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The *opsX* Locus of *Xanthomonas campestris* Affects Host Range and Biosynthesis of Lipopolysaccharide and Extracellular Polysaccharide†

MARK T. KINGSLEY,‡ DEAN W. GABRIEL,* GARY C. MARLOW, AND PAMELA D. ROBERTS

Plant Pathology Department, University of Florida, Gainesville, Florida 32611-0680

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Xanthomonas campestris **pv. citrumelo strain 3048 is the causal agent of citrus bacterial leaf spot disease and has a wide host range that includes rutaceous and leguminous plants. A spontaneous prototrophic mutant of strain 3048 (strain M28) that had lost virulence on citrus but retained virulence on bean plants was recovered. Growth studies in planta showed that M28 cells died rapidly in citrus leaves but grew normally in bean leaves. In addition to the loss of citrus-specific virulence, M28 displayed the following mutant phenotypes in culture: decreased growth rate, reduction of the amount of exopolysaccharide (to ca. 25% of the amount in 3048), loss of capsules, and significant alterations of the two 3048 lipopolysaccharide (LPS) bands visualized by silver stain on polyacrylamide gels, consistent with a defect(s) in LPS assembly. A 38-kb DNA fragment from a 3048 total DNA library that complemented the mutant phenotypes of M28 was identified. The 38-kb fragment did not hybridize to two similarly sized fragments carrying different *hrp* (hypersensitive response and pathogenicity) genes cloned from 3048. Subcloning, DNA sequence analyses, and gene disruption experiments were used to identify a single gene, *opsX* (for outer-membrane polysaccharide), responsible for the mutant phenotypes of M28. At least one other gene downstream from *opsX* also affected the same phenotypes and may be part of a gene cluster. We report here the DNA sequence and transcriptional start site of *opsX*. A search of protein sequence data bases with the predicted 31.3-kDa OpsX sequence found strong similarity to Lsi-1 of *Neisseria gonorrhoeae* and RfaQ of *Escherichia coli* (both are involved in LPS core assembly). The host-specific virulence function of *opsX* appears to involve biosynthesis of the extracellular polysaccharide and a complete LPS. Both may be needed in normal amounts for protection from citrus, but not bean, defense compounds.**

Bacterial cell-surface molecules compose the glycocalyx (8) and play critical roles in the survival of free-living and parasitic prokaryotes. In gram-negative bacteria, the outermost molecules include extracellular polysaccharide (EPS), which is secreted to form an outer layer in the form of capsules or loose slime, and lipopolysaccharide (LPS), a component of the outer membrane. The ability of bacteria to colonize a specific niche may depend upon the EPS layer, which serves a variety of roles, including protecting pathogens from desiccation, toxic metals, and host defense responses (6, 7, 31, 42). The outer membrane LPS acts as a permeability barrier to various toxic molecules such as hydrophobic antibiotics, detergents, and hydrophobic dyes (39, 42, 45). Since they have many charged substituents, the polysaccharides of the LPS are believed to be involved in the barrier function, including protecting both animal and plant pathogens from toxic hydrophobic host molecules (42, 43, 45).

With some plant-associated microbes, cell surface molecules have been shown to be crucial for determining the success or failure of parasitism (6, 24, 43). For example, numerous studies have revealed that EPS production is necessary for *Rhizobium* spp. to nodulate alfalfa, peas, clovers, and *Leuceana* spp., although EPS production is not essential for bean and soybean nodulation (14, 43). It has been suggested that the EPS may play a role in the ability of *Xanthomonas* spp. to infect plants (58), but many groups have induced mutations affecting its EPS (2, 6, 26, 28, 33, 38, 60, 65) and no definitive role of its EPS or cell surface molecules in virulence has been determined (2, 9, 17, 29, 36, 44, 48, 59, 61). Defects in the LPS of *Rhizobium*, *Erwinia*, and *Pseudomonas* species can lead to partial or complete loss of virulence (30, 43, 51). Although mutations affecting the LPS of *X. campestris* have been reported (65), to our knowledge mutations affecting both the *Xanthomonas* LPS and virulence have not been reported.

Members of the genus *Xanthomonas* are all plant associated, and strains exhibit a high degree of host specificity. A variety of different virulence factors and genes have been described (9, 66). Both host range and disease severity of *Xanthomonas* strains can be determined by host-specific virulence genes (21, 64), but no functions for these genes have been reported. *Xanthomonas campestris* pv. citrumelo strain 3048 is closely related to xanthomonads that cause leaf spot diseases of leguminous plants, such as *X. campestris* pv. alfalfae, but strain 3048 has a wide host range which also includes citrus (19, 20, 22). On citrus, strains of *X. campestris* pv. citrumelo are opportunistic and capable of causing limited pandemics on juvenile citrus in nurseries (22). This study was initiated in an attempt to identify, isolate, and characterize the gene(s) in 3048 that allows a legume-associated xanthomonad to achieve a host range on citrus. We report here the isolation, characterization, and DNA sequence of one such gene, *opsX* (for outer membrane polysaccharide), which is required for the virulence of 3048 on citrus but not on bean plants. We further report (i) that *opsX* pleiotropically alters the LPS and affects the production of capsular slime and the EPS, (ii) that *opsX* is not an *hrp* (hypersensitive response and pathogenicity) gene, (iii) that

* Corresponding author.

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‡ Present address: Environmental Microbiology, Battelle Pacific Northwest Laboratory, Richland, WA 99352.

opsX does not affect the export of plant maceration enzymes, and (iv) that the translated DNA sequence of *opsX* revealed significant homology to genes involved in LPS biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, culture conditions, and tests. *Escherichia coli* and *X. campestris* strains, mutants, and plasmids with their relevant characteristics are listed in Table 1. Cultures of *E. coli* were grown at 37°C in Luria broth (LB) and on LB-agar plates and were maintained and stored according to standard protocols (50). For *E. coli*, LB medium was supplemented with the following antibiotics as required at the concentrations indicated: ampicillin (sodium salt), 100 μg/ml; chloramphenicol, 50 μg/ml; gentamicin sulfate, 3 μg/ml; kanamycin sulfate, 50 μg/ml; naladixic acid (sodium salt), 100 μg/ml; rifampin, 50 μg/ml; and spectinomycin dihydrochloride, 50 μg/ml. Except for LPS extraction, cultures of *X. campestris* were grown at 30°C in tryptone-yeast extract-MOPS (morpholinepropanesulfonic acid) medium (TYM) (19) and on TYM LB-agar plates. *X. campestris* cultures were maintained at room temperature (ca. 22°C) for up to 2 weeks on lima bean agar (Difco Laboratories, Detroit, Mich.) (pH adjusted to 7.4). For long-term storage, turbid TYM broth cultures were supplemented with glycerol to 15% (vol/vol) and frozen at −80°C. For *X. campestris*, TYM medium was supplemented with following antibiotics as required at the concentrations indicated: chloramphenicol, 35 μg/ml; gentamicin, 3 μg/ml; kanamycin, 35 μg/ml; rifampin, 50 μg/ml; and spectinomycin, 35 μg/ml. Mutants were screened for auxotropy by plating on MOPS minimal medium (MM) (20) solidified with 1.5% (wt/vol) Noble agar (Difco). Bacteriological tests for amylase, protease, and lipase were done as described previously (20). Capsules were examined by using India ink (16), after growth in TYM. Carbohydrate utilization tests were performed by using GN Microplates (Biolog, Inc., Hayward, Calif.).

The quantity of EPS in culture supernatants was determined spectrophotometrically by the phenol-sulfuric acid method with glucose as the standard (25). For these assays, cells were grown in TYM broth supplemented with 5% glucose and were then removed by centrifugation. The EPS was precipitated from the supernatant by the addition of 3 volumes of 95% ethyl alcohol, pelleted, washed with 70% ethyl alcohol, dried, and resuspended in sterile distilled H_2O. The amount of EPS was normalized to the amount of cell protein present. Cell protein was determined by the Folin reaction with bovine serum albumin as the standard (25). All buffers and other reagents were prepared with double-distilled water (distilled in glass).

LPS extraction, gel electrophoresis, and staining. *X. campestris* cells were grown overnight at 30°C in peptone-yeast extract-glycerol-MOPS (PYGM) medium (12). Approximately 2×10^9 CFU was harvested at the mid- to late-log phase of growth, pelleted, washed twice with 0.7% aqueous NaCl, and washed once with 5 mM $MgCl_2$–10 mM 2-mercaptoethanol–10 mM Tris-HCl (pH 7.5). Each pellet was resuspended, and cells lysed in 50 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–0.5% sodium dodecyl sulfate (SDS). DNase I (180 Kunitz units) and RNase A (5 Kunitz units) were added, and the suspension was incubated at 25°C for 6 h. Proteinase K (200 μg) was added, and the incubation was continued at 25°C for 16 h. Cell debris was pelleted at 13,000 × *g* for 15 min, and 50 μl of supernatant was mixed with an equal volume of sodium deoxycholate sample buffer (0.5% sodium deoxycholate, 20% glycerol, 350 mM Tris-HCl [pH 6.8]) (34) and heated to 100°C for 5 min prior to loading. Separation by polyacrylamide gel electrophoresis was done exactly as described previously (34), except that the upper buffer for preelectrophoresis was 125 mM Tris-HCl (pH 6.8) and the gels were 15% acrylamide and 0.75 mm thick. The gels were prestained in Alcian Blue (49) and then silver stained (27) exactly as described previously.

