Osmoregulated Periplasmic Glucan Synthesis Is Required for *Erwinia chrysanthemi* Pathogenicity

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Erwinia chrysanthemi* is a phytopathogenic enterobacterium causing soft rot disease in a wide range of plants. Osmoregulated periplasmic glucans (OPGs) are intrinsic components of the gram-negative bacterial envelope. We cloned the *opgGH* operon of *E. chrysanthemi*, encoding proteins involved in the glucose backbone synthesis of OPGs, by complementation of the homologous locus *mdoGH* of *Escherichia coli*. OpgG and OpgH show a high level of similarity with MdoG and MdoH, respectively, and mutations in the *opgG* or *opgH* gene abolish OPG synthesis. The *opg* mutants exhibit a pleiotropic phenotype, including overproduction of exopolysaccharides, reduced motility, bile salt hypersensitivity, reduced protease, cellulase, and pectate lyase production, and complete loss of virulence. Coinoculation experiments support the conclusion that OPGs present in the periplasmic space of the bacteria are necessary for growth in the plant host.

Osmoregulated periplasmic glucans (OPGs) are a family of oligosaccharides found in the periplasmic space of gram-negative bacteria. Their two common features are the presence of glucose as the sole constituent sugar and their increased level in media of low osmolarity (5).

Members of the family *Enterobacteriaceae* and related bacteria synthesize a family of linear and branched OPGs that are variously substituted. The linear backbone is constituted by glucose units joined by β,1-2 linkages, and the branches are made of one glucose unit linked to the main chain by a β,1-6 linkage. In *Escherichia coli*, the backbone, containing 7 to 13 glucose units, is substituted with phosphoglycerol, phosphoethanolamine, and succinyl residues (19). In *Erwinia chrysanthemi*, the backbone contains 5 to 12 glucose units substituted with succinyl and acetyl residues (9), and in *Pseudomonas syringae*, the backbone, consisting of 6 to 13 glucose units, is not substituted (38). In *E. coli*, the OPG backbone is synthesized by the products of the *mdoGH* operon located in the vicinity of *pyrC*, a gene involved in the biosynthesis of uracil (6). In this bacterium, the defect in OPG synthesis does not confer an easily selectable phenotype in laboratory conditions. Thus, the *mdoGH* locus was cloned using the linked selectable genetic marker *pyrC* (23).

Many factors are involved in the virulence of pathogenic bacteria, and OPGs appear to be among them. In *P. syringae* pv. syringae, the causal agent of brown spot disease of the common bean (*Phaseolus vulgaris*), the *hrpM* mutant, obtained after transposon mutagenesis, was isolated because it failed to incite disease on the host plant and to cause the hypersensitive reaction on a non-host plant such as tobacco (29). The *hrpM* mutant does not synthesize OPGs, and the *hrpM* locus complements the OPG synthesis defect of the *mdoH200*::Tn*10* mutant of *E. coli*. The amino acid sequences of HrpM and MdoH are 75.5% identical and 87.5% similar (25). More recently, a transposon insertion in a gene similar to *hrpM*/*mdoH* was isolated because it severely reduces the virulence in *Pseudomonas aeruginosa* PA14, an opportunistic pathogen for human, mice, and the worm *Caenorhabditis elegans* (26).

The pectinolytic bacterium *E. chrysanthemi*, a member of the *Enterobacteriaceae* family, causes soft rot disease in a wide range of plant species. The virulence of this bacterium is strongly associated with the synthesis and secretion of a set of enzymes that degrade plant cell wall components, such as pectinases, cellulases, and proteases. The action of these enzymes, particularly the pectinases, leads to the extension of the disease, causes maceration of the plant tissues, and supplies the bacteria with carbon sources derived from the degraded polymers. The combination of pectin lyase, polygalacturonase, pectate lyase, pectin acetyl esterase, and pectin methyl esterase activities is needed for the degradation of pectin, reflecting the structural complexity of this polymer (2, 36). Among these proteins, endo pectate lyases play the most important role in the maceration process.

The variability of the plant cell wall is reflected by the great number of pectinase genes and correlated with the great number of stimuli affecting the differential expression of the *pel* genes (15). These genes seem to be, at least in part, the reason for the broad host range of this bacterium (3). *E. chrysanthemi* 3937 synthesizes five major endo pectate lyases, encoded by *pelA* to *pelE* (39), and a set of minor ones. Transcription of the *pel* genes is induced by pectin catabolic products, the stationary growth phase, host plant metabolites (7), and iron depletion (28). Transcription of these genes is repressed by nitrogen starvation, high temperature, and high osmolarity and is under

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TABLE 1. Bacterial strains.

Strain	Relevant genotype	Source[a] or reference
E. chrysanthemi		
3937	Wild type	20
A350 (= L2)	*lacZ2*	16
A655	*lacZ2 arg-10 met-2 outC::lacZ-amp*	18
A1383	*ura-2 arg-1 pro-546 rpsL*	UMG collection
A1703	*lacZ2 pelE::lacZ-cml*	UMG collection
A1706	*lacZ2 pelC::lacZ-cml*	UMG collection
A2575	*lacZ2 epsG::lacZ-tet*	10
A3144	3937, *outS::kan*	UMG collection
RH7006	3937, *msrA::cml*	
NFB3500	3937, *opgG500::uidA-kan*	This study
NFB3501	3937, *opgH501::uidA-kan*	This study
NFB3502	A1703, *opgG500::uidA-kan*	This study
NFB3503	A1706, *opgG500::uidA-kan*	This study
NFB3504	A655, *opgG500::uidA-kan*	This study
NFB3511	A2575, *opgG500::uidA-kan*	This study
E. coli		
NFB223	F⁻ *pyrC46 argE3* Δ(*lac*)*U169 thi araD139 mdoH200::*Tn*10 flbB5301 ptsF25 relA1 rpsL150 rbsR deoC1 rpoB*	20
NFB216	*pyrC46* Δ(*lac-pro*) *thi ara mdoH200::*Tn*10 rpsL* (φ80Δ*lacZ*ΔM15)	23
NFB702	*pyrC46* Δ(*lac-pro*) *thi ara mdoG202::neo rpsL* (φ80Δ*lacZ*ΔM15)	20

