levels of relative humidity (RH). Each value represents the average number of lesions observed on 25 plants. Values followed by different letters were significantly different: LSD(0.05) = 0.64.

^z Not tested.

Fig. 3. Epiphytic (EREB) populations of *Xanthomonas campestris* pv. *campestris* parent strain B-24 and *pigB* mutant strain B24-B2 detected on cauliflower plants incubated at 95% relative humidity. Each data point is the mean of five replicates. Bars at each point represent the standard error of the mean.

Although bacterial pheromones have been implicated in the control of virulence or pathogenicity in a number of phytopathogenic bacteria (Jones et al. 1993; Pirhonen et al. 1993; Clough et al. 1994; Beck von Bodman and Farrand 1995), this is the first example of a bacterial pheromone that may be involved in epiphytic survival or host plant infection. The partial restoration of the epiphytic fitness of the *pigB* mutant strain by co-inoculation with the parent strain B-24 is consistent with a role for DF in epiphytic survival. Production of DF by B-24 may extracellularly restore the *pigB* mutant on the leaf surface. Extracellular complementation could also explain the isolation of naturally occurring *pigB* mutant strains from seed (Poplawsky and Chun 1995, 1997a), since pigmented DF⁺ strains were also isolated from this seed lot (A. R. Poplawsky, *unpublished results*). However, there are alternative explanations for these observations. Strain B-24 might establish a favorable micro-habitat on the leaf through production of EPS or other DF-controlled products, which might then be available for occupation by strain B24-B2. But, since co-application of partially purified DF with strain B24-B2 also partially restored the epiphytic fitness of this strain (Chun et al. 1997), the explanation of extracellular complementation on the leaf surface seems most likely.

The simplest explanation for the reduced infection frequencies observed with mutant strain B24-B2 is that reduced epiphytic survival of this strain resulted in reduced infection. For

a number of reasons, this is probably not the case. Previous investigators have concluded that *X. campestris* pv. *campestris* infects via wounds or the hydathodes found on the margins of the host leaves (Russell 1898; Cook et al. 1952; Bretschneider et al. 1989). In extensive studies, infection was never observed via stomata under natural conditions, and it was concluded that a continuous layer of liquid was required for infection at the hydathodes (Cook et al. 1952). In this investigation, it is likely that the infection we observed at non-wound sites was via the hydathodes, since the observed lesions originated at the margins of the leaves, and we took care to use plants without prior wound damage. Additionally, it is likely that infection occurred during the 16-h period after leaves were sprayed with bacterial suspensions, when the plants were incubated in a dew chamber where continuous leaf wetness and hydathode exudation droplets were maintained. Neither leaf wetness nor exudation droplets were ever observed during the subsequent incubation of plants at 40 to 50% RH for the following 3 weeks. Our observations of black rot lesions 2 weeks after plants were sprayed with bacterial suspensions is consistent with prior observations of disease following hydathode infection (Cook et al. 1952; Staub and Williams 1972). Since symptoms were observed in our experiments with artificial inoculation approximately 1 week after injection, natural infection could not have occurred later than 1 week after plants were sprayed with bacterial suspensions.

Thus, if reduced epiphytic populations were the reason for the B24-B2 reduced infection frequency, one would expect

Fig. 4. Epiphytic (EREB) populations of *Xanthomonas campestris* pv. *campestris* parent strain B-24, *pigB* mutant strain B24-B2, and these two strains when mixed together in equal numbers (suffix M), detected on cauliflower plants incubated at 40 to 50% relative humidity. Each data point is the mean of five replicates. Bars at each point represent the standard error of the mean.

Fig. 5. Growth in planta of *Xanthomonas campestris* pv. *campestris* parent strain B-24 and *pigB* mutant strain B24-B2. Each data point is the mean of four replicates. Bars at each point represent the standard error of the mean. After 7 days, black rot symptoms included extensive chlorosis, blackened veins, and wilting for both strains.

to see these population reductions at the sampling periods 16 h or 1 week after plants were sprayed. However, there was not a significant correlation between EREB populations levels and infection frequencies at these or at any other time periods. Additionally, in one experiment, the levels of both populations of strain B-24 were very similar to those of B24-B2 after either 16 h or 1 week (see Figure 1), yet the B24-B2 infection frequency was only approximately 3% of the B-24 frequency. Also, EREB populations of both strains B-24 and B24-B2 were greatly increased under conducive conditions (95% RH; see Figure 3), compared with moderately stressful conditions (40% RH; see Figure 2). Yet the infection frequencies of both strains were unaffected by these increased EREB population levels. Finally, in experiments with natural infection via the spraying of bacterial suspensions onto wounded leaves, although plants sprayed with strain B-24 developed a 33-fold greater number of lesions at nonwound sites than plants sprayed with strain B24-B2, the frequencies of infection of these two strains at wound sites were very similar. If the reduction in infection frequency of strain B24-B2 at nonwound sites were simply due to reduced levels of epiphytic bacteria, one would expect to have seen this same reduction in infection at wound sites.