Recombinant DNA methods, transposon Tn*5* transfer, and plasmid constructions. Plasmid DNA was extracted from *E. coli* and *X. campestris* by the SDS-alkaline lysis procedure (50). Isolation of *X. campestris* chromosomal DNA, restriction digestions, gel electrophoresis, random priming, and DNA hybridization were done as previously described (35, 57). Restriction fragment length polymorphism analyses were done exactly as described previously (19). All enzymes were used according to standard methods (50) or the manufacturers' protocols. Southern blot and colony hybridizations, performed at 68°C, were followed by washing at high stringency (68°C, 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.5% SDS) (50).

Transposon Tn*5* was introduced into strain 3048Sp by overlaying 10^7 to 10^8 3048Sp cells spotted onto tryptone-yeast extract (TY) plates with 10^6 to 10^7 *E. coli* SM10/pSUP1011 (54) cells and incubating the mixture overnight at 28°C as described previously (64). The 3048Sp cells were obtained from late-log-phase liquid cultures that were washed with sterile tap water, and the SM10/pSUP1011 cells were obtained from late-log-phase liquid cultures that were grown without antibiotics. Kanamycin-resistant exconjugants were selected on TYM plates supplemented with spectinomycin and kanamycin. Purified exconjugants were stored frozen in 15% glycerol at −80°C.

A library of 3048 size-fractionated genomic DNA was constructed according to standard protocols (50) in the wide-host-range cosmid shuttle vector pUFR043 (11) and transduced into *E. coli* HB101, selecting for kanamycin and gentamicin resistance. The DNA library appeared to carry randomly cloned DNA inserts, with a mean size of 35 kb. Wide-host-range shuttle vector pUFR047 (57) was used for subcloning in *E. coli* and activity localization assays in *X. campestris*. *E. coli* vectors pUFR004 (12) and pUFR012 (11a) were used as integrative plasmids in *X. campestris*. DNA fragments cloned in *E. coli* by using pUFR004, pUFR012, pUFR043, and pUFR047 were transferred by conjugation to *X. campestris* strains by using pRK2013 or pRK2073, as described previously (56). To construct marker exchange plasmid pMK74.2, the internal 1.4-kb *Pst*I fragment of pMK29.28 was deleted by partial digestion with *Pst*I and replaced with the 1.3-kb Km^r cassette cloned from pUC4K as a *Pst*I fragment, forming pMK54.4. This deleted most of *opsX* plus an additional 700 bp downstream from *opsX*. The resulting 4.3-kb *Sal*I fragment from pMK54.4 (containing the Km^r cartridge) was inserted into the *Sal*I site of pUFR004, forming pMK74.2.

Marker disruption and exchange mutagenesis. Marker disruption plasmid pPR001 carried a 348-bp *Sph*I fragment, internal to *opsX*, cloned in pUFR012. Marker disruption of *opsX* was achieved by conjugation and selection of stable transconjugants in 3048Sp on TYM containing kanamycin. Integration was confirmed by Southern blots of total DNA extracted from stable transconjugants, cut with *Bgl*II and *Sal*I (separate digests), and probed with pMK41.1. Marker exchange mutagenesis of 3048Rf was performed in two steps. After introducing plasmids pMK28.4 or pMK74.2

TABLE 1. List of strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
DH5α	*f80dlacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* I^- *thi-1 gyrA96 relA1*	GIBCO-BRL
HB101	*supE44 hsdS20* (r_B^- m'_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	50
ED8767	*supE44 supF58 hsdS3* (r_B^- m'_B^-) *recA56 galK2 galT22 metB1*	41
SM10	C600 *recA hsdR thi-1 thr-1 leuB6* [*chr*::RP4-2-Tc::Mu]	54
PCT800	ED8767 [*chr*::Tn*5* (Nm^r)]	61
C600-387	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21 hflA150* [*chr*::Tn*5-gusA* (Nm^r Tc^r)]	56
C2110	*polA* Nal^r	55
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F'(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	67
X. campestris pv. citrumelo		
3048^H	Holopathotype strain (ATCC 49120); causes leaf spot on citrus and bean	20
3048Sp	Spc^r derivative of 3048^H; otherwise, wild type	56
3048Rf	Rif^r derivative of 3048^H; otherwise, wild type	This study
ME23	Rif^r relative of 3048^H; *hrp*::Tn*5*	G. Minsavage
ME70	Rif^r relative of 3048^H; *hrp*::Tn*5*	G. Minsavage
M28	*opsX* derivative of 3048Sp [*chr*::Tn*5* (Nm^r)]	This study
M28Rf	Rif^r derivative of M28	This study
MX3028	Km^r Δ*opsXY* derivative of 3048Rf	This study
X. campestris pv. malvacearum NSp2	Wild-type strain; causes cotton blight	12
X. campestris pv. vesicatoria		
88-5	Wild-type strain; causes bacterial spot on tomato and pepper	40
M103	Hrp^-; chemically-induced mutant derivative of 88-5	4
Plasmids		
pRK2013	ColE1 Km^r Tra^+ (conjugation helper plasmid)	15
pRK2073	*npt*::Tn*7*, Km^s Sp^r derivative of pRK2013	37
pSUP1011	pACYC184::Tn*5* ColE1 Cm^r Nm^r Mob^+ (suicide plasmid)	54
pUC4K	ColE1 Km^r	62
pUC18	ColE1 Ap^r *lacZ*α$^+$	67
pUC118	ColE1 Ap^r *lacZ*α$^+$	63
pUC119	ColE1 Ap^r *lacZ*α$^+$	63
pUFR004	ColE1 Cm^r Mob^+ (integrative vector)	12
pUFR012	ColE1 Cm^r Nm^r Mob^+ (integrative vector)	11a
pUFR042	IncW Nm^r Gm^r Mob^+ *lacZ*α$^+$	10
pUFR043	IncW Nm^r Gm^r Mob^+ *lacZ*α$^+$ *cos*	11
pUFR047	IncW Ap^r Gm^r Mob^+ *lacZ*α$^+$	57
pUFT-1	30-kb DNA fragment carrying repetitive DNA useful for *X. campestris* taxonomy and strain identification	19
pXV9	*hrp* cluster from *X. campestris* pv. vesicatoria 75-3 cloned in pLAFR3	4
pUFM2110	*hrp* gene(s) from *X. campestris* pv. citrumelo 3048 cloned in pUFR043	This study
pUFM3016	*hrp* gene(s) from *X. campestris* pv. citrumelo 3048 cloned in pUFR043	This study
pMK28	20-kb *Eco*RI M28::Tn*5* Km^r junction fragment cloned in pUC18	This study
pMK28.4	*Eco*RI reclone of pMK28 in pUFR004	This study
pUFM1140	*opsXY* on 38-kb fragment from *X. campestris* pv. citrumelo 3048 cloned in pUFR043	This study
pMK29.26	*opsXY* on 10-kb *Sal*I subclone of pMK11.40 in pUFR047	This study
pMK29.28	*opsXY* on 4.4-kb *Sal*I subclone of pMK29.26 in pUFR047	This study
pMK33.9	*opsX* on 3.8-kb *Sst*I subclone of pMK29.26 in pUFR047	This study
pMK40.1	1.9-kb *Sal*I-*Eco*RI fragment from pMK29.28 in pUFR047	This study
pMK41.1	*opsX* on 2.3-kb *Sal*I-*Sst*I fragment from pMK29.28 in pUFR047	This study
pMK42.1	1-kb *Sal*I-*Pst*I from pMK41.1 in pUFR047	This study
pMK44.1	0.7-kb *Pst*I-*Eco*RI fragment from pMK41.1 in pUFR047	This study
pMK45.1	1.2-kb *Pst*I-*Sst*I fragment from pMK41.1 in pUFR047	This study
pMK47.6	*opsX* on 2.3-kb *Sst*I fragment from pMK41.1, recloned in pUFR042 in reverse orientation	This study
pMK54.4	pMK29.28 Δ*opsXY* (1.4-kb *Pst*I fragment replaced by 1.3-kb Km^r fragment from pUC4K)	This study
pMK60.1	2.3-kb *Sst*I fragment from pMK47.6 in pUC119	This study
pMK60.2	As pMK60.1, reverse orientation	This study
pMK60.11	pMK60.1, 600-bp *Eco*RI-*Sst*I fragment deleted	This study
pMK60.14	pMK60.2, 1.5-kb *Eco*RI-*Sst*I fragment deleted	This study
pMK62.1	2.3-kb *Sst*I fragment from pMK47.6 in pUC118	This study
pMK62.2	As pMK62.1, reverse orientation	This study
pMK62.51	pMK62.2, with 1.3-kb *Pst*I fragment deleted	This study
pMK62.52	pMK62.1, with 900-bp *Pst*I fragment deleted	This study
pMK74.2	4.3-kb *Sal*I fragment (Km^r) from pMK54.4 recloned in pUFR004 for marker exchange	This study
pPR001	348-bp *Sph*I fragment of pMK41.1, recloned in pUFR012 for marker interruption	This study

from *E. coli* DH5α into 3048Rf by conjugation, exconjugants were obtained by selecting for kanamycin resistance, indicating integration via a single recombination event. These were subcultured en masse twice on TYM agar plates with kanamycin, and then individual colonies were screened for sensitivity to chloramphenicol. Total DNA from colonies which were Km^r Cm^s were examined by Southern hybridizations for verification of marker exchange (two homologous recombination events). For verification of marker exchange by using pMK28.4, the DNA was cut with *Eco*RI and probed with pMK28. For verification of marker exchange by using pMK74.2, the DNA was cut in separate digests with *Eco*RI, *Sal*I, and *Hin*dIII and separately probed with pMK41.1 and pUC4K.

