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Phenotypic Switching Affecting Chemotaxis, Xanthan Production, and Virulence in *Xanthomonas campestris*

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The chemotaxis towards sucrose and yeast extract of nine strains of *Xanthomonas campestris* representing pathovars campestris, armoraciae, translucens, vesicatoria, and pelargonii was analyzed by using swarm plates. Unexpectedly, each of these strains formed small or reduced swarms typical of nonmotile or nonchemotactic bacteria. With time, however, chemotactic cells appeared on the swarm plates as blebs of bacteria. These cells were strongly chemotactic and were concomitantly deficient in exopolysaccharide production. The switch from the wild type (exopolysaccharide producing and nonchemotactic) to the swarmer type (exopolysaccharide deficient and chemotactic) appeared irreversible ex planta in bacteriological medium. However, in radish leaves swarmer-type strains of *X. campestris* pv. campestris were able to revert to the wild type. Swarmer-type derivatives of two *X. campestris* pv. campestris wild-type isolates showed reduced virulence and growth in the host plants cauliflower and radish. However, exocellular complementation of *X. campestris* pv. campestris Hrp (nonpathogenic) mutant was achieved by coinoculation with a swarmer-type strain.

Xanthomonas campestris pv. campestris is a motile bacterium that bears a single polar flagellum (3). The chemotactic properties of *X. campestris* pv. campestris as well as other *Xanthomonas* pathovars have not been extensively studied. In planta observations by Zoller (B. G. Zoller, Ph.D. dissertation, University of California, Davis, 1972) showed that *X. campestris* pv. campestris is nonmotile and nonflagellated in the xylem fluid of infected cabbage leaves and suggested that the movement of the bacteria in the xylem was due to diffusion rather than to motility. On the other hand, studies by Feng and Kuo (8) on the rice pathogen *X. campestris* pv. oryzae showed that chemotaxis occurs towards a synthetic medium and towards water exudates of susceptible rice plants, whereas no chemotaxis towards hydathode exudates of resistant plants was observed. This suggested that chemotaxis may play a role before the penetration of the bacteria inside the rice leaf. However, the role of chemotaxis in the movement of the bacteria inside the plant remains unknown.

When supplemented with an exogenous sugar source, *X. campestris* pv. campestris produces large amounts of an exopolysaccharide (EPS) known as xanthan gum, which consists of a cellulosic (1→4)-β-D-glucose backbone with trisaccharide side chains composed of two mannose and one glucuronic acid residue attached to alternate glucose residues in the backbone (11, 17, 19). Genes involved in the synthesis of xanthan gum have been cloned recently and found to be clustered in a 13.5-kb region of DNA (2, 10, 26). Colony type variants affected in EPS production have been shown to occur in *X. phaseoli*, *X. campestris* pv. oryzae, and *X. campestris* pv. pruni (5, 6, 9), and variants affected in EPS were also isolated at high frequency from old slants or chemostats of *X. campestris* NRRL B-1459 (4). Whereas some of these strains have been shown to be affected in virulence, a number of spontaneous or transposon-induced EPS-deficient mutants of *X. campestris* pv. campestris still induced disease symptoms, suggesting that xanthan gum is not required for pathogenicity (20, 25).

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In this study, the motility and chemotaxis of several *X. campestris* isolates have been analyzed, using semisolid agar swarm plates. Curiously, all strains examined appeared nonmotile or nonchemotactic, but a few days later these nonmotile or nonchemotactic cells converted to swarmers that appear as blebs on the swarm plates. Phenotypic characterization of the swarmer-type strains was conducted, along with analysis of their virulence and growth in planta. Exocellular complementation of a *X. campestris* pv. campestris Hrp (nonpathogenic) mutant was also achieved by coinoculation with a swarmer-type strain. The possible occurrence and role of swarmer types in the life cycle of xanthomonads are discussed.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are described in Table 1.

Media and antibiotics. *X. campestris* pathovars were routinely grown in rich medium 523 or minimal medium 925 (12) in liquid culture or on 1.5% agar plates at 29°C. Semisolid (0.3%) agar swarm plates were made either as minimal medium 925 with 0.05% sucrose or as nutrient agar medium, composed of 0.7% NaCl, 0.115% K_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl, and 0.01% yeast extract. The antibiotics used were rifampin (50 μg/ml), chloramphenicol (50 μg/ml), and tetracycline (5 μg/ml), purchased from Sigma Chemical Co., St. Louis, Mo. All antibiotics were filter sterilized and added to freshly prepared medium prior to pouring in petri plates.