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catabolic repression (16). Several proteases and the cellulase EGZ, encoded by the *celZ* gene, are secreted by *E. chrysanthemi* 3937, but they seem to play a minor role in pathogenesis. The cellulase and most of the pectinases are secreted in the medium by the specific Out secretion system encoded by the 15 *out* genes (34). Synthesis and secretion of pectinases are tightly associated. Thus, expression of the *out* genes is regulated in the same way and by the same factors as the *pel* genes (11). Because of iron depletion found in plant fluids, two iron-scavenging systems are needed for full virulence (27).

In this paper, we describe the molecular analysis of the *opgGH* operon involved in OPG backbone synthesis in *E. chrysanthemi*. *opgG* and *opgH* mutants were obtained by reverse genetic manipulation and analyzed for the synthesis and secretion of plant cell wall-degrading enzymes and for virulence.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. chrysanthemi* and *E. coli* K-12 strains are listed in Table 1. Plasmids used for subcloning experiments were pYZ4 (kanamycin resistance) and pUC18 (ampicillin resistance) (25).

Media and growth conditions. Bacteria were grown at 30°C (*E. chrysanthemi*) or 37°C (*E. coli*) in Luria-Bertani broth (LB) or in minimal medium M63 supplemented with the required metabolites and a carbon source added at a concentration of 2 g/liter (29). Polygalacturonate was added at a concentration of 4 g/liter. Potato extract was added at a concentrations of 4 or 8% (vol/vol). When low-osmolarity medium was required, LB without NaCl or low-osmolarity medium (LOS) was used (23). Solid media were obtained by adding agar at 15 g/liter.

Potato extract was obtained as follows. Potato tubers were crushed in a domestic mixer, and the resulting suspension was passed through a French pressure cell at 20,000 lb/in² (1.4 × 10⁷ Pa) and filter sterilized (0.22-μm; Millipore).

Motility tests were made on LB plates containing agar at 0.4 g/liter. The MIC of bile salts (no. 3; Difco Laboratories) was determined on LB plates. The solid media used to test the pectinase and cellulase activities have been described previously (16, 28), and protease activity was detected by adding 1% milk to LB solid medium.

Antibiotics were used in media at the following concentrations: streptomycin,

100 μg/ml; ampicillin and kanamycin, 50 or 10 μg/ml; and tetracycline, 25 and 5 μg/ml for *E. coli* and *E. chrysanthemi*, respectively. X-Gal (5-bromo-4-chloro-3-indolyl-β-ᴅ-galactopyranoside) was used in media at a concentration of 20 μg/ml.

Transduction and transformation. Transduction of *E. chrysanthemi* with phage φEC2 was carried out according to Resibois et al. (32).

Transformation of *E. coli* and *E. chrysanthemi* cells was carried out by the rubidium chloride technique (29) and by electroporation (35), respectively.

Transfer of RP4 derivative plasmids by mating. Plasmid pULB110, a kanamycin-sensitive RP4::mini-Mu derivative (41), was used to generate R-prime derivatives containing bacterial DNA. Matings between recipient and donor strains carrying plasmids were performed by spreading 0.2 ml of overnight cultures of the strains on 63 minimal medium plates and incubating for 5 h at 30°C. Bacteria were resuspended in 1 ml of 63 minimal medium and spread on the appropriate selective media.

Recombinant DNA techniques. Standard procedures were performed for genomic and plasmid DNA extraction (35). Restriction enzymes (Eurogentec) and T4 DNA ligase (Gibco-BRL) were used according to the manufacturer's recommendations. DNA sequences were determined with the Sequenase version 2.0 kit (U.S. Biochemical Corporation). For DNA hybridization, 10 μg of DNA was digested with the appropriate restriction enzyme(s) and treated as previously described (35). DNA fragments were transferred and fixed onto a Biotrans nylon membrane as recommended by the manufacturer (ICN). The digoxigenin DNA-labeling and detection kit was used for labeling of probes, hybridization, and detection of hybrids according to the manufacturer's recommendations (Boehringer Mannheim).

The DNA sequences and deduced amino acid sequences were analyzed by using computer programs made available from Infobiogen (http://www.infobiogen.fr/).

Construction of *opgG::uidA*-kan and *opgH::uidA*-kan insertions and marker exchange recombination. The *uidA*-kan cassette was liberated by *Sph*I digestion of plasmid pUIDK11 (1). In order to inactivate the *opgG* or *opgH* gene, this *Sph*I DNA fragment was then introduced into pNFW32, containing the *opgGH* operon, partially digested by *Sph*I, either in the *Sph*I site located in *opgG* or in the first of the two *Sph*I sites located in *opgH* (Fig. 1). The resulting plasmids were introduced into *E. chrysanthemi* cells by electroporation. The insertions were then integrated into the *E. chrysanthemi* chromosome by marker exchange recombination after successive cultures in low-phosphate medium in the presence of kanamycin (33).