Nucleotide sequence and transcriptional start site analysis of the *opsX* region. Subclones for sequencing both DNA strands were obtained by directionally cloning defined restriction fragments from pMK47.6 into pUC118 and pUC119 and transforming *E. coli* JM109 with the resulting plasmids. Single-stranded DNA templates were prepared with helper phage VCSM13 (Stratagene, La Jolla, Calif.) by using the single-stranded rescue technique of Vieira and Messing (63). Single-stranded templates were sequenced by the dideoxy chain termination technique and the Sequenase (version 2.0) DNA sequencing kit (U.S. Biochemical, Cleveland, Ohio) with ^{35}S-dATP. Sequencing reactions utilized the −40 universal primer supplied with the kit and the following oligonucleotide primers (synthesized by the Interdisciplinary Center for Biotechnology Research DNA synthesis core, University of Florida, Gainesville): DG1 (5′-GCGGCGATT GAT-3′), DG2 (5′-CAGGTCCTTGGA-3′), DG3 (5′-AGGGT GTCCTTG-3′), DG4 (5′-TTGCCGAGCCGC-3′), and DG9 (5′-AAGGCATCGAAGCGGCCC-3′). Assembly, editing, and analyses of the completed sequence were performed by using the Sequence Analysis Software Package of the University of Wisconsin, Genetics Computer Group (version 7.0), Madison, Wis. (13). Computation was performed at the Interdisciplinary Center for Biotechnology Research Biological Computing Facility, University of Florida, Gainesville. The Swiss-Prot (23.0; August 1992), PIR (34.0 [complete], 30 September 1992), and CDS translation from GenBank (release 73.1, 1 October 1992) data bases were searched by using the predicted amino acid sequence of *opsX* and the BLAST program (1), run at the National Center for Biotechnology Information network service in Bethesda, Md.

The starting sites for transcription of *opsX* were determined by primer extension with total RNA from 10-ml cultures of 3048 and M28 grown to late-log phase in TYM. Cells were harvested by centrifugation and washed twice with 10 ml of 0.7% NaCl in water. Total RNA was then extracted as described by Gilman (23). Primer extension was done as described by Kingston (32), except that Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Bethesda, Md.) and ^{35}S-α-dATP were used. A single-stranded preparation of pMK60.1 DNA was used as the DNA template for sequencing and comparison of the size of the extension product to the DNA sequence run on the same gel. Primer DG9 was used for both primer extension and sequencing.

Plant assays. All *Citrus paradisi* cv. Duncan (grapefruit), *Phaseolus vulgaris* cv. California Light Red (kidney bean), *Gossypium hirsutum* L. cv. Acala 44 (cotton), and *Capsicum annuum* cv. Early Cal Wonder (pepper) plants were grown and maintained in greenhouses. The nonhost hypersensitive response was assayed in cotton leaves. For assays of macroscopic symptoms, plants were inoculated by pressure infiltration of the abaxial leaf surface with the blunt end of a tuberculin syringe as described previously (20, 57). Washed cell suspensions were standardized in sterile tap water to 10^8 CFU/ml for these inoculations. No antibiotics were used in the plant assays; the shuttle vectors used replicate stably in many *Xanthomonas* strains without selection (12). Plant reactions were assayed visually 1 to 5 days postinoculation, depending on the plant-microbe interaction (10). All plant inoculation tests of macroscopic symptoms were repeated at least three times. Growth in planta assays were performed as previously described (20, 56, 57); the titer of starting inocula was adjusted to 10^5 CFU/ml. The resulting titer in leaves at the time of inoculation was approximately 10^3 CFU/cm^2 of bean or citrus leaf tissue. The standard error of the mean of three replicate samples was calculated and plotted; each experiment was repeated at least twice.

Nucleotide sequence accession number. The nucleotide sequence of the *opsX* gene has been submitted to GenBank and assigned accession number L21026.

RESULTS

Multiple mutant phenotypes of M28. Kanamycin-resistant exconjugants of *X. campestris* pv. citrumelo strain 3048Sp were recovered at frequencies between 10^{-7} and 10^{-8} per recipient after conjugation with *E. coli* SM10/pSUP1011 (Tn*5*). Southern blot analyses of total DNA from these exconjugants demonstrated random insertion of Tn*5* in the 3048Sp genome (data not shown). In the process of screening these 3048Sp::Tn*5* strains for defects in virulence, one of the Tn*5* marked strains, M28, was observed to be fully virulent on bean leaves but had lost all virulence on citrus leaves (Fig. 1). By contrast, the parental strain 3048Sp was fully virulent on both bean and citrus and elicited typical water-soaked responses on both hosts (compare results in Fig. 1 and 2). Even high-level inoculations of M28 ($\geq 10^{10}$ CFU ml^{-1}) cells elicited no symptoms on citrus (data not shown); by contrast, inoculations with 3048, 3048Sp, or 3048Rf at 10^8 CFU ml^{-1} led to confluent water soaking within 3 to 5 days (depending on leaf condition), followed by necrosis in the inoculation zone. Growth studies in planta showed that M28 died rapidly when inoculated into grapefruit leaves and were undetectable by 7 days postinoculation, while growth in bean leaves was only slightly affected relative to that of the wild type (Fig. 2).

In addition to the loss of virulence on citrus, M28 exhibited mutant phenotypes in culture. The colonies of 3048, 3048Sp, 3048Rf, and M28 growing on TYM plates were similar in morphology (convex, uniform, round, and entire), but the colonies of M28 were smaller. In addition, the gums of 3048, 3048Sp, and 3048Rf were viscous and sticky when touched with a bacteriological loop, while the gum of mutant M28 was relatively mucoid (much less viscous; watery). M28 displayed an increase in lipolytic activity and grew more slowly in complex media (Table 2). Exopolysaccharide analysis of 3048Sp and M28 culture supernatants demonstrated that M28 produced levels that were ca. one-fourth of wild-type levels (Table 2). Microscopic examination of TYM broth-grown cells stained by India ink showed that the wild type possessed capsules which were missing in the mutant. Mutant M28 was prototrophic; no differences were observed between wild-type 3048Sp and M28 in the ability to grow on MM plates, to utilize carbohydrates (Biolog plates), to hydrolyze starch, casein, or gelatin, to tolerate NaCl (up to 3%), or to survive in sterile tap or double-distilled water.

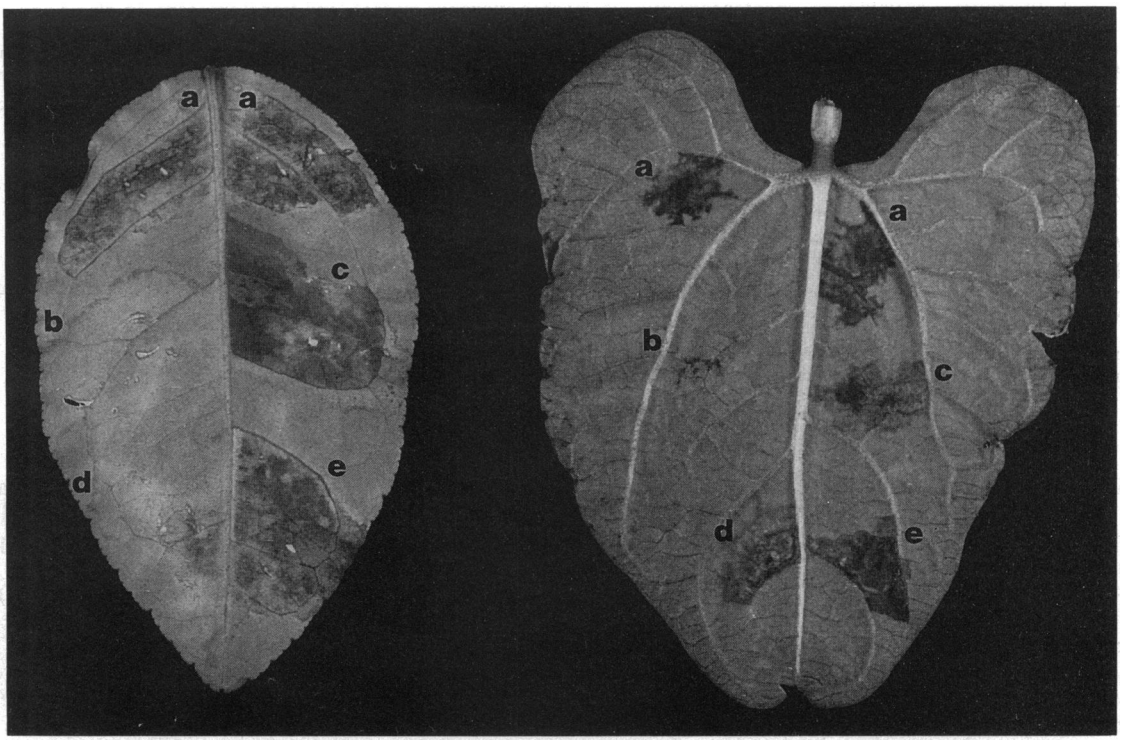

FIG. 1. Host responses of citrus (left) and bean (right) inoculated with wild-type *X. campestris* pv. citrumelo 3048Sp (a), Hrp$^-$ mutant ME23 (b), ME23/pUFM3016 (c), M28 (*opsX*) (d), or M28/pUFM1140 (e).