Swarm plate assays. Cultures were grown in medium 523 overnight to early stationary phase, and 1 ml of each strain was harvested by centrifugation (15,000 × *g*, 5 min, 24°C) and washed three times with 1 ml of PME buffer (10 mM potassium phosphate [pH 7.2], 1 mM $MgCl_2$, 0.1 mM EDTA). Small samples of the washed cells were stabbed in swarm agar plates and incubated at 29°C. When strains containing a luciferase reporter were used, "swarm blots" were obtained from incubated swarm plates after an X-ray film was placed under the petri dish, and dishes were wrapped with aluminum foil. Exposure time varied from 15 min to 2 h depending on the luminescence of the strains tested.

TABLE 1. Phenotypic analysis of *X. campestris* strains

Strain	Description	Reference or source	Chemotaxis (swarm diam, mm)[a]	EPS production (μg/10^9 cells)[b]
X. campestris pv. campestris				
2D520	Wild-type isolate	25	4.0 ± 0.8	71
2D520X1	Swarmer type of 2D520	This paper	13.3 ± 2.1	10
2D520TL	2D520 containing one copy of pUCD607 integrated in the chromosome	23	ND	142
2D520TL1	Swarmer type of 2D520TL	This paper	ND	18
JS111	Tn*4431* mutant of 2D520, Hrp$^-$, *hrpXc-lux* fusion	25	5.5 ± 1.1	74
JS111R35	Swarmer type of JS111	This paper	12.2 ± 2.1	25
JS111R64	Swarmer type of JS111	This paper	16.8 ± 0.7	3
2D540R	Wild-type isolate	14	4.5 ± 0.8	132
2D540R4	Swarmer type of 2D540R	This paper	9.0 ± 1.0	12
X. campestris pv. armoraciae				
756C	Wild-type isolate	A. Alvarez	9.5 ± 0.5	96
756C1	Swarmer type of 756C	This paper	17.7 ± 2.1	13
X. campestris pv. vesicatoria				
6D53R	Wild-type isolate	14	3.8 ± 0.4	289
6D53R1	Swarmer type of 6D53R	This paper	6.3 ± 0.5	31
X. campestris pv. translucens				
10D5R	Wild-type isolate	14	3.3 ± 0.5	133
10D5R1	Swarmer type of 10D5R	This paper	8.3 ± 0.9	45
X. campestris pv. pelargonii				
11D51	Wild-type isolate	This paper	2.0 ± 0.0	52
11D5V1	Swarmer type of 11D51	This paper	9.7 ± 0.7	17
11D52	Wild-type isolate	This paper	2.0 ± 0.0	53
11D52V2	Swarmer type of 11D52	This paper	10.3 ± 0.5	14

[a] Diameter 48 h after inoculation on sucrose swarm plates. Numbers represent means ± standard deviations of six replicates. ND, Not determined.
[b] Acidic EPS content. Numbers represent average of duplicate experiments.

Isolation of swarmer cells. Swarmer cells were isolated from single blebs of highly chemotactic bacteria that appeared on the swarm plates 2 to 3 days after inoculation of a wild-type isolate. The swarmer cells were first grown in 523 broth and then purified to single colonies on 523 agar plates with the appropriate antibiotics. Sucrose and yeast extract swarm plates yielded phenotypically similar swarmer-type strains.

Acidic EPS assays. Acidic EPS (xanthan gum) was quantitatively determined as described previously, using precipitation with cetyltrimethylammonium bromide followed by an anthrone assay for total carbohydrates (13).

Pathogenicity assays. *X. campestris* pv. campestris strains were assayed for pathogenicity by whole-plant wound inoculation (24) on cauliflower (*Brassica oleracea* var. *botrytis* cv. Early Super Snowball; Park Seed Co.) and white radish (*Raphanus sativus* cv. White Icicle; Burpee Co.).