OPG analysis. To measure OPG synthesis in *E. chrysanthemi*, cells were grown overnight in LB without NaCl (50 ml). Bacteria were collected by centrifugation at 4°C for 15 min at 8,000 × g. Cell pellets were resuspended in 1 ml of distilled water and extracted with 5% trichloroacetic acid (TCA). The TCA extracts were neutralized with ammonium hydroxide 10%, desalted on a Sephadex G15 column (Pharmacia), and concentrated by rotary evaporation. The resulting material (2 ml) was then fractionated by gel filtration on a Bio-Gel P-4 (Bio-Rad). The column (55 by 1.6 cm) was equilibrated with 0.5% acetic acid. The column was eluted in the same buffer at a flow rate of 15 ml/h, and fractions of 2.5 ml were collected. Sugar content in each fraction was determined colorimetrically by the anthrone reagent procedure (37).

To measure OPG synthesis in *E. coli*, cultures (5 ml) were made in LOS containing 0.45 mM [2-³H]-glycerol (296 MBq/mmol; New England Nuclear), and labeled OPGs were extracted by the charcoal adsorption procedure; 0.2 ml from the 2 ml of pyridine extract obtained by this procedure was used for counting (23). When necessary, 1.5 ml of pyridine extract was chromatographed on a Sephadex G25 medium column (Pharmacia). The column (2 by 45 cm) was equilibrated with 0.15 M ammonium acetate in 7% (vol/vol) aqueous propanol. The column was eluted with the same buffer at a rate of 17 ml/h. Fractions (1.5 ml) were collected, and radioactivity was counted.

Determination of enzyme activities. Enzymatic assays were performed on toluene-treated cells. Pectate lyase activity was determined by monitoring spectrophotometrically the formation of unsaturated products from polygalacturonate at 230 nm. The assay mixture consisted of 0.1 M Tris-HCl (pH 8.5), 0.1 mM CaCl₂, and polygalacturonate at 0.5 mg/ml. One unit of activity was defined as the amount of enzyme required to produce 1 μM unsaturated product per min (30). β-Galactosidase activity was determined in Z buffer (29).

Pathogenicity test. Potato tubers were inoculated as previously described (24). Sterile pipette tips containing a bacterial suspension of 10⁷ cells in 5 μl were inserted into the tuber parenchyma. After 24 to 72 h of incubation in a dew chamber, tubers were sliced vertically through the inoculation point, and the weight of decayed tissue was taken as the characteristic of disease severity. Moreover, in coinoculation tests containing both Kanʳ (Opg⁻) and Kanˢ (Opg⁺) bacteria, all the macerated tissues (ranging from 0.3 to 2.0 g) were collected, weighed, and diluted to a concentration of 1 g of macerated tissue in 2 ml of
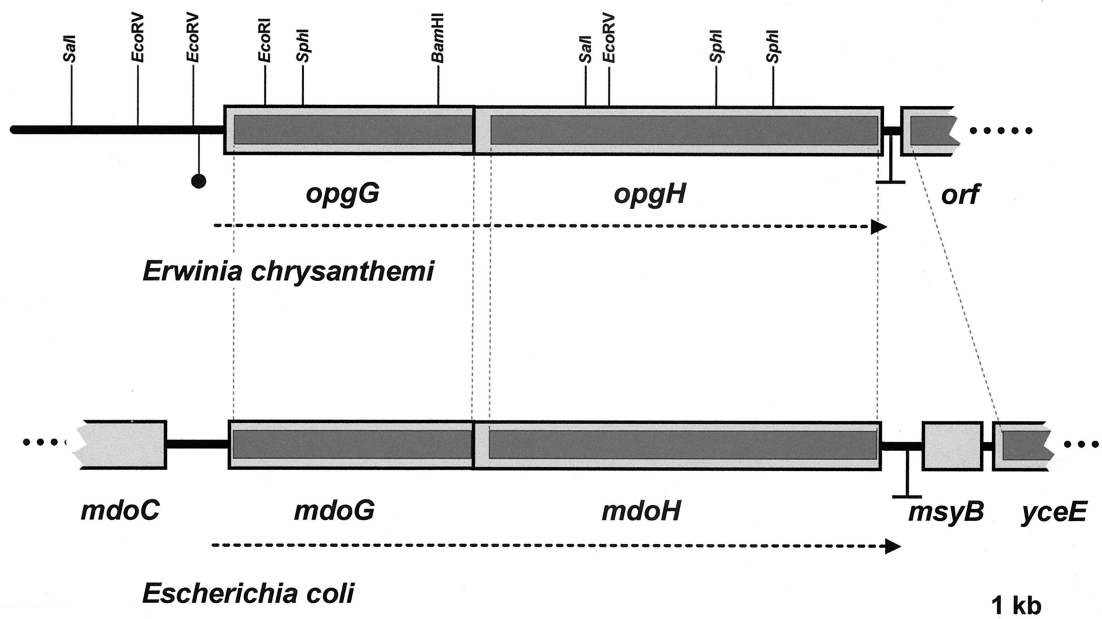
FIG. 1. Genetic structure of homologous regions of the chromosomes of *E. chrysanthemi* and *E. coli* in the vicinity of the *opgGH* and *mdoGH* operons. Boxes represent open reading frames. Dark boxes represent the regions where the nucleotide sequences are 75% identical (on average) between both organisms. A restriction map is given at the top of the *opgGH* region of *E. chrysanthemi*. Transcriptional terminators of the *opgGH* and *mdoGH* operons are indicated by an inverted T. The horizontal dotted arrows indicate the location and direction of transcription of the genes. The solid circle upstream from *opgG* indicates the location of 13-bp direct repeats (see text).

sterile physiological distilled water. The numbers of bacteria originating from each of the two strains were determined by spreading appropriate dilutions of the cell suspensions onto LB plates with or without kanamycin. Chicory leaves were slightly wounded prior to inoculation (10^6 bacteria). After 24 h of incubation in a dew chamber, the length of rotted tissue was measured to estimate disease severity. These tests were repeated in at least three independent experiments.