Complementation of M28 phenotypes. The mutant phenotypes of M28 were initially assumed to be due to the Tn*5* insertion, but clones recovered from the 3048 DNA library by hybridization with probe pMK28 (which contained Tn*5* and flanking DNA from M28) did not restore M28 to virulence on citrus. Furthermore, marker exchange of wild-type 3048Rf with pMK28.4 yielded exoconjugants that were indistinguishable from the wild type in culture media or in pathogenicity tests; marker exchange was verified by DNA hybridizations (data not shown). These results indicated that the phenotypes displayed by M28 on culture media and citrus were unrelated to the Tn*5* insertion. Therefore, individual clones from a 3048 DNA library were screened in M28 for the ability to complement either the mucoid gum or virulence phenotypes. Both the gum and virulence phenotypes were restored by cosmid clone pUFM1140. This clone did not hybridize to pMK28. M28 was verified to be derived from 3048 by restriction fragment length polymorphism

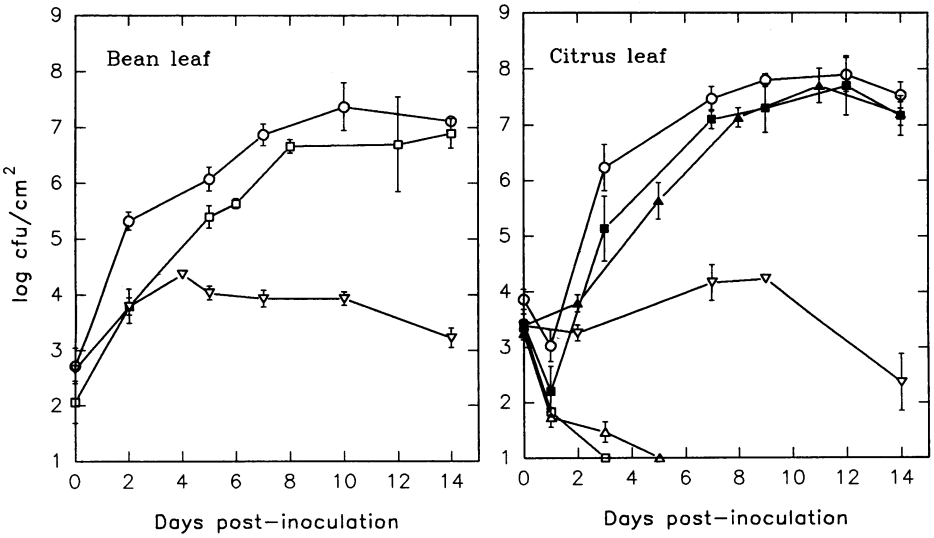

FIG. 2. Growth of *X. campestris* pv. citrumelo 3048Sp and derivatives in bean and citrus leaves. ○, 3048Sp (wild type); □, M28 (*opsX*); ▽, *X. campestris* pv. malvacearum NSp2 (negative control); △, MX3028 (Δ*opsXY*); ■, M28/pMK41.1; ▲, MX3028/pMK29.28.

TABLE 2. Phenotypes of wild-type, spontaneous mutant, marker exchange mutant, and transconjugants in plants and culture

Strain	Virulence on[a]:		Capsules	Exopolysaccharide[b] (μg/μg of protein)	Doubling time[c] (min)	Lipase[d]	
	Citrus	Bean				2 days	7 days
3048	++	++	+	1.8	84	−	+
M28	−	+	−	0.5	120	+	++
M28/pMK29.28	++	++	+	ND[e]	ND	−	+
M28/pMK41.1	++	++	+	2.3	120	−	+
MX3028	−	+	−	0.6	115	+	++
MX3028/pMK29.28	++	++	+	2.2	ND	−	+
MX3028/pMK41.1	−	+	−	0.5	ND	+	++

[a] Virulence based on symptoms: ++, wild-type symptom expression; +, reduced symptom expression; −, null, little, or no symptoms.
[b] In TYM broth.
[c] Determined by counting CFUs on TYM plates.
[d] Detection of lipase: −, no detectable reaction; +, precipitate detectable; ++, strong precipitate reaction around colonies.
[e] ND, not determined.

analyses using probe pUFT1 and was indistinguishable from 3048.

Gene localization and DNA sequence analysis. The gene responsible for the observed phenotypes was localized on pUFM1140 by subcloning to a 2.3-kb *Sal*I-*Sst*I DNA fragment cloned in pMK41.1 (Fig. 3). Plasmid pMK41.1 fully restored capsules, EPS, and virulence on citrus to M28 (refer Table 2 and Fig. 2). Deletions of either the *Sal*I-*Pst*I fragment (pMK45.1) or the *Eco*RI-*Sst*I fragment (pMK40.1) resulted in clones unable to complement M28 for any mutant phenotype. We consistently observed complementation for the gum phenotype in vitro (detectable on plates) to correlate with restoration of pathogenicity in planta. No subclone separated the two phenotypes. There were no differences detected between pMK41.1 (reverse orientation relative to the vector *lacZ* promoter) and pMK47.6 (forward orientation) in their ability to complement M28 (gum and virulence on citrus), indicating that the complementing gene was transcribed from its own promoter.

The DNA sequence of a 1,975-bp fragment of pMK41.1 was determined from an internal *Pvu*II site to the *Sst*I site and is presented in Fig. 4. Although several open reading frames (ORFs) were evident, computer-assisted analysis of codon preference and third position GC bias (13) indicated that an ORF from positions 703 to 1545 had a high probability of encoding a protein translation product. The ORF was preceded by consensus sequences for a putative ribosomal binding site at positions 693 to 696 and putative −10 and −35 regions as indicated. Primer extension (data not shown) of total RNA extracted from both 3048 and M28 cells showed transcription beginning at positions 646 and 650, confirming the −10 and −35 promoter regions.

Three *Sph*I sites were found internal to the ORF as indicated in Fig. 4; these were used to construct marker interruption plasmid pPR001. This plasmid was introduced into 3048Sp by conjugation, and all resulting colonies on selective media containing appropriate antibiotics appeared to exhibit the reduced gum phenotype characteristic of M28. Six exconjugants were randomly selected for further testing. Integration of pPR001 was confirmed by Southern hybridizations (data not shown). All six mutants retained virulence on bean but had lost virulence on citrus. Plasmid pMTK41.1 fully restored the gum phenotype and virulence on citrus; there was no evidence of a second functional gene on pMTK41.1.

The deduced peptide product of the ORF indicated in Fig. 4 consists of 280 amino acids with a calculated molecular mass of 31.3 kDa and an estimated isoelectric point of 10.74. A search of genetic data bases with the deduced amino acid sequence, via the National Center for Biotechnology Information BLAST network server (1), revealed potentially significant homology to two genes (Fig. 5). The predicted peptide sequence of *lsi-1* of *N. gonorrhoeae* (47) was 27% identical and 50% similar to the predicted peptide sequence of *opsX*, compared over their entire lengths. The DNA coding sequences were 41% identical. The predicted peptide sequence of *rfaQ* of *E. coli* (45, 46) was 26% identical and 52% similar to the predicted peptide sequence of *opsX*, compared over their entire lengths. The DNA coding sequences were 42% identical. The average similarity observed among all three peptide sequences as aligned in Fig. 5 was 54%. No significant homology was found with gene products from any plant-associated microbes.

M28 is not an *hrp* mutant. Mutant M28 had lost virulence on citrus, but not bean, and retained ability to elicit a hypersensitive response on cotton line Acala 44 (data not shown). By comparison, *X. campestris* pv. citrumelo Hrp$^-$ mutants (4) ME23 and ME70 were not host specific but were defective in virulence on both bean and citrus (compare results in Fig. 1) and did not elicit a hypersensitive response on cotton. Mutants ME23 and ME70 were examined by restriction fragment length polymorphism analyses using probe pUFT1 and were indistinguishable from 3048 (blot not shown). Cosmid clones from the *X. campestris* pv. citrumelo 3048 DNA library carrying a potentially homologous *hrp* gene cluster were identified by colony hybridization with the *hrp* cluster from *X. campestris* pv. vesicatoria on pXV9 (4) as a probe. Cosmid clones pUFM2110 and pUFM3016 were identified in activity assays to differentially complement ME23 and ME70 to full virulence on both bean and citrus (Table 3). Although both pUFM2110 and pUFM3016 were able to complement M103 of *X. campestris* pv. vesicatoria, only pUFM2110 restored ME23 to virulence and only pUFM3016 restored ME70 to virulence (Fig. 1 and Table 3), indicating that these two cosmids carried overlapping but not identical regions of an *X. campestris* pv. citrumelo *hrp* gene cluster. Neither pUFM2110 nor pUFM3016 restored host-specific virulence or any other observed mutant phenotype to M28, and pUFM1140 did not restore the Hrp$^+$ phenotype to M103, ME23, or ME70 (Table 3). Cosmid pUFM1140 did not hybridize to strain 3048 insert fragments cloned in either pUFM2110 or pUFM3016.