If the difference in infection frequencies between strains B-24 and B24-B2 was not simply the result of differences in epiphytic population levels, then it must have been the result of a difference in the behavior of the two strains during infection. This difference was not a result of behavioral or growth differences after entry into the host, since the two strains showed nearly identical growth characteristics and symptomatology in planta after artificial infection. The difference in infection frequency of these two strains was not due to a difference in the general movement of the bacteria on the leaf surface to the infection point, since if this were the case one would expect to have seen a difference in the frequencies of infection of strains B-24 and B24-B2 at the leaf margin wound sites, as well as the nonwound sites. Assuming that most of the infection at nonwound sites was via the hydathodes (as discussed above), the difference in behavior between these two strains was directly related to entry via the hydathodes. These results indicate that there may

be a specific infection process that takes place via the hydathodes and does not occur during infection at wound sites. This is consistent with our observation that lesions at wound sites were much larger than those at nonwound sites after 2 weeks. The postulated specific infection process at hydathodes may require more time than the opportunistic infection at wound sites. Strain B24-B2 may be in some way compromised in this specific infection process, perhaps in its ability to congregate at, or actually enter, the hydathodes. This evidence for a hydathode-specific infection process is of particular interest, since f-gene resistance to black rot in cabbage is fully and rapidly expressed only when the bacteria enter via the hydathodes, as opposed to entry via wounds (Staub and Williams 1972).

The possibility of xanthomonadins, EPS, DF pheromone, or other pheromone-controlled products playing a direct role in bacterial host plant infection via natural openings is completely unexplored, and should provide a fertile field for future research.

MATERIALS AND METHODS

Bacterial strains and culture.

E. coli strains used in this study were TB1 and HB101 (Yanisch-Perron et al. 1985). Plasmid pRK2013 (Ditta et al. 1980) was used as a helper plasmid in the bacterial matings. Strain B-24 is a pathogenic, cephalixin-resistant, wild-type strain of *X. campestris* pv. *campestris* that was previously used to clone and characterize the xanthomonadin encoding region (*pig*) (Poplawsky et al. 1993; Poplawsky and Chun 1997a). pIG102 (tetracycline-resistant) is a pLAFR3 cosmid clone of the entire *pig* region that includes *pigB* (Poplawsky et al. 1993). Strains B24-B2 and B24-79 are derivatives of strain B-24 with Tn3HoHo1 insertions in *pigB* and a genomic region to the right of *pig*, respectively (Poplawsky and Chun 1997a; Fig. 6). Tn3HoHo1 is a derivative of the transposon Tn3 with a promoter-less β -galactosidase gene and a disarmed transposase gene (Stachel et al. 1985). This transposon can be used to inactivate and simultaneously construct transcriptional fusions with target genes.

Fig. 6. Partial map of the *Xanthomonas campestris* pv. *campestris* *pig* region showing the locations of Tn3HoHo1 insertions in strains B24-B2 and B24-79. Numbers represent distances in kilobase pairs from the left border of *pig* (see Poplawsky and Chun 1997a). Abbreviations of restriction endonuclease sites are as follows: Sa, *Sall*; Sm, *Sma*I; X, *Xho*I, and E, *Eco*RI.

Xanthomonas strains were grown at 28°C in nutrient yeast glycerol (NYGB) medium (Turner et al. 1984), nutrient starch agar (NSA) medium (Schaad and Kendrick 1975), or minimal medium (Chatterjee 1980) supplemented with 1 mg of methionine per liter (MAKC). *E. coli* was grown at 37°C in Luria Bertani (LB) medium (Davis et al. 1980). Antibiotics were added to the medium when appropriate at the following concentrations: kanamycin (Km), 50 mg/liter; tetracycline (Tc), 12 mg/liter; cephalixin (Ce) and ampicillin (Ap), each at 50 mg/liter.

Biochemical, molecular, and genetic techniques.

Previously described methods were used for conjugal plasmid transfer and xanthomonadin quantification (Poplawsky et al. 1993) and Southern hybridization (Poplawsky and Chun 1995), as well as EPS quantification and determination of DF production (Poplawsky and Chun 1997a). Chromosomally restored *pigB* mutant strains were selected in the following manner. The *pig* region-encoding cosmid pIG102 was transferred to the *pigB* mutant strain B24-B2, and a single, pigmented transconjugant was selected and purified (B24-B2[pIG102]). This strain was grown for three cycles on NSA(Tc) agar medium, which selected for retention of pIG102 and yellow pigmentation. It was then transferred to continuous NYGB broth culture with no antibiotics for five cycles, at which point the broth culture had turned from yellow to white due to the loss of pIG102. Dilutions were then plated on NSA agar medium with no antibiotics, and occasional yellow pigmented colonies were tested for resistance to tetracycline and ampicillin. Pigmented isolates sensitive to both antibiotics were presumed to be chromosomally restored at *pigB*. This was verified by Southern hybridization with pIG102 as a probe.

Epiphytic survival and in planta growth.