Nucleotide sequence accession number. Sequence data will appear in the EMBL/GenBank/DDBJ nucleotide sequence data libraries under accession number AJ294718.

RESULTS

Complementation of the *mdoH200*::Tn*10* mutation of *E. coli* with an RP4 derivative of *E. chrysanthemi*. Since plasmids harboring the *P. syringae hrpM* gene complemented the *E. coli mdoH200*::Tn*10* mutation, we postulated that the genes involved in OPG backbone synthesis in *E. chrysanthemi* may also complement such mutations. The absence of a selectable phenotype in *mdoGH* mutants of *E. coli* prevented direct selection for Opg$^+$ complementation. We decided to use the selectable marker *pyrC*, located 13 kb downstream from the *mdoGH* locus in *E. coli*. Despite some important genomic rearrangements, the genetic organization of several genomic regions is quite similar in *E. coli* and *E. chrysanthemi* (17).

Plasmid pULB110, a kanamycin-sensitive RP4::mini-Mu derivative (41), was used to generate R-prime derivatives containing an insert of bacterial DNA from *E. chrysanthemi* complementing the *pyrC* mutation of *E. coli* strain NFB223 (*pyrC mdoH200*::Tn*10*). Ura$^+$ (Pyr$^+$) transconjugants were isolated on M63 medium plates supplemented with streptomycin. The plasmid contents of 12 Pyr$^+$ transconjugants were analyzed. Four plasmids with a DNA insert of 15 to 44 kb were tested for the Opg phenotype that they conferred. One of them, pR24, complemented the *mdoH* mutation and conferred an Opg$^+$

phenotype. Moreover, the amount of OPGs produced by the *mdoH200*::Tn*10* strain harboring pR24 was 1.5 times higher than in the wild-type *E. coli* strain.

Cloning of *opgGH* locus of *E. chrysanthemi*. Plasmid pR24 was digested with *Eco*RI, and the resulting DNA fragments were ligated into the *Eco*RI site of pYZ4. Plasmid pNFW3, containing a 9.5-kb DNA insert, was Opg$^+$ (Fig. 1) and thus complemented the *mdoH200*::Tn*10* mutation of NFB216, but only at the wild-type level. The sequences of both ends of the insert were established. The region adjacent to the *lac* promoter present in the vector was highly similar to the beginning of the open reading frame of the *E. coli mdoG* gene (starting at amino acid number 72). Then, a truncated promoterless version of the *E. chrysanthemi* locus has been cloned, and complementation of the *mdoH200*::Tn*10* mutation was most probably the result of transcription from the *lac* promoter. A similar observation was made during the cloning of the *mdoGH* operon (23).

In order to restore a complete version of the operon, a 3.3-kb *Sal*I DNA fragment of pR24 overlapping the pNFW3 insert was detected by Southern blot using the 1.1-kb *Eco*RI-*Bam*HI fragment of the pNFW3 insert as a probe (Fig. 1) and cloned into pUC18. The resulting plasmid, pNFW27, introduced into NFB702 complemented the *mdoG202*::neo mutation. The reconstitution of the whole operon was done as follows. pNFW3 was digested with *Bam*HI and partially digested with *Eco*RV to obtain a 4.1-kb *Bam*HI-*Eco*RV DNA fragment (Fig. 1). This DNA fragment was ligated into pNFW27 digested with *Bam*HI and *Sma*I (using a site originating from the vector polylinker) to give pNFW32 containing a 6.4-kb DNA insert. Complementation of *mdoG202*::neo and *mdoH200*:: Tn*10* was observed when pNFW32 was introduced into strains

NFB702 and NFB216, respectively. Moreover, strains harboring pNFW32 contained an OPG level twice that of the wild type. The same increasing effect was observed when *E. coli mdoGH* was cloned into a multicopy plasmid (23). These results clearly indicated that the pNFW32 insert contains the entire *E. chrysanthemi* locus necessary for complementation of the *mdoGH* operon of *E. coli*.

Analysis of nucleotide sequence of *opgGH* genes of *E. chrysanthemi*. The 5.0 kb starting from the first *Eco*RV (Fig. 1) and encompassing the *opgGH* locus were sequenced in both strands. The analysis of this sequence confirmed the genetic analysis, and two open reading frames were detected. The first open reading frame, *opgG*, encoding a 522-amino-acid polypeptide, begins with a putative start codon (GTG) and ends at a TAA codon. The GTG start codon represents 11.5% (versus 86.5% for ATG) of the start codons found in the 88 sequences of *E. chrysanthemi* available in the EMBL sequence database (release 62, March 2000). The second open reading frame, *opgH*, encoding an 850-amino-acid polypeptide, begins with a putative start codon (ATG) and ends at a TGA codon. Moreover, the end of the *opgG* sequence overlaps the start of *opgH* (ATGAATAA). An identical sequence was observed in *E. coli*, leading to the same overlapping between *mdoG* and *mdoH* (25). The putative ribosome-binding site is located 8 nucleotides upstream from the GTG start codon of *opgG*. The −10 and −35 regions of the putative promoter are separated by 18 nucleotides, and the GTG start codon is located 32 nucleotides downstream from the putative transcriptional start site. In *E. coli*, the same promoter sequence was found except for one nucleotide in the −10 region. Thus, the two genes most probably form an operon functionally homologous to the *mdoGH* operon.