A *Xanthomonas ops* gene cluster. In order to determine whether *opsX* was in a gene cluster involved in polysaccharide production and/or host-specific virulence, a marker-exchanged mutant of 3048Rf was constructed by the intro-

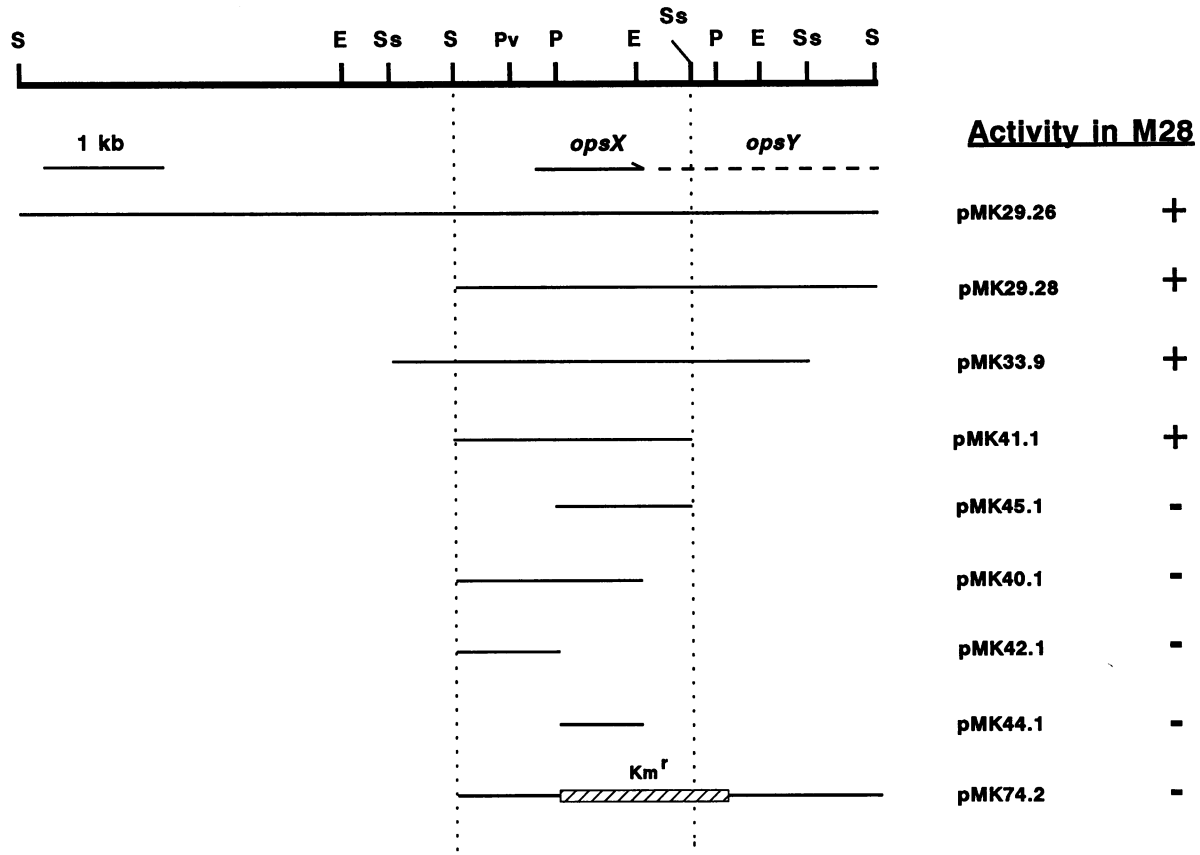

FIG. 3. Restriction map and activity assays of pUFM1140 and subclones in M28. Restoration of virulence on citrus always correlated with restored gum phenotypes. The precise location of *opsX* and the approximate location of *opsY* are indicated. Also shown is marker exchange plasmid pMK74.2; the hashed bar indicates the internal 1.4-kb *Pst*I fragment of pMK29.28, which was deleted and replaced with the 1.3-kb Km^r cartridge from pUC4K. S, *Sal*I; E, *Eco*RI; Ss, *Sst*I; Pv, *Pvu*II; P, *Pst*I.

duction of plasmid pMK74.2. This plasmid carried the regions of DNA flanking *opsX*, but most of *opsX* (downstream from the *Pst*I site) plus an additional 700 bp downstream region were deleted and replaced by a Km^r cartridge (Fig. 3). Verification of marker exchange of the region in one of the resulting mutants, MX3028, was confirmed by Southern blot hybridizations of 3048, M28, and MX3028 DNA (data not shown). MX3028 behaved similarly to M28 in pathogenicity tests (Table 2 and Fig. 2). Furthermore, MX3028/pMK41.1 (*opsX*$^+$) behaved similarly to M28; pMK41.1 was not observed to complement any mutant phenotype of MX3028. A longer strain 3048 DNA fragment was required for complementation; pMK29.28 fully complemented all mutant phenotypes of MX3028 tested (Table 2). At least one additional gene, downstream of *opsX*, present on pMK29.28, and designated *opsY*, was also required for virulence on citrus and normal gum production.

***opsX* pleiotropically affects LPS.** Because of the apparent homology of the deduced gene product OpsX with LPS-related gene products from other pathogenic microorganisms, we examined the LPS of 3048, M28, MX3028, and complementing clones. In these comparisons, the wild-type 3048 and both mutants complemented with pMK29.28 (*opsXY*) exhibited two very distinct LPS bands (labeled I and II; Fig. 6). Spontaneous mutant M28 (*opsX*) lost most of LPS I, and the single LPS band of M28 exhibited greater mobility than LPS II (Fig. 6B). Marker exchange mutant MX3028 (Δ*opsXY*) lost all evidence of LPS I (Fig. 6E), even when overloaded (Fig. 6G). Like M28, MX3028 also had a single LPS band with greater mobility than LPS II. Plasmid pMK41.1 (*opsX*) in M28 restored LPS I but did not fully restore LPS II. Strain M28/pMK41.1 exhibited a second band with intermediate mobility between that of LPS II and the single LPS band of M28 or MX3028.

Although production of EPS was also clearly restored when M28 or MX3028 was complemented with pUFM1140 or subclones pMK29.26 or pMK29.28 (Table 2), none of these clones complemented any one of a collection of 90 non- or low-mucoid mutants of *X. campestris* NRRL B-1459 (ATCC 13951) (47a).

DISCUSSION

X. campestris pv. citrumelo was first isolated in 1984 as the causal agent of a new form of citrus canker disease (52). Despite an intense eradication effort and the destruction of over 23 million citrus trees (52), every year since 1984 there have been new outbreaks of the disease. Of particular interest is the fact that citrus does not appear to be the source of the repeated pandemics. Since all *X. campestris* strains are plant associated, we hypothesized that some strains of a population found on another host carried a variant gene(s) which allowed virulence on citrus and selective amplification of strains carrying the gene(s), resulting in citrus-specific clonal groups (19). In extensive surveys of citrus plants, pathovar citrumelo was found to be comprosed