In planta growth. In planta growth was determined in cauliflower leaves as described previously (14, 25). Bacteria were inoculated into petioles, and at different times leaves were harvested (no petiole), mascerated, serially diluted, and plated. No bacteria were recovered at zero time. A 1:1 (cell-cell) mixture of two strains was used for the coinoculation experiments. The number of bacteria per leaf for each strain was determined by serially diluting mascerated leaves in PM buffer (10 mM potassium phosphate [pH 7.2], 1 mM $MgCl_2$) and plating on medium 523 agar plates supplemented with the appropriate antibiotics. Statistical analysis of the data was performed with Duncan's multiple range test (18).

RESULTS

Isolation and phenotypic analysis of swarmer cells. The chemotactic properties of nine strains of *X. campestris* representing pathovars campestris, armoraciae, translucens, vesicatoria, and pelargonii were investigated. On sucrose or yeast extract swarm plates, each of these strains formed small or reduced swarms typical of nonmotile or nonchemotactic bacteria (Table 1; Fig. 1 and 2), but 2 to 3 days after inoculation blebs of chemotactic populations emerged from the original swarm (Fig. 1). Chemotactic bacteria were isolated from the swarming populations, grown in 523 broth, and purified to single colonies before retesting on swarm plates. In all cases they retained their chemotactic properties and continued to form large swarms, unlike the parental wild-type isolates. These results suggest that the swarmers represent chemotactic variants of the original population (Table 1; Fig. 1 and 2). Surprisingly, the swarmer cells not only differed from the parental isolate in chemotaxis but also were defective in the production of xanthan gum, the EPS produced by *X. campestris*. On sucrose-rich medium, all swarmer-type strains were nonmucoid (dry), unlike the copiously slimy colony character of the wild-type isolate. Thus, little EPS was detected from the supernatant of the variants by cetyltrimethylammonium bromide precipitation followed by an anthrone assay (Table 1).

Virulence and growth in planta of swarmer cells. The virulence of swarmer-type derivatives of *X. campestris* pv. campestris strains 2D520 and 2D540R has been determined

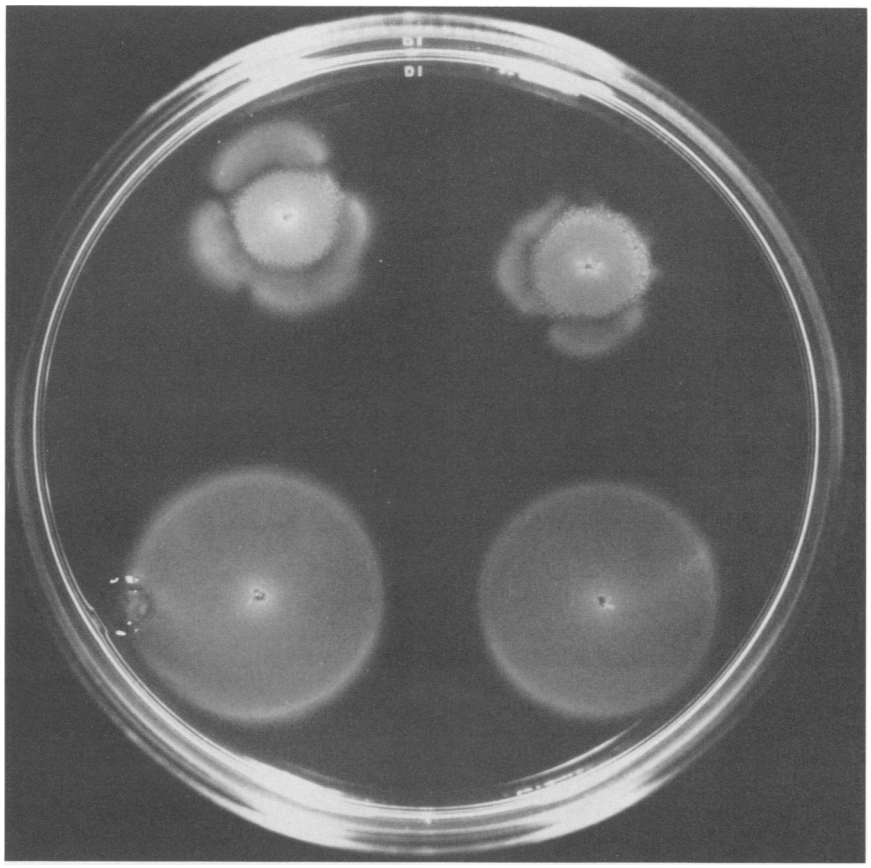

FIG. 1. Swarm formation by *X. campestris* pv. armoraciae wild-type 756C (top) and swarmer-type 756C1 (bottom) 72 h after inoculation on a sucrose swarm plate. Note the typical pattern formed by the wild-type strain showing blebs of chemotactic variants.