Comparison of the nucleotide sequences of *opgGH* (*E. chrysanthemi*) and *mdoGH* (*E. coli*) shows strong sequence alignments beginning just before the promoter and ending just after the stop codon of the second gene. Two 13-bp directly repeated sequences (TTAAGCGCGTCAATTAAGCGCGTCAA) are found upstream from the putative promoter of *opgGH*, and the nucleotide sequence found upstream from these direct repeats does not show similarity to any sequence present in the nucleotide sequence data libraries. Nineteen nucleotides downstream from the stop codon of *opgH* are found two inverted repeats of 11 nucleotides (AAGCAACGGCCAGTCCGTT GCTT), followed by a stretch of 5 T's characteristic of an intrinsic transcription terminator. However, the terminator sequences are divergent (Fig. 1). The *opgH* gene is followed by an open reading frame transcribed in the opposite orientation which shows similarity to the *E. coli yceE* gene of unknown function.

Alignment between the MdoG and OpgG proteins shows 75.5% identity (386 of 511) and 87.5% similarity (447 of 511) over the whole sequence, and alignment between MdoH and OpgH shows 74.7% identity (625 of 829) and 85.8% similarity (711 of 829). Similar results were previously observed between MdoH and HrpM of *P. syringae* (25). Comparison between OpgH and HrpM shows 68.1% (524 of 769) identity and 78.5% (623 of 769) similarity.

Construction of *opgG* and *opgH* mutants and localization of *opgGH* locus on chromosome of *E. chrysanthemi*. Both gene disruptions were obtained by insertion of a *uidA*-kan cassette into pNFW32 followed by transfer into the chromosome (see Materials and Methods) to give NFB3500 (*opgG500::uidA*-kan) and NFB3501 (*opgH501::uidA*-kan). Recombination of these constructions with the homologous locus into the chromosome was confirmed by Southern blot hybridization using the 3.3-kb *Sal*I DNA fragment of pNFW27 (data not shown).

OPG synthesis was measured in both mutant strains. OPGs were extracted from overnight cultures grown in LB without NaCl, and the extracts were loaded onto a Bio-gel P-4 column (see Materials and Methods). The peak containing OPGs, normally observed in extracts of the wild-type strain 3937 (9), was absent in both extracts, indicating that these mutants are affected in OPG backbone synthesis (data not shown). Chromosomal mobilization mediated by plasmid pULB110 was used for conjugation with various polyauxotrophic recipients to localize the *opgGH* locus on the *E. chrysanthemi* chromosome. Using the *opgG500::uidA*-kan fusion, the Kan^r marker cotransferred at 4% with *ade-377*, 10% with *ogl*, and 96% with *ura-2*. Thus, the *opgGH* locus is located very near the *ura-2* marker on the *E. chrysanthemi* chromosome (17). In addition, the auxotrophy of the *ura-2* mutant is complemented by the four *pyrC*^+ R-prime plasmids isolated above, strongly suggesting that *ura-2* is a mutation affecting the *pyrC* gene of *E. chrysanthemi*. Moreover, in a generalized transduction by phage φEC2, the Kan^r resistance of the *opgG500::uidA* mutant was cotransduced at 80% with *ura-2*.

Pleiotropic phenotypes of *opg* mutants of *E. chrysanthemi*. Colonies of the *opg* mutants have a mucoid aspect on minimal medium agar plates, a typical sign of increased exopolysaccharide (EPS) biosynthesis. We analyzed the effect of the *opgG* mutation on the transcription of the EPS biosynthetic operon of *E. chrysanthemi* using the *epsG::lacZ* fusion present in the *eps-29* mutant (A2575). The β-galactosidase activity was increased about threefold in the *opgG500::uidA*-kan derivative (data not shown) grown with either glycerol or glucose as the sole carbon source. Induction of transcription of the EPS genes was previously observed in *opg* mutants of *E. coli* (12) and *Agrobacterium tumefaciens* (8).

On semisolid agar plates, swarm diameters of *opgG* and *opgH* mutants and of wild-type strains were 0.50 ± 0.25 cm and 2.25 ± 0.25 cm, respectively (data not shown). Thus, even if we cannot exclude a defect in flagellum functioning, we consider that flagellum apparatus synthesis or assembly is most probably affected in *opg* mutants of *E. chrysanthemi*, since this was previously observed in *opg* mutants of *E. coli* (14) and *Agrobacterium tumefaciens* (8).

Since the *opg* mutations seemed to have a pleiotropic effect on several envelope components, we decide to test the bile salt sensitivity of the mutants. The MIC was 2.5 ± 0.3 g/liter for both *opg* mutants, while it was 18.3 ± 0.3 g/liter for the wild-type strain. Thus, the *opg* mutants were sevenfold more sensitive to bile salts than the wild-type strain, confirming that the envelope structure was severely affected in these mutants.