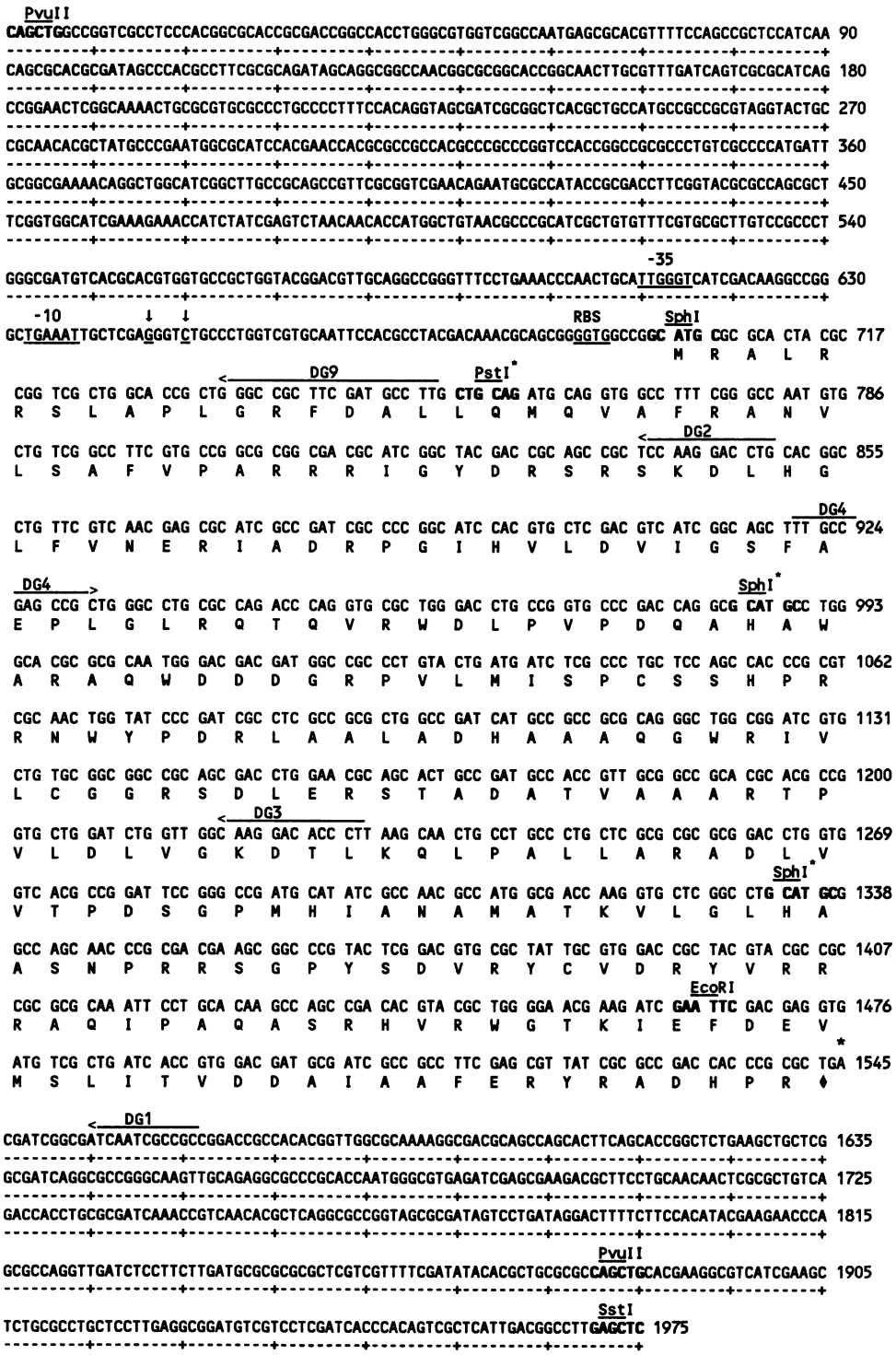

FIG. 4. DNA sequence of a 2,075-bp fragment (*Pvu*II-*Sst*I) containing the *opsX* region and the deduced *opsX* peptide sequence (GenBank accession no. L21026). The oligonucleotide primers used for DNA sequencing are indicated; DG9 was also used to determine the transcriptional start site. The two experimentally determined transcription start sites are indicated by the arrows above and the underline below the appropriate base. The putative ribosomal binding site (RBS) and −10 and −35 promoter regions are indicated. Some restriction sites are indicated in bold type. The *Sph*I sites flanking the fragment used to create marker interruption plasmid pPR001 are marked *Sph*I*. The *Pst*I site used to create marker exchange plasmid pMK74.2 is also marked by an asterisk.

```
          1                                                  50
 OpsX     .......... .......... .....mralr rslapLgrFd alLqMqvaFR
 RfaQ     mqksiccfir tpslfclktR klmplygisN kgagtFdkiK nvLsLiktLR
 Lsi-1    .......... ....mavfeR mpevneileN pfghgaleLK rrwrvgreLg
Consensus ---------- ---------R ---------N ---------K --L-----LR

          51                                                 100
 OpsX     AN........ .......VLs afvPARrRIG Ydrsrskdlh glfvneriAd
 RfaQ     ANnYDlVInL tdqwmvAlLv rclPARmkIs qlyghRqhgi wkksfthlAp
 Lsi-1    rrgYDrVIvL pgslksAVia latgigkRtG YvgesRyfLl ndirrldker
Consensus AN-YD-VI-L ------AVL- ---PAR-RIG Y----R--L- --------A-

          101                                                150
 OpsX     rPGiHVlDvi gSfaEPLGLr qtqvrwDLpV pDqahawaRa qWDddG..rp
 RfaQ     ihGtHIvErn LSvlEPLGit dfytdttMSy aEdcWkkmRr eLDalGvKdh
 Lsi-1    lPlmvdryta LahqsqedFd ghsrfpEFSI dErrReisve tFglnlgKpv
Consensus -PG-H----- LS--EPLG-- --------S- -E------R- --D--G-K--

          151                                                200
 OpsX     vlmIsPcssh PrrnWypDrL AaLaDHaaaq GWrIVLcGGr SDlErstaDa
 RfaQ     yvvIqPtarq ifKcWdnDkF skviDalqqr GYQVVLtcGp SadDlAcvDe
 Lsi-1    lafcpgaefg PaKrWparhF AeLgkHysea GWQVwLfGsq kDnEiAeein
Consensus ---I-P---- P-K-W--D-F A-L-DH---- GWQVVL-GG- SD-E-A--D-

          201                                                250
 OpsX     tvaaarT.PV ldLvGKdtLk QLpALLarAD LVVtpDSGPM HIAnAMaTKV
 RfaQ     iargceTkPI tgLaGKTrFp eLgALidhAv LfIgvDSaPg HIAAAvkTpV
 Lsi-1    clsdgm...c vnLcGKTdLs QamdLLslAD tVVcnDSGlM HlAAALgrKV
Consensus ------T-P- --L-GKT-L- QL-ALL--AD LVV--DSGPM HIAAA--TKV

          251                                                300
 OpsX     lgLhaASnPr rsgPYSDvry cVdryvrrra ........qI PaqAsrhVrW
 RfaQ     IsLFGAtdhv fwrPWtE... .........n ........iI qfwAgnYqkm
 Lsi-1    VavYGsSsPt htpPlSDrak iVslhlecsp cfkrecplgI PtastgCIpR
Consensus --L-GAS-P- ---P-SD--- -V-------- ---------I P--A------

          301                                          342
 OpsX     gTkiEFD... EvMSLItvDD aIAAfEryra Dhpr*..... ..
 RfaQ     pTrhELDRnk kyLSvIpaED vIAAtEkllp Edapsadrna ql
 Lsi-1    rlcrrLkRry DgFcFriytv *......... .......... ..
Consensus -T--ELDR-- ---S-I---D -IAA-E---- ---------- --
```

FIG. 5. Alignment of the predicted amino acid sequences of *opsX*, *rfaQ*, and *lsi-1*. The consensus sequence shown was based on matching amino acids occurring at a given position in any two of the three aligned peptide sequences. Similarity values were 52% for *opsX* and *rfaQ*, 50% for *opsX* and *lsi-1*, and 43% for *rfaQ* and *lsi-1*.

of clonal groups (19, 22). A clonal population structure is typical of several bacterial species and is thought to be due to (i) low rates of recombination and (ii) selective amplification of strains with specific virulence factors (53).

X. campestris pv. citrumelo mutant M28 was of interest because it had lost virulence on citrus specifically. Even inoculations of M28 at concentrations of $\geq 10^8$ CFU ml^{-1} elicited no response in citrus, and cells died rapidly in citrus after inoculation (Fig. 2). Growth of M28 in bean tissue was only slightly affected relative to that of the wild type (Fig. 2), and inoculations of M28 on nonhosts resulted in a typical hypersensitive response (Table 3). Therefore, the mutation(s) affecting virulence in M28 was unlike *hrp* gene mutations, which abolish the nonhost hypersensitive response and virulence on all hosts (66). In addition, the mutation(s) affecting host range in M28 was unlike *avr* gene mutations, which can increase the host range of affected strains (18). The rapid death in citrus of M28 cells indicated either an unusually inhospitable host response to M28 or an unusual sensitivity of M28 to its host.

Mutant M28 was also affected in its growth rate, colony morphology, capsular slime, EPS production level, and LPS. Most mutant phenotypes of M28 were fully restored to those of the wild-type 3048 when pMK41.1 was introduced into M28. A single uninterrupted 842-bp ORF that had a high probability of encoding a translation product along its entire length was identified on pMK41.1. Putative promoter consensus sequences were identified, and their positions were confirmed by primer extension analysis of total RNA extracted from strain 3048 cells grown in minimal and complex media. Marker interruption experiments confirmed that physical interruption of the 842-bp region in 3048 resulted in mutants with pleiotropic phenotypes indistinguishable from those of M28. The 842-bp ORF, designated *opsX*, appeared responsible for all pleiotropic mutant phenotypes observed in M28. On the basis of complementation assays using the marker exchange mutant MX3028, from which *opsX* and an adjacent region have been deleted, at least one gene adjacent to *opsX* is also required for the same phenotypes. Even though MX3028 was not complemented by pMK41.1 (*opsX*), it was complemented by pMK29.28, which carries *opsX* and at least one other gene, designated *opsY*. MX3028/pMK41.1 (*opsX*$^+$ *opsY*) was nearly identical in phenotype to M28 (*opsX opsY*$^+$) (Table 2); therefore, *opsX* and *opsY* are clustered and appear to be involved in the same biochemical pathway.

There was no evidence that the *opsXY* region was immediately adjacent to *hrp* genes found in *X. campestris* pv. citrumelo. Two cosmid-sized clones with different *hrp* genes (pUFM2110 and pUFM3016) were cloned from 3048, and

TABLE 3. *X. campestris* pv. citrumelo *hrp* (for hypersensitive response on nonhosts and pathogenicity on hosts) genes, clones, and mutational phenotypes

Strain	*X. campestris* response on[a]:			
	Citrus	Bean	Pepper	Cotton
X. campestris pv. vesicatoria				
88.5 (wild type)	NT	NT	+	HR
M103 (*hrp*)	NT	NT	−	−
M103/pUFM2110	NT	NT	+	HR
M103/pUFM3016	NT	NT	+	HR
X. campestris pv. citrumelo				
3048 (wild type)	+	+	NT	HR
ME23 (*hrp*)	−	−	NT	−
ME23/pUFM2110	−	−	NT	−
ME23/pUFM3016	+	+	NT	HR
ME70 (*hrp*)	−	−	NT	−
ME70/pUFM2110	+	+	NT	HR
ME70/pUFM3016	−	−	NT	−
M28 (*opsX*)	−	+	NT	HR
M28/pUFM2110	−	+	NT	HR
M28/pUFM3016	−	+	NT	HR

[a] Citrus and bean plants are hosts for *X. campestris* pv. citrumelo. Pepper is a host plant for *X. campestris* pv. vesicatoria. Cotton plants were used as a common nonhost plant to assay the nonhost hypersensitive response. +, pathogenic symptoms (water soaking); HR, plant hypersensitive response (rapid cellular collapse followed by necrosis); −, no pathogenic symptoms: NT, not tested.

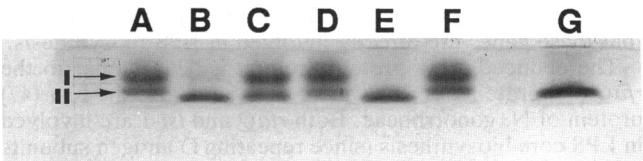

FIG. 6. Silver-stained LPS gel showing complementation of *opsX* and Δ*opsXY*. Lanes: A, LPS from wild-type 3048; B, M28 (*opsX*); C, M28/pMK41.1 (*opsX*$^+$); D, M28/pMK29.28 (*opsXY*$^+$); E and G, MX3028 (Δ*opsXY*); F, MX3028/pMK29.28 (*opsXY*$^+$). Lane G was loaded with twice the LPS concentration as lane E.