on cauliflower and radish. All swarmer-type strains showed a significant delay in the appearance of black rot symptoms that varies from about 4 days for 2D540R derivatives to >1 week for 2D520 swarmer types, suggesting that swarmer-type strains of *X. campestris* pv. campestris have reduced virulence.

To further investigate the attenuation in virulence of the swarmer-type strains, growth curves in cauliflower leaves were established for *X. campestris* pv. campestris 2D520, 2D520X1, 2D540R, 2D540R4, JS111, and JS111R64. Both swarmer-type strains, 2D520X1 and 2D540R4, displayed a slower growth than the wild-type isolates 2D520 and 2D540R, respectively. The effect was markedly stronger in 2D540R4. However, both swarmer-type variants displayed growth curves characterized by a lag phase between 72 and 120 h, followed by a substantial increase in the growth rate that contrasts with the exponential growth of the wild-type strains (Table 2; Fig. 3). The Hrp mutant JS111 and a swarmer-type isolate of this mutant, JS111R64, displayed similar attenuated growth in cauliflower leaves (Fig. 3C). Thus, the attenuation of the growth in planta of swarmer-type strains confirms the reduction in virulence described earlier.

Reversion to wild-type phenotype. To determine the stability of the switch from a nonchemotactic and EPS-producing to a chemotactic and EPS-deficient cell type, several swarmer-type strains were continuously subcultured on either minimal or rich medium. Meanwhile, the strains were visually scored for EPS production and periodically checked for chemotaxis. All strains appeared totally stable for both phenotypes, and reversion to the parental type was never observed on bacteriological medium.

To determine whether swarmer-type strains are also stable in planta, 12 independently purified swarmer-type derivatives of *X. campestris* pv. campestris 2D520 were inoculated on radish plants, kept in the greenhouse, and examined for black rot symptoms. Two weeks after inoculation, 10 strains caused disease lesions and the bacteria isolated from these lesions were identical to the wild-type strain, i.e., nonchemotactic and EPS producing. To eliminate the possibility of contamination, a similar experiment was conducted with 2D520TL1, a swarmer-type derivative of 2D520TL, a transconjugant of 2D520 with a chromosomal copy of plasmid pUCD607. In radish leaves, 2D520TL1 also yielded at variable frequencies bacteria identical to the parental strain 2D520TL; these revertants were Km^r, Sp^r, and Ap^r (the antibiotic markers of pUCD607), which eliminates the possibility of cross-contamination by another *X. campestris* pv. campestris strain. These results suggest that swarmer-type strains can switch back to the parental type in planta. The results also suggest that some plant signal could cause the reversion to the nonchemotactic or nonmotile phenotype.

Coinoculation experiments. The *X. campestris* pv. campestris Hrp (nonpathogenic) mutant, JS111, is unable to grow in cauliflower leaves (25). In another study, we have shown that the growth deficiency of JS111 can be complemented by coinoculation with the wild-type strain 2D520 (14). To determine whether swarmer-type strains can rescue JS111 growth