Exoenzyme synthesis and secretion in *opgG* mutant of *E. chrysanthemi*. Pectate lyase activity was first estimated on plates containing polygalacturonate and a second carbon source. No halo derived from polygalacturonate degradation was observed when the *opg* mutants were grown in the presence of glycerol, glucose, or sucrose. A reduced halo, compared with that of the wild-type strain, was observed when

TABLE 2. Expression of *pel* and *out* genes in the presence of an *opgG* mutation in *E. chrysanthemi*[a]

Enzyme	Fusion	Inducer	Sp act (nmol of product liberated/min/mg (dry wt) ± SD)	
			opgG+	*opgG*
Pectate lyase		−	0.05 ± 0.02	0.02 ± 0.01
		+	19.7 ± 3.2	8.4 ± 2.1
β-Galactosidase	*pelE-lacZ*	−	7 ± 3	5 ± 2
		+	132 ± 18	55 ± 9
	pelC-lacZ	−	5 ± 2	2 ± 2
		+	863 ± 82	432 ± 25
	outC-lacZ	−	87 ± 5	58 ± 6
		+	340 ± 20	156 ± 32

[a] Cultures were grown in minimal medium to late log phase in the presence of either glycerol (−) or the inducing compounds polygalacturonate and galacturonate (+). The results reported are averages of at least three independent experiments ± standard deviation. Pectate lyase specific activity reflects the expression of the five major *pel* genes (*pelA* to *pelE*). β-Galactosidase specific activity reflects the expression of the corresponding *lacZ* fusion.

succinate or galacturonate was added or in the absence of a second carbon source. Thus, pectate lyase activity appeared to be reduced at various levels in the *opg* mutants, depending on the carbon source used by the bacteria (data not shown).

Assay of the global pectate lyase activity demonstrated that production of these enzymes decreased by twofold in the presence of the *opgG* mutation in either the presence or absence of an inducer (Table 2). A similar decrease was observed when *pelC*::*lacZ* or *pelE*::*lacZ* transcriptional fusions were used to analyze the expression of these genes (Table 2).

We also tested the effect of the *opgG* mutation on the efficiency of the Out secretion machinery (Fig. 2). While more than 80% of the pectate lyase activity was found in the supernatant of the parental strain, only 30 to 50% of the pectate lyase activity was observed in the supernatant of the *opgG* and *opgH* mutants. Comparison with a nonsecretory *outC* mutant indicated that secretion is only partially affected by the *opg* mutations. When the transcription of the *out* operon was analyzed using an *outC*::*lacZ* transcriptional fusion (Table 2), a 30 to 50% decrease was observed in the presence of the *opgG* mutation.

Cellulase and protease activities were only estimated on plates. In the cellulase plate test, haloes derived from carboxymethylcellulose degradation by *opg* and *out* mutants were similar, showing severely reduced sizes compared to that of the wild-type strain (data not shown). In the protease plate test, no halo derived from milk protein degradation could be detected around the *opg* mutants (data not shown). This suggested that cellulase and protease secretions, like pectinase secretion, were affected in the *opg* mutants.

Pathogenicity of *opg* mutants of *E. chrysanthemi*. Inoculation with the *opgG* mutant incited no tissue maceration on potato tubers after 24 h, while an *outS* mutant produced reduced symptoms (Fig. 3A). Identical results were obtained when the *opgG* and *opgH* mutants were used to inoculate chicory leaves (Fig. 3B). Moreover, after a 10-day incubation, plants inoculated with *opg* mutants did not exhibit any symptoms. The *opg* mutants were thus completely nonvirulent.

Among the various phenotypic characters altered in the *opg* mutants, two are necessary for virulence: secretion of cell wall-

degrading enzymes (2) and motility, since a nonmotile *msrA* mutant strain exhibits reduced symptoms on chicory leaves (13). Coinoculation experiments were done in order to determine if the defect in virulence of the *opg* mutants could be complemented by wild-type bacteria present in the close environment. Potato tubers were inoculated with a mix of wild-type and mutant strains in various relative concentrations. Three different mutants were tested: *opgG*, *outS*, and *msrA*. The three different initial percentages of mutant bacteria were 10, 90, and 99%. After 24 to 72 h of incubation, depending on the wild-type strain concentration, wild-type and mutant cells present in the macerated tissue were counted (see Materials and Methods).

The percentages of *outS* and *msrA* mutant cells were similar in the inoculum and in the macerated tissue (Fig. 4). This indicates that growth occurred at a similar rate for the *outS*, *msrA*, and wild-type bacteria. Thus, intercellular complementation occurred even when the inoculum contained only 1% wild-type cells. The percentage of *opgG* mutant cells was reduced by a factor of 22 to 330 depending on the ratio of mutant cells in the inoculum (Fig. 4). Nevertheless, the viability of the *opg* cells was unaffected, because the number of bacteria was similar in the inoculum and in the macerated tissue. For example, when the inoculum contained 90% *opg* cells, the total macerated tissue contained an average of $1.2 \times 10^7 \pm 0.3 \times 10^7$ Opg− bacteria, while the inoculum contained 9×10^6 Opg− bacteria. Since the population would have increased only by a factor of 1.3 (in the range of error), one can consider that no growth of the mutant strain occurred in the plant tissues. During the same time, the wild-type population increased by a factor of 4,580. Thus, intercellular complementation did not occur between the *opgG* mutant and wild-type strains, indicating that the loss of virulence of *opgG* mutants did not result simply from motility or exoenzyme deficiencies.
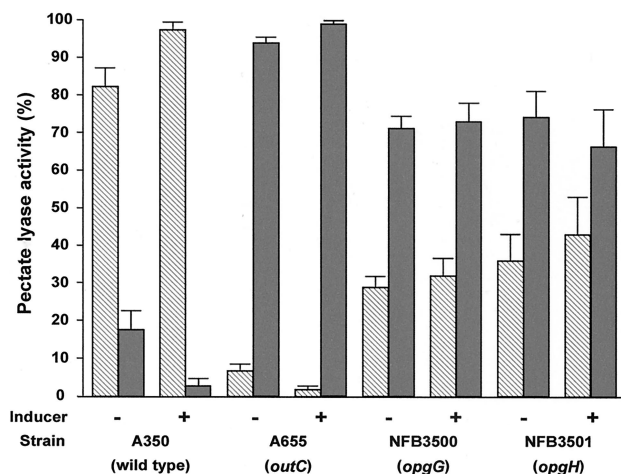

