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## The Oligogalacturonate-specific Porin KdgM of *Erwinia chrysanthemi* Belongs to a New Porin Family\*

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The phytopathogenic Gram-negative bacteria *Erwinia chrysanthemi* secretes pectinases, which are able to degrade the pectic polymers of plant cell walls, and uses the degradation products as a carbon source for growth. We characterized a major outer membrane protein, KdgM, whose synthesis is strongly induced in the presence of pectic derivatives. The corresponding gene was characterized. Analysis of transcriptional fusions showed that the *kdgM* expression is controlled by the general repressor of pectinolytic genes, KdgR, by the repressor of hexuronate catabolism genes, ExuR, by the pectinase gene repressor, PecS, and by catabolite repression via the cyclic AMP receptor protein (CRP) transcriptional activator. A *kdgM* mutant is unable to grow on oligogalacturonides longer than trimers, and its virulence is affected. Electrophysiological experiments with planar lipid bilayers showed that KdgM behaves like a voltage-dependent porin that is slightly selective for anions and that exhibits fast block in the presence of trigalacturonate. In contrast to most porins, KdgM seems to be monomeric. KdgM has no homology with currently known porins, but proteins similar to KdgM are present in several bacteria. Therefore, these proteins might constitute a new family of porin channels.

The phytopathogenicity of the pectinolytic *Erwinia* species, reclassified *Pectobacterium* (1), is mainly due to their ability to macerate the major component of plant cell walls, pectin. Pectin consists of  $\alpha$ -1,4-linked galacturonate residues (PGA)<sup>1</sup> with some methyl-esterified and acetyl-esterified residues. *Erwinia chrysanthemi* (synonym *Pectobacterium chrysanthemi*), which causes soft-rot disease of various plants, is able to use pectin as a carbon source for growth. Pectin catabolism involves a variety of pectinases, including esterases and depolymerases (Fig. 1), among which endo-pectate lyases play a crucial role in the

soft-rot disease (2, 3). Pectinases are secreted in the extracellular medium by a type II secretion pathway, the Out system (4, 5). The end products of pectin degradation by these extracellular pectinases are mainly dimers to tetramers of galacturonides (GA<sub>2</sub> to GA<sub>4</sub>) with some longer oligomers. Most oligogalacturonides (GA<sub>n</sub>) resulting from a lyase activity have a 4,5-unsaturated bond at their non-reducing end (6, 7).

Pectin degradation products are known to enter the *E. chrysanthemi* cells (8, 9) (Fig. 1). Enzymes able to cleave GA<sub>n</sub> exist inside the bacteria. GA<sub>4</sub> is the best substrate for the periplasmic exo-pectate lyase PelX, but this enzyme is also able to degrade longer oligomers (10). GA<sub>3</sub> and GA<sub>4</sub> are good substrates for the cytoplasmic pectate lyase PelW, and GA<sub>2</sub> is the preferential substrate for the cytoplasmic oligogalacturonate lyase Ogl (11). After being internalized into the cytoplasm, GA<sub>n</sub> are catabolized by PelW and Ogl into monomers of GA and 5-keto-4-deoxyuronate, which are next degraded into 2-keto-3-deoxygluconate and then into pyruvate and 3-phosphoglyceraldehyde, products that finally enter the general cellular metabolism (2, 11). Recently, two transport systems allowing GA<sub>n</sub> translocation through the inner membrane, TogMNAB and TogT, have been characterized (12, 13). TogMNAB is a member of the carbohydrate uptake transporter-1 family, included in the ATP-binding cassette (ABC) transporter superfamily. TogT is a member of the glycoside-pentoside-hexuronide transporter family. The simultaneous inactivation of both transporters is required to prevent growth with pectin as the sole carbon source (13).