Cauliflower plants (variety 3303 from Tanimura & Antle, Salinas, CA) were germinated in 10.16-cm-diameter, plastic pots in Sunshine Mix #1 in the greenhouse, thinned to five plants per pot, and treated after 3 weeks with Rapid-Gro fertilizer. The abaxial and adaxial surfaces of leaves from 5- to 6-week-old plants were then misted with bacterial suspensions of strains B-24, B24-B2, B24-B2R, or B24-79, or buffer controls. Suspensions were prepared by washing bacteria from 2-day-old NSA medium plates with 0.01 M PO₄ buffer (pH 7.0, with 0.01% polyoxyethylene-sorbitan monolaurate [Tween 20] added as a wetting agent), adjusting the turbidity at A₆₀₀ to 1.0, and diluting 1:1000 in PO₄ buffer. This resulted in a bacterial suspension of approximately 10⁷ CFU/ml. Misted plants were left in the dew chamber in the dark for 16 to 20 h, during which leaf wetness was maintained. Plants were subsequently incubated for 3 weeks at 25 to 28°C and 40 to 50% RH or 95% RH, with a 16-h photoperiod at an intensity of approximately 4,000 lx. There were five pots per treatment, and pots were arranged in a completely randomized design. Upon transfer to less conducive conditions and each week thereafter, leaves were sampled for populations of the pathogen. A mature but nonsenescent leaf from each pot was selected and removed with the petiole. The leaves were weighed and then submerged individually in 18 × 150 mm borosilicate glass culture tubes, each containing 15 ml of PO₄ buffer. The end of the petiole was always kept well above the surface of the liq-

uid. Between each leaf sampling, all equipment was thoroughly cleaned with 95% ethanol. When all of the samples from a particular sampling period were collected, the tubes were submerged in a Branson model #3210 sonicator for 10 min. Subsequently, each tube was vortexed for 5 s and dilutions (10⁰, 10⁻¹, and 10⁻²) were plated on FS(M) medium, selective for the pathogen (Schaad 1989). Pathogen numbers were enumerated after 3 to 4 days of incubation at 28°C. Since epiphytic bacterial populations on leaf surfaces are lognormally distributed (Hirano et al. 1982), data were log-transformed and means and standard errors of the means were then calculated and plotted. Three weeks after the plants were sprayed, the number of typical black rot lesions found originating at the margins of plant leaves was recorded.

In the case of combination treatments with both B-24 and B24-B2, the two strains were mixed in equal numbers to achieve the same inoculum level as described above. Leaf sonicate dilutions were then plated on both FS(M) and FS(M) with ampicillin media. Strain B24-B2 was enumerated on the FS(M)(Ap) medium, and the B-24 population levels were calculated by subtracting the numbers observed on FS(M)(Ap) medium from those observed on FS(M) medium. Strains B-24 and B24-B2 could also be visually differentiated on FS(M) medium due to differences in pigmentation and mucoidy, although this became difficult on plates with greater than approximately 50 colonies. Presumably this difficulty was due to extracellular complementation of B24-B2 colonies by B-24 colonies on the same plates.

Experiments for the comparison of different bacterial populations of the same strain were conducted as described above with some slight differences. In these experiments, two leaves per pot were harvested from eight pots of plants per treatment each week. Leaves from one set were individually sonicated as described above, rinsed in fresh, sterile, phosphate buffer, and then homogenized with a Tissue Tearor (Biospec Products, Racine WI). Dilutions of sonicate solutions and homogenates were plated on FS(M) medium for colony enumeration. Colonies arising from homogenates were considered to be total bacteria remaining after sonication. Total bacteria remaining after sonication and bleach treatment were determined in the same manner as above except that, after sonication, leaves were treated with 0.525% sodium hypochlorite for 3 min, and then rinsed in excess volumes of distilled water for 5 min, prior to homogenization. Preliminary analysis indicated that log-transformed data more closely approximated a normal distribution than nontransformed data. Thus, log-transformed data were used to calculate and plot means and standard errors of the means.

For in planta growth and assessment of symptomatology, the first true leaves of 4-week-old plants were injected in the mid-vein with fresh bacterial suspensions (approximately 10⁶ CFU/ml) of strain B-24 or B24-B2 as previously described (Schaad and Stall 1988). Subsequently for each treatment, 2.54 cm² leaf sections were removed from four, inoculated, sodium-hypochlorite-treated (as described above) leaves at 2-day intervals, weighed, and separately homogenized in phosphate buffer (0.01 M, pH 7.0). These homogenates were then diluted and plated on FS(M) medium. Bacterial numbers were enumerated after 3 to 4 days, and symptomatology was assessed at 6 and 8 days after inoculation. Alternatively, a sterile hole punch was used to make a tiny wound on the terminal

edge of each leaf, and plants were then sprayed with bacterial suspensions and incubated as described for the epiphytic experiments above (28°C, 40 to 50% RH). Lesions at wound sites, and independent of wound sites originating at the margins of leaves, were then enumerated after 2 weeks.

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