FIG. 2. Pectate lyase activity of *outC*, *opgG*, and *opgH* mutants compared to the wild-type strain. Cultures were grown in minimal medium to late log phase in the presence of either glycerol (−) or the inducing compounds polygalacturonate and galacturonate (+). Cells were harvested, and pectate lyase activities in the pellet (gray bars) and the culture supernatant (hatched bars) were determined (see Materials and Methods). The results reported are averages of three independent experiments. Pectate lyase activity reflects the expression of the five major *pel* genes (*pelA* to *pelE*).
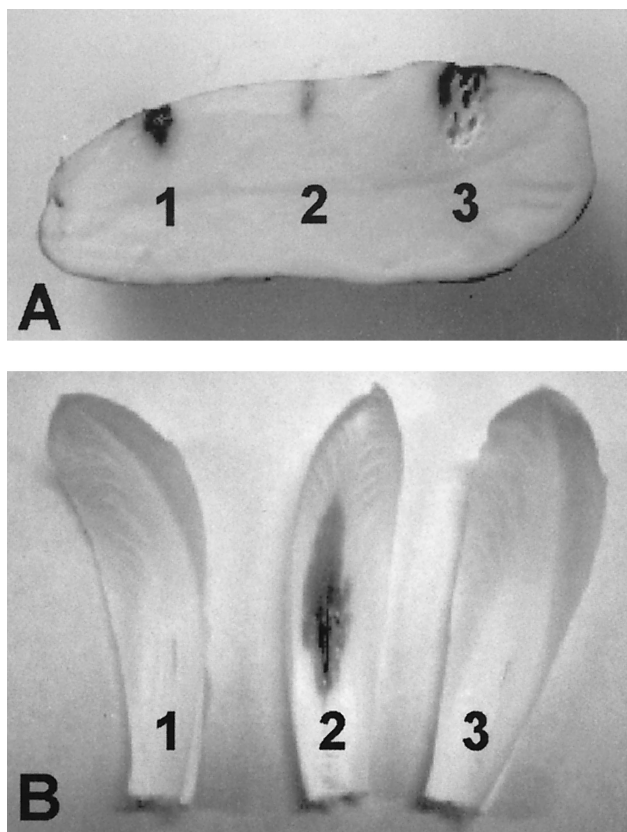
FIG. 3. Pathogenicity of *outS, opgG,* and *opgH* mutant strains on potato tubers and chicory leaves. (A) Bacteria (10^7) of the *outS* strain A3144 (spot 1), the *opgG* strain NFB3500 (spot 2), and the wild-type strain 3937 (spot 3) were inoculated into holes on potato tubers. Disease symptoms were observed after 72 h. (B) Bacteria (10^6) of the *opgG* strain NFB3500 (spot 1), the wild-type strain 3937 (spot 2), and the *opgH* strain NFB3501 (spot 3) were inoculated into scarified chicory leaves. Disease symptoms were observed after 48 h.

After plant infection, the *opg* mutant stopped its growth but remained viable.

In order to determine whether the wild-type strain could inhibit the growth of the *opgG* mutant, we performed coinoculation tests in M63 minimal liquid medium. In these conditions, both strains grew equally, and the mutant/wild-type ratio remained constant throughout the experiment, indicating that the wild-type strain did not inhibit growth of NFB3500 (data not shown).

In order to determine whether some potato tuber molecules could inhibit the growth of NFB3500, M63 minimal liquid medium was supplemented with potato tuber extracts (see Materials and Methods). The growth rate and the stationary-phase plateau were identical when various amounts of potato tuber extracts were present, indicating that potato tuber does not contain factors that inhibit the growth of the *opg* mutant (data not shown).

DISCUSSION

This paper describes the molecular organization of the *opgGH* operon from *E. chrysanthemi*, the phenotypes observed

when these genes are disrupted, and their implication in its pathogenicity.

As in *E. coli*, the two genes necessary for OPG backbone synthesis in *E. chrysanthemi* are organized in an operon, *opgGH*, in the vicinity of the *pyrC* gene. In both organisms, *yceE*, a gene of unknown function, is found downstream from *mdoH* or *opgH*. In *E. coli*, this gene is transcribed with *msyB*, which is only known to encode a suppressor of a *secY* thermo-sensitive mutation. Nevertheless, in *E. chrysanthemi, msyB* is not present at this locus (Fig. 1). The DNA sequences of the two organisms are conserved over the entire sequences of the *opgGH* and *mdoGH* operons, including the promoter region. In *E. coli*, the *mdoC* gene, involved in OPG succinylation, is located just upstream from the *mdoGH* operon (21). Despite the fact that the OPGs of *E. chrysanthemi* are succinylated (9), no gene homologous to *mdoC* was found upstream of the *opgGH* operon. The 13-bp direct repeats found upstream from *opgGH* could be the limit of a chromosomal rearrangement mediated by a transposable element. Moreover, the gene encoding the OPG succinylation activity could differ strongly from *mdoC*, since several attempts to obtain PCR amplification of that gene failed (data not shown).