M28 was not complemented by either of them. Neither of these cosmids cross-hybridized with pMK1140, which carries *opsX* on a 38-kb DNA fragment from 3048. These results indicated that *opsXY* is not part of the *hrp* cluster.

Mutants of *X. campestris* regulatory and export genes can pleiotropically affect virulence, gum production, and NaCl and chloramphenicol tolerance (9, 44, 59). Such regulatory and export mutants exhibit reduced virulence and are often deficient in various extracellular enzymes, such as protease and pectinase, in addition to low exopolysaccharide production. By contrast with such mutants, M28 and MX3028 were similar to wild-type 3048 for a range of functions including amylase and protease production, carbohydrate utilization, and osmotic tolerance in high- and low-salt solutions. Both M28 and MX3028 exhibited increased lipase activity in plate assays; to our knowledge, increased lipase activity has not been reported to negatively affect virulence. These results indicate that *opsXY* is not directly involved in gene regulation or export functions.

Xanthomonas genes involved in EPS biosynthesis have not been reported to affect virulence. The *opsXY* locus affected the EPS of 3048 but was not observed to complement any of the 90 non- or low-mucoid mutants of *X. campestris* tested in the Kelco, Inc., collection. The mutational lesions in these 90 mutants were mapped to eight different regions of the chromosome and are involved in the synthesis of the pentasaccharide (26), synthesis of the sugar nucleotide precursors (38), or to unknown functions of xanthan biosynthesis (25a). These results indicated that the *opsXY* locus was not primarily involved in EPS biosynthesis.

Xanthomonas genes involved in LPS biosynthesis have not been identified. Although confirmation by chemical analysis is required, two LPS bands, designated LPS I and LPS II, appear to be produced by wild-type *X. campestris* pv. citrumelo 3048. These two highly reproducible bands appeared upon silver staining of proteinase K-treated whole-cell extracts of 3048; silver-stained LPS profiles of proteinase K-digested whole-cell extracts of other organisms are similar to those of homologous purified LPS samples (27). Purified LPS of *X. campestris* pv. begoniae also revealed two major bands upon silver staining (3). M28 produced a very slight level of LPS I, which was not evident in the deletion mutant MX3028. Since transcription of *opsX* was observed in both 3048 and M28, it seems likely that the mutational lesion in M28 is in the coding region of *opsX*, but transcriptional levels were not determined. Plasmid pMK41.1 appeared to fully restore LPS I in M28 but did not fully restore LPS II in terms of mobility, while pMK29.28 fully restored both LPS I and LPS II in M28. There are several possible explanations, including promoter interference, since pMK41.1 carried *opsX* in reverse orientation relative to the functional *lacZ* promoter of the vector (pMK29.28 carried *opsXY* downstream from the *lacZ* promoter). Alternatively, a slightly polar mutation in *opsX* could affect *opsY* as well. The loss of LPS I and the apparent increase in mobility of LPS II affected by *opsXY* indicate that these genes are directly involved in LPS biosynthesis.

The deduced *opsX* protein product is 52% similar to the *rfaQ* (46) protein of *E. coli* and is 50% similar to the *lsi-1* (47) protein of N. gonorrhoeae. Both *rfaQ* and *lsi-1* are involved in LPS core biosynthesis (since repeating O antigen subunits are absent in gonococcal LPS, it is often called lipo-oligosaccharide, or LOS). Gene *rfaQ* is the first of three overlapping ORFs in an operon consisting at least of *rfaQ*, *rfaP*, and *rfaG* (46). While the function of RfaQ is unknown (45), it shares homology with RfaC and RfaF, which are involved in the synthesis of the heptose region of the LPS core (46). The same region of homology is shared by OpsX (from positions 236 to 257 in Fig. 5). It is possible that the gene *opsX* is the first gene in an operon consisting at least of *opsXY*. Since the kanamycin cartridge in the marker exchange mutant MX3028 does not create polar mutations, it is possible that an *ops* operon extends beyond two genes. The homology of OpsX with genes involved in the synthesis of the LPS core is consistent with the hypothesis that LPS II of strain 3048 represents the LPS core oligosaccharide, while LPS I may represent complete LPS containing O polysaccharide repeats.

The LPS of some pathogens is thought to function as an essential permeability barrier against toxic host compounds produced by both animals and plants (42, 43). On the basis of homology with RfaQ and Lsi-1, the lack of capsules, the defective LPS, and the rapid death of *opsX* or *opsY* cells in citrus, it seems plausible that *opsXY* is involved in both LPS core and EPS biosynthesis. Mutations that simultaneously affect EPS, LPS, and virulence have been reported for both *R. leguminosarum* (14) and *P. solanacearum* (30). The fact that mutations in *opsX* caused the affected cells to die off rapidly in citrus strongly indicates that the cells were being killed by citrus (possibly defense) compounds which are not found in bean tissue and which can penetrate the defective LPS barrier. Complete LPS structures appear necessary for *R. leguminosarum* infection of bean plants (5). With *X. campestris* pv. citrumelo, either complete LPS structures or normal amounts of EPS, or both, appear necessary for infection of citrus. The fact that some LPS-specific antigens of *X. campestris* are also pathovar specific (3) may indicate that specific modifications of the LPS barrier may also be needed for virulence on specific hosts.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Meyers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Barrere, G. C., C. E. Barber, and M. J. Daniels.** 1986. Molecular cloning of genes involved in the production of the extracellular polysaccharide xanthan by *Xanthomonas campestris* pv. campestris. Int. J. Biol. Macromol. **8**:372–374.
3. **Benedict, A. A., A. M. Alvarez, and L. W. Pollard.** 1990. Pathovar-specific antigens of *Xanthomonas campestris* pv. begoniae and *X. campestris* pv. pelargonii detected with monoclonal antibodies. Appl. Environ. Microbiol. **56**:572–574.
4. **Bonas, U., R. Schulte, S. Feneseleau, G. V. Minsavage, B. J. Staskawicz, and R. E. Stall.** 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. vesicatoria that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interact. **4**:81–88.
5. **Cava, J. R., P. M. Elias, D. A. Turowski, and K. D. Noel.** 1989. *Rhizobium leguminosarum* CFN42 genetic regions encoding lipopolysaccharide structures essential for complete nodule development on bean plants. J. Bacteriol. **171**:8–15.
6. **Coplin, D. L., and D. Cook.** 1990. Molecular genetics of extra-

cellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. Mol. Plant-Microbe Interact. **41**:459–472.
7. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41**:435–464.
8. **Costerton, J. W., R. T. Irvin, and K.-J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **39**:299–324.
9. **Daniels, M. J., J. M. Dow, and A. E. Osbourn.** 1988. Molecular genetics of pathogenicity in phytopathogenic bacteria. Annu. Rev. Phytopathol. **26**:285–312.
10. **DeFeyter, R., and D. W. Gabriel.** 1991. At least six avirulence genes are clustered on a 90-kilobase plasmid in *Xanthomonas campestris* pv. malvacearum. Mol. Plant-Microbe Interact. **4**:423–432.
11. **DeFeyter, R., and D. W. Gabriel.** 1991. Use of cloned DNA methylase genes to increase the frequency of transfer of foreign genes into *Xanthomonas campestris* pv. malvacearum. J. Bacteriol. **173**:6421–6427.
11a. **DeFeyter, R., and D. W. Gabriel.** Unpublished data.
12. **DeFeyter, R., C. I. Kado, and D. W. Gabriel.** 1990. Small stable shuttle vectors for use in *Xanthomonas*. Gene **88**:65–72.
13. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the vax. Nucleic Acids Res. **12**:387–395.
14. **Diebold, R., and K. D. Noel.** 1989. *Rhizobium leguminosarum* exopolysaccharide mutants: biochemical and genetic analyses and symbiotic behavior on three hosts. J. Bacteriol. **171**:4821–4830.
15. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacterial construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
16. **Doetsch, R. N.** 1981. Determinative methods of light microscopy, p. 21–33. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
17. **Fett, W. F., and S. F. Osman.** 1985. Purification and characterization of *Xanthomonas campestris* pv. glycines exopolysaccharide. Plant Sci. **40**:99–103.
18. **Gabriel, D. W.** 1989. The genetics of plant pathogen population structure and host-parasite specificity, p. 343–379. *In* T. Kosuge and E. W. Nester (ed.), Plant-microbe interactions: molecular and genetic perspectives, vol. 3. Macmillan Publishing Co., New York.
19. **Gabriel, D. W., J. E. Hunter, M. T. Kingsley, J. W. Miller, and G. R. Lazo.** 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol. Plant-Microbe Interact. **1**:59–65.
20. **Gabriel, D. W., M. T. Kingsley, J. E. Hunter, and T. R. Gottwald.** 1989. Reinstatement of *Xanthomonas citri* (ex Hasse) and *X. phaseoli* (ex Smith) to species and reclassification of all *X. campestris* pv. citri strains. Int. J. Syst. Bacteriol. **39**:14–22.
21. **Gabriel, D. W., M. T. Kingsley, Y. Yang, J. Chen, and P. Roberts.** Host-specific virulence genes of *Xanthomonas*. *In* C. I. Kado and J. Crosa (ed.), Molecular mechanisms of bacterial virulence, in press. Kluwer Academic Publishers, Hingham, Mass.
22. **Gottwald, T. R., A. M. Alvarez, J. S. Hartung, and A. A. Benedict.** 1991. Diversity of *X. campestris* pv. citrumelo strains associated with epidemics of citrus bacterial spot in Florida citrus nurseries: correlation of detached leaf, monoclonal antibody and restriction fragment length polymorphism assays. Phytopathology **81**:749–753.
23. **Gilman, M.** 1987. Rapid isolation of RNA from gram-negative bacteria, p. 4.4.2–4.4.4. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
24. **Gray, J. X., R. A. de Maagd, B. G. Rolfe, A. W. B. Johnston, and B. J. J. Lugtenberg.** 1992. The role of the *Rhizobium* cell surface during symbiosis, p. 359–376. *In* D. P. S. Verma (ed.), Molecular signals in plant-microbe communications. CRC Press, Boca-Raton, Fla.
25. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
25a. **Harding, N. E.** Personal communication.
26. **Harding, N. E., J. M. Cleary, D. K. Cabanas, I. G. Rosen, and K. Kang.** 1987. Genetic and physical analysis of a cluster of genes essential for xanthan gum biosynthesis in *Xanthomonas campestris*. J. Bacteriol. **169**:2854–2861.
27. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
28. **Hötte, B., I. Rath-Arnold, A. Pühler, and R. Simon.** 1990. Cloning and analysis of a 35.3-kilobase DNA region involved in exopolysaccharide production by *Xanthomonas campestris* pv. campestris. J. Bacteriol. **172**:2804–2807.
29. **Kamoun, S., and C. I. Kado.** 1990. A plant inducible gene of *Xanthomonas campestris* pv. campestris encodes an exocellular component required for growth in the host and hysensitivity on nonhosts. J. Bacteriol. **172**:5165–5172.
30. **Kao, C. C., and L. Sequeira.** 1991. A gene cluster required for coordinated biosynthesis of lipopolysaccharide and extracellular polysaccharide also affects virulence of *Pseudomonas solanacearum*. J. Bacteriol. **173**:7841–7847.
31. **Kingsley, M., and B. B. Bohlool.** 1992. Extracellular polysaccharide is not responsible for aluminum tolerance of *Rhizobium leguminosarum* bv. phaseoli CIAT899. Appl. Environ. Microbiol. **58**:1095–1101.
32. **Kingston, R. E.** 1987. Primer extension, p. 4.8.1–4.8.3. *In* F. M. Ausebel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
33. **Koplin, R., W. Arnold, B. Hotte, R. Simon, G. Wang, and A. Puhler.** 1992. Genetics of xanthan production in *Xanthomonas campestris*: the *xanA* and *xanB* genes are involved in UDP-glucose and GDP-mannose biosynthesis. J. Bacteriol. **174**:191–199.
34. **Krauss, J. H., J. Weckesser, and H. Mayer.** 1988. Electrophoretic analysis of lipopolysaccharides of purple nonsulfur bacteria. Int. J. Syst. Bacteriol. **38**:157–163.
35. **Lazo, G. R., R. Roffey, and D. W. Gabriel.** 1987. Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment length polymorphisms. Int. J. Syst. Bacteriol. **37**:214–221.
36. **Leigh, J. A., and D. L. Coplin.** 1992. Exopolysaccharides in plant-bacterial interactions. Annu. Rev. Microbiol. **46**:307–346.
37. **Leong, S. A., G. S. Ditta, and D. R. Helinski.** 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for aminolevulinic acid synthetase from *Rhizobium meliloti*. J. Biol. Chem. **257**:8724–8730.
38. **Marzocca, M. P., N. E. Harding, A. Petroni, J. M. Cleary, and L. Ielpi.** 1991. Location and cloning of the ketal pyruvate transferase gene of *Xanthomonas campestris*. J. Bacteriol. **173**:7519–7524.
39. **Maurelli, A. T., and P. J. Sansonetti.** 1988. Genetic determinants of *Shigella* pathogenicity. Annu. Rev. Microbiol. **42**:127–150.
40. **Minsavage, G. V., D. Dahlbeck, M. C. Whalen, B. Kearney, U. Bonas, B. J. Staskawicz, and R. E. Stall.** 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. vesicatoria-pepper interactions. Mol. Plant-Microbe Interact. **3**:41–47.
41. **Murray, N. E., W. J. Brammer, and K. Murray.** 1977. Lambdoid phages that simplify the recovery of in vitro recombinants. Mol. Gen. Genet. **150**:53–61.
42. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1–32.
43. **Noel, K. D.** 1992. Rhizobial polysaccharides required in symbioses with legumes, p. 341–357. *In* D. P. S. Verma (ed.), Molecular signals in plant-microbe communications. CRC Press, Boca Raton, Fla.
44. **Osbourn, A. E., B. R. Clarke, B. J. H. Stevens, and M. J.**

Daniels. 1990. Use of oligonucleotide probes to identify members of two-component regulatory systems in *Xanthomonas campestris* pathovar campestris. Mol. Gen. Genet. **222:**145–151.

45. **Parker, C. T., A. W. Kloser, C. A. Schnaitman, M. A. Stein, S. Gottesman, and B. W. Gibson.** 1992. Role of the *rfaG* and *rfaP* genes in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12. J. Bacteriol. **174:**2525–2538.
46. **Parker, C. T., E. Pradel, and C. A. Schnaitman.** 1992. Identification and sequence of the lipopolysaccharide core biosynthetic genes *rfaQ*, *rfaP*, and *rfaG* of *Escherichia coli* K-12. J. Bacteriol. **174:**930–934.
47. **Petricoin, E. F., III, R. J. Danaher, and D. C. Stein.** 1991. Analysis of the *lsi* region involved in lipooligosaccharide biosynthesis in *Neisseria gonorrhoeae*. J. Bacteriol. **173:**7896–7902.

47a. **Puester, M. K., and N. Harding (Kelco, Inc.).** Personal communication.

48. **Ramirez, M. E., L. Fucikovsky, F. Garcia-Jimenez, R. Quintero, and E. Galindo.** 1988. Xanthan gum production by altered pathogenicity variants of *Xanthomonas campestris*. Appl. Microbiol. Biotechnol. **29:**5–10.
49. **Reuhs, B. L., R. W. Carlson, and J. S. Kim.** 1993. *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-D-*manno*-2-octulosonic acid-containing polysaccharides that are structurally analogous to Group II K antigens (capsular polysaccharides) found in *Escherichia coli*. J. Bacteriol. **175:**3570–3580.
50. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
51. **Schoonejans, E., D. Expert, and A. Toussaint.** 1987. Characterization and virulence properties of *Erwinia chrysanthemi* lipopolysaccharide-defective, ϕEC2-resistant mutants. J. Bacteriol. **169:**4011–4017.
52. **Schoulties, C. L., E. L. Civerolo, J. W. Miller, R. E. Stall, C. J. Krass, S. R. Poe, and E. P. DuCharme.** 1987. Citrus canker in Florida. Plant Dis. **71:**388–395.
53. **Selander, R. K.** 1985. Protein polymorphism and the genetic structure of natural populations of bacteria, p. 85–106. *In* T. Ohta and K. Oaki (ed.), Population genetics and molecular evolution. Japan Scientific Societies Press, Tokyo.
54. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
55. **Stachel, S. E., G. An, C. Flores, and E. W. Nester.** 1985. A Tn*3* *lacZ* transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. EMBO J. **4:**891–898.
56. **Swarup, S., R. DeFeyter, R. H. Brlansky, and D. W. Gabriel.** 1991. A pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *X. campestris* to elicit canker-like lesions on citrus. Phytopathology **81:**802–809.
57. **Swarup, S., Y. N. Yang, M. T. Kingsley, and D. W. Gabriel.** 1992. An *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhosts. Mol. Plant-Microbe Interact. **5:**204–213.
58. **Takahashi, T., and N. Doke.** 1984. A role of extracellular polysaccharides of *Xanthomonas campestris* pv. citri in bacterial adhesion in citrus leaf tissues in preinfectious stage. Annu. Rev. Phytopathol. **50:**565–573.
59. **Tang, J.-L., C. L. Gough, and M. J. Daniels.** 1990. Cloning of genes involved in negative regulation of production of extracellular enzymes and polysaccharide of *Xanthomonas campestris* pv. campestris. Mol. Gen. Genet. **222:**157–160.
60. **Thorne, L., L. Tansey, and T. J. Pollock.** 1987. Clustering of mutations blocking synthesis of xanthan gum by *Xanthomonas campestris*. J. Bacteriol. **169:**3593–3600.
61. **Turner, P., C. Barber, and M. J. Daniels.** 1985. Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. campestris. Mol. Gen. Genet. **199:**338–343.
62. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
63. **Vieira, J., and J. Messing.** 1987. Production of single stranded plasmid DNA. Methods Enzymol. **153:**3–11.
64. **Waney, V. R., M. T. Kingsley, and D. W. Gabriel.** 1991. *Xanthomonas campestris* pv. translucens genes determining host specific virulence and general virulence on cereals identified by Tn*5*-*gusA* insertion mutagenesis. Mol. Plant-Microbe Interact. **4:**623–627.
65. **Whitefield, C., I. W. Sutherland, and R. E. Cripps.** 1981. Surface polysaccharides in mutants of *Xanthomonas campestris*. J. Gen. Microbiol. **124:**385–392.
66. **Willis, D. K., J. J. Rich, and E. M. Hrabak.** 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact **4:**132–138.
67. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19. Gene **33:**103–119.