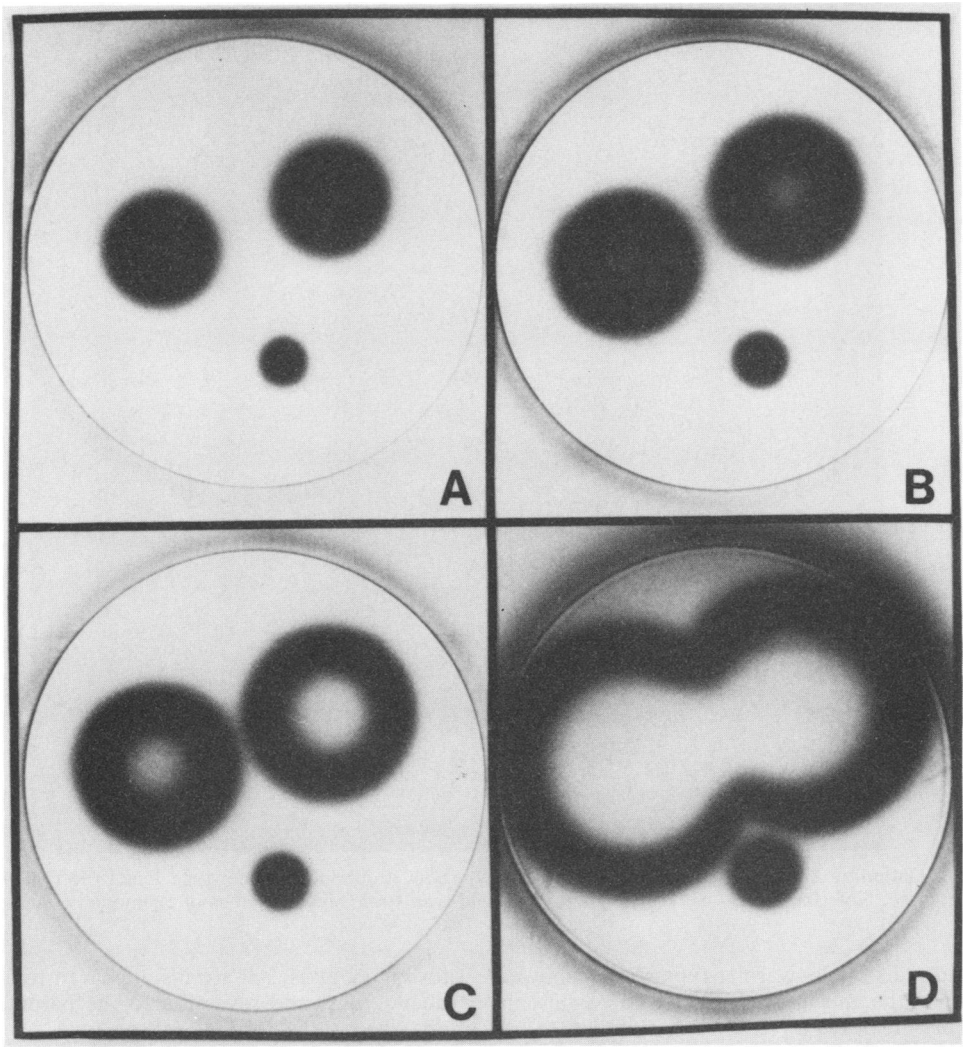

FIG. 2. Light swarm blots of *X. campestris* pv. campestris Hrp mutant JS111 (bottom) and swarmer-type derivatives JS111R35 (top left) and JS111R64 (top right) inoculated on yeast extract swarm plates: (A) 12 h after inoculation; (B) 24 h; (C) 30 h; (D) 48 h. Bioluminescence reflects the presence of metabolically active bacteria.

deficiency, coinoculation of JS111 and 2D520X1 was performed in cauliflower, and the growth of both strains was monitored for several days. In coinoculated leaves, both strains showed a lag phase between days 1 and 3 before growing to high levels, suggesting that the exocellular complementation of JS111 by 2D520X1 occurs but is subject to a lag phase that superposes to the growth of the swarmer-type strain (Fig. 4).

TABLE 2. Growth of *X. campestris* pv. campestris strains in cauliflower leaves

Strain	No. of bacteria per leaf at given time after inoculation[a]	
	72 h	120 h
2D520	4.0×10^{7} A	2.1×10^{8} A
2D520X1	2.3×10^{7} AB	1.7×10^{7} AB
2D540R	2.1×10^{8} AB	2.0×10^{9} A
2D540R4	1.4×10^{7} B	9.0×10^{6} B

[a] Values followed by the same superscript letter (A or B) did not differ significantly ($P = 0.05$) by Duncan's multiple range test.