The *opg* mutants exhibit a pleiotropic phenotype. Colonies of *opg* mutants are mucoid, indicating increased biosynthesis of EPS. This correlates with a threefold increase in the expression of the *epsG::lacZ* fusion in an *opgG* mutant background. This increase could be the result of a direct action of OPGs on *eps* gene transcription via a regulatory protein(s). Actually, in *E. coli*, the *cps* genes, involved in colanic acid capsular polysaccharide biosynthesis, are regulated by a two-component sensor-regulator system, RcsB/RcsC. In an *mdoH* mutant, *cps* gene transcription is increased but remains regulated in an RcsB/RcsC-dependent fashion. This suggests that RcsC, the sensor of the two-component system, could sense the level of OPG in the periplasm in a still unknown way (12). In *Erwinia amylovora* and *Erwinia stewartii*, the RcsB and RcsC proteins
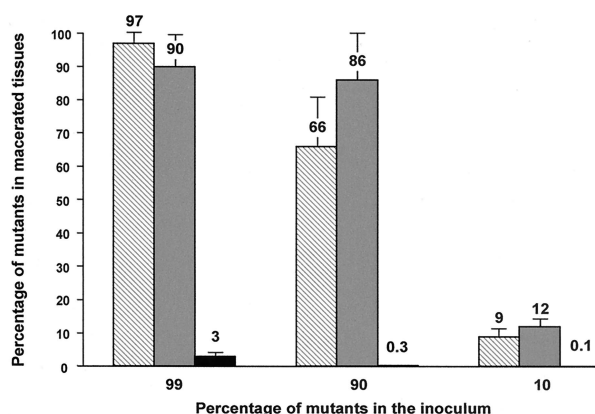

FIG. 4. Coinoculation of potato tubers with wild-type strain 3937 and *outS* strain A3144 (hatched bars), *msrA* strain RH7006 (gray bars), or *opgG* strain NFB3500 (black bars). Mixed bacteria (10^7) were inoculated into holes in potato tubers. After 72 h, macerated tissue was collected, weighed, and diluted in physiological distilled water. The percent mutant bacteria was determined by the ratio of the number of mutant bacteria (Kan[r]) to the number of total bacteria per gram of tissue. The results reported are averages of three independent experiments performed on at least two potato tubers.

are involved in the regulation of EPS biosynthesis and show strong similarities with the RcsB and RscC proteins of *E. coli*, indicating the existence of a family of related capsule activator proteins (4). Thus, in *E. chrysanthemi*, OPGs could also participate in EPS biosynthesis regulation by acting via a similar two-component regulatory system. Alternatively, this increase could be one of the responses resulting from a disorganization of the cell envelope induced by the lack of OPGs. Disorganization of the cell envelope is suggested by the other phenotypes of the *opg* mutants, particularly bile salt hypersensivity and defective chemotaxis. Lack of OPGs could result in abnormal assembly of several envelope components due to an unknown structural function of the glucans, or OPG concentration could be detected and used to regulate the synthesis of several envelope components, especially those which are subjected to osmotic regulation, like the flagellum apparatus (14) and colanic acid synthesis (12) in *E. coli*.

Among the various effects observed in *opg* mutants, the most important seems to be the reduction in pectate lyase production and secretion, because they are known to play a crucial role in virulence. *opg* mutations result in a twofold decrease in pectate lyase production associated with a similar decrease in *pel* gene transcription. OPGs could modulate *pel* gene expression via the two-component sensor-regulator system PecM/PecS (15) in a way which remains to be elucidated, but the existence of new regulatory factors cannot be excluded. The reduction in Pel protein synthesis is associated with a reduction in their secretion through the cell envelope. This defect in the Out secretory apparatus could be explained in part by a decrease in its synthesis and in part by an alteration in its assembly and/or functioning resulting from the cell envelope disorganization.

The *opg* mutants are completely nonvirulent when inoculated into potato tubers or chicory leaves. This phenotype has already been observed in planta with the *hrpM* mutant of *P. syringae* pv. syringae (28) and for the *hrpM* mutant of *P. aeruginosa* PA14 (26). The major consequence of *pel* and *out* mutations is the severely reduced virulence of strains harboring these mutations. However, the complete loss of virulence of the *opg* mutants cannot be explained only by a defect in pectate lyase secretion, since the *outS* mutant (severely impaired in pectate lyase secretion) induces reduced symptoms, while the *opg* mutant, which secretes more pectate lyases than the *outS* mutant, induces no symptoms.

The *outS* (altered secretion) and *msrA* (altered motility and peptide methionine sulfoxide reductase) mutant cells could be sustained by coinoculation with no more than 1% of the wild-type strain in potato tubers. These mutant strains grew poorly in planta and produced few macerated tissues by themselves, but they are able to use the macerated tissues produced by the wild-type strain for their growth. This clearly suggests that only the plant-bacterium interaction is disrupted in these mutants. On the other hand, the *opg* mutant cells cannot grow when coinoculated in potato tubers with as much as 90% wild-type cells, while growth remains unaffected when the coinoculation occurs in laboratory conditions. However, the *opg* mutants which are unable to use macerated tissues for their growth can survive in these conditions. These results indicate that OPGs are necessary not only for plant colonization, but also for bacterial multiplication in macerated tissues. When *E. chrysan-*

themi is grown in a liquid medium, a large fraction of the OPGs produced can be recovered in the medium, at least under certain circumstances (9). We have no indication whether this is true when bacteria grow in planta. However, our experiments clearly show that if OPGs are released in the plant tissues during co-inoculation with wild-type cells, these external OPGs cannot complement the growth defect of the *opg* mutants. Moreover, OPG-defective mutants of *A. tumefaciens* remain nonvirulent when inoculated in planta with purified OPGs (8). This strongly suggests that OPGs need to be inside the periplasmic space and not in the external environment during the infection process.

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