DISCUSSION

The chemotaxis towards sucrose and yeast extract of nine wild-type isolates of *X. campestris* representing pathovars campestris, armoraciae, translucens, vesicatoria, and pelargonii was analyzed with swarm plates. Unexpectedly, all strains formed small swarms and appeared poorly motile, suggesting that these isolates of *X. campestris* are not actively chemotactic. With time, however, chemotactic cells appeared on the swarm plates as blebs of bacteria (Fig. 1). These cells were strongly chemotactic to sucrose or yeast extract and were also deficient in EPS production, suggesting a relation between EPS deficiency and chemotaxis (Table 1). This correlation is unlikely to be an artifact of the swarm plate assay since transposon-induced, EPS-deficient mutants of *X. campestris* pv. campestris 2D520 are, similarly to the wild type, nonchemotactic (data not shown). However, it is not clear why chemotaxis and EPS production appear ex-

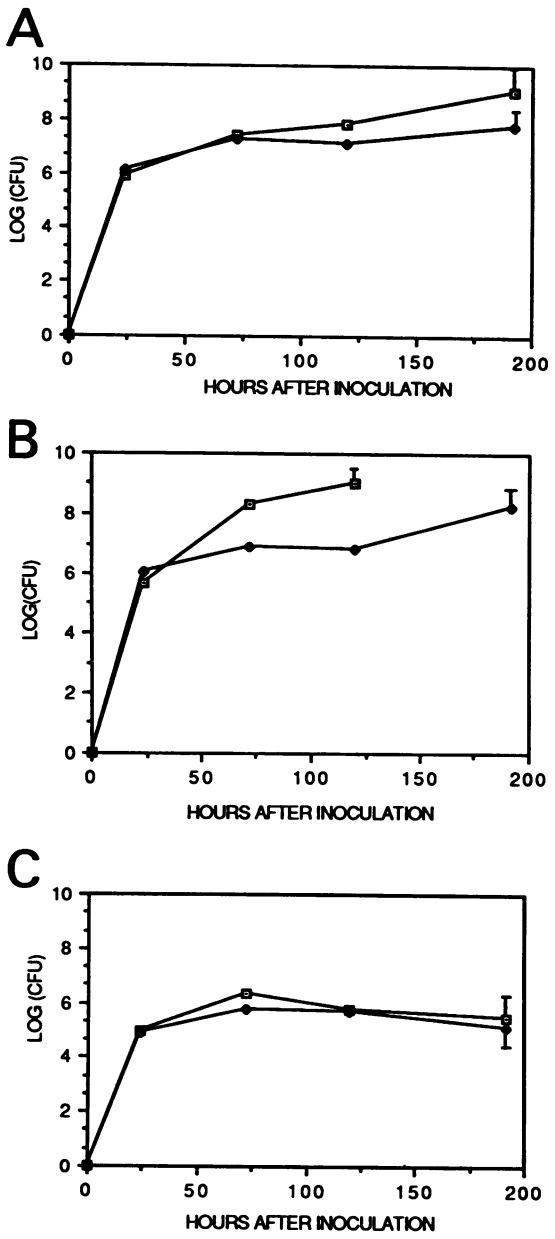

FIG. 3. Time course of growth of *X. campestris* pv. campestris strains in cauliflower leaves: (A) 2D520 (□) and 2D520X1 (■); (B) 2D540R (□) and 2D540R4 (■); (C) JS111 (□) and JS111R64 (■). Each point represents the average of three to six replicas. Bars reflect the standard deviation throughout the experiment.

clusive. Since both processes are energetically expensive for the bacterium, it could be deleterious for *X. campestris* to chemotact and produce EPS simultaneously.

The switch from the wild type (EPS producing and nonchemotactic) to the swarmer type (EPS deficient and chemotactic) appeared irreversible ex planta in bacteriological medium, even after continuous subculturing. However, several days after inoculation on plants, swarmer-type strains were able to revert to cell types that are like the wild-type strain in EPS production and chemotaxis, suggesting that the switch from one cell type to another could be triggered by specific signals in the host plant.

Virulence and growth analysis of swarmer-type derivatives of *X. campestris* pv. campestris 2D520 and 2D540R showed a strong delay in the initiation of black rot symptoms along with a reduction of lesion size on cauliflower and radish. Also, these swarmer-type strains displayed reduced growth rates in cauliflower leaves that correlate well with the reduction in virulence. The growth in planta of the swarmer-type strains was characterized by an extended lag phase of 2 days (Table 2; Fig. 3). A logical explanation for the lag phase in planta would be the appearance of revertant forms of the swarmer type that are able to grow to high populations in the host.

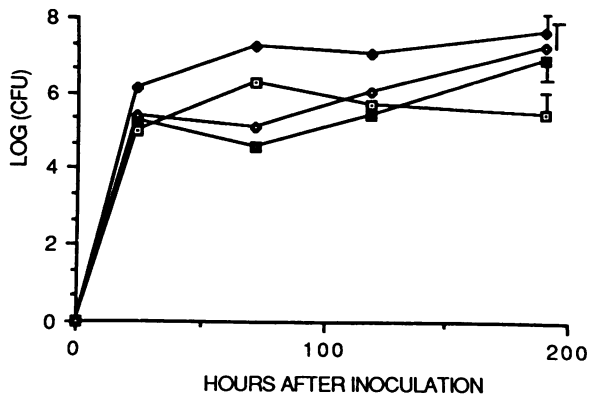

FIG. 4. Time course of growth of *X. campestris* pv. campestris strains 2D520X1 and JS111 in separate and mixed inoculations in cauliflower leaves: 2D520X1 (◆); JS111 (□); mixed inoculations—2D520X1 (◇) and JS111 (■). Each point represents the average of four to six replicas. Bars reflect the standard deviation throughout the experiment.

The Hrp (nonpathogenic) mutant of *X. campestris* pv. campestris, JS111, is unable to grow in the plant and has been shown previously to be complemented by coinoculation with the wild-type strain (14). In this study, we showed that JS111 can also be complemented by coinoculation with a swarmer-type strain. However, the complementation by the swarmer type differed from that by the wild type in two features. (i) In coinoculated leaves the swarmer type always reached higher population levels than JS111. (ii) The growth of JS111 was subject to a lag phase that superposes to the growth of the swarmer type. These observations suggest that the *hrp* gene mutated in JS111 (*hrpXc*) is still expressed in the swarmer-type strain 2D520X1. We are currently investigating whether the *hrpXc* locus along with other loci involved in EPS production are differentially expressed in swarmer-type strains.

The observation that wild-type isolates of a number of plant-pathogenic *X. campestris* strains are unable to show active chemotaxis to sucrose or yeast extract confirms a previous study that showed that *X. campestris* pv. campestris is nonmotile in bacteriological medium and in cabbage leaves and that diffusion, rather than motility, is responsible for the movement of the bacterium in the plant xylem (Zoller, Ph.D. dissertation). Moreover, wild-type isolates of the plant-pathogenic bacteria *Pseudomonas solanacearum* have been shown to be nonmotile, and motile, avirulent, and EPS-deficient variants arise at high frequency in still cultures (15, 16). Our results show that a similar phenomena occurs in *X. campestris*. On swarm plates nonchemotactic wild-type strains can yield cell types that are consistently chemotactic and also defective in EPS production and virulence. This switch does not occur immediately after inoculation of the swarm plate, and the addition of an excess of a nitrogen

source (ammonium sulfate) to the yeast extract swarm plates delayed the appearance of swarmer-type strains for several days without affecting chemotaxis (data not shown). This observation, along with the appearance of nonmucoid variants in chemostats or old slants (4, 6, 9), suggests that the phenotypic switch could be triggered by poor nutrient conditions. This could explain why previous investigators have noted loss of virulence after prolonged culture of virulent plant-pathogenic bacteria (9).

Several microbial pathogens of different types have evolved the ability to vary the expression of both physiological processes and virulence genetically (7, 22, 21). Such variations can change the properties of the microbe, yielding cell types that are better adapted to new environments inside or outside the host (7). Whether swarmer-type xanthomonads occur in nature is unknown. However, since *X. campestris* strains are likely to require chemotaxis at one stage of their life cycle, swarmer-type cells possibly arise at that point. Chemotaxis is known to be advantageous in environments that are scarce in nutrients (1), suggesting that swarmer-type cells may be better fit for the survival in the soil or in the phyllosphere. Then, chemotaxis could play an important role in leading the bacterium to plant opening (8). Studies on the genetics of the phenotypic switching as well as on the ecology of xanthomonads will help determine the occurrence and role of the swarmers in the life cycle of *X. campestris* pathovars.

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