The transport of GA<sub>n</sub> through the outer membrane is the only step of the pectin catabolism pathway that remains unknown. In Gram-negative bacteria, carbohydrates enter the periplasm by passing through porins. Porins form water-filled channels that permit the diffusion of hydrophilic solutes across the outer membrane. They are divided in two classes as follows: (i) the non-specific porins of the general bacterial porin family, such as OmpF and OmpC of *Escherichia coli*, permit the diffusion of molecules below about 600 Da and are responsible for the exclusion limit of the outer membrane (14, 15); (ii) the substrate-specific porins can facilitate the diffusion of specific substrates. Complex sugars can enter the cell by specific porins, like the maltoporin LamB of *E. coli* or *Salmonella typhimurium* which is specific for maltose and maltodextrin uptake (16). In this paper, we describe the identification of a new porin, KdgM, involved in GA<sub>n</sub> translocation across the outer membrane of *E. chrysanthemi*.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Culture Conditions**—Bacterial strains and plasmids used in this work are described in Table I. *E. chrysanthemi* and *E. coli* cells were grown at 30 and 37 °C, respectively, in LB or M63 medium (25) supplemented with a carbon source at 2 g l<sup>-1</sup>

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<sup>1</sup> The abbreviations used are: PGA, polygalacturonate; GA, galacturonate; GA<sub>2</sub>, digalacturonate; GA<sub>3</sub>, trigalacturonate; GA<sub>4</sub>, tetragalacturonate; GA<sub>n</sub>, oligogalacturonides; ABC, ATP-binding cassette; Km, kanamycin; Ap, ampicillin; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tricine, N-tris(hydroxymethyl)methylglycine; CRP, cyclic AMP receptor protein.

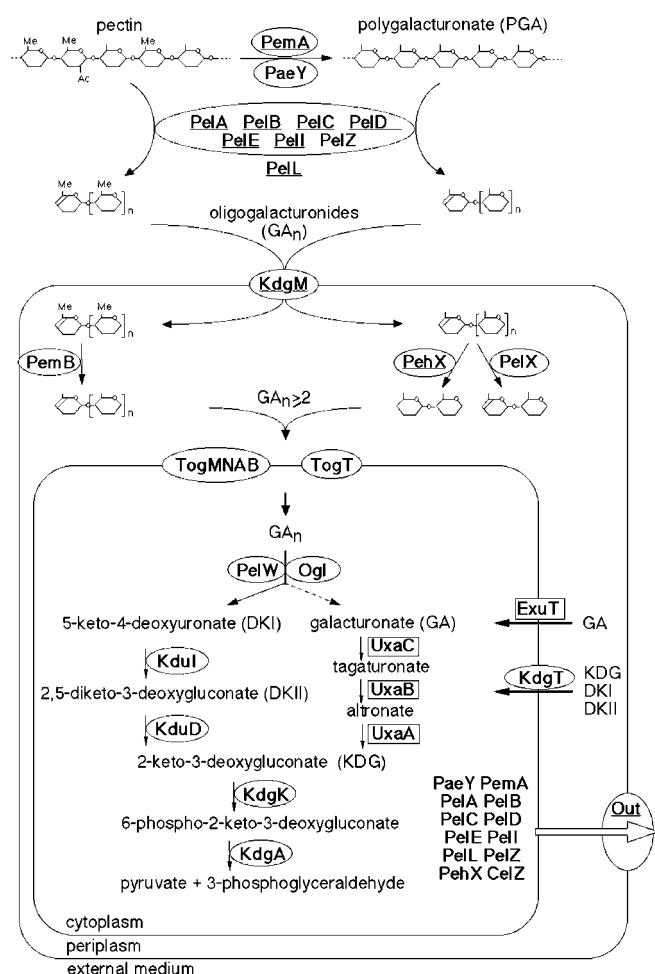


FIG. 1. **Pectin catabolism in *E. chrysanthemi*.** Extracellular pectinases (*Pae*, pectin acetyltransferase; *Pem*, pectin methyltransferase; *Pel*, pectate lyase; and *Peh*, polygalacturonase) degrade the pectin and the polygalacturonate (PGA) into oligogalacturonides ( $GA_n$ ) which then enter the cell.  $GA_n$  are degraded by the periplasmic pectinases and pass through the inner membrane via one of the two transport systems, *TogMNAB* or *TogT*.  $GA_n$  are then catabolized in the cytoplasm, producing pyruvate and 3-phosphoglyceraldehyde which integrate the general cellular metabolism. Proteins whose biosynthesis is controlled by *KdgR*, *PecS*, and *ExuR* are in ovals, underlined, and boxed, respectively.

for glycerol and glucose,  $1 \text{ g} \cdot \text{l}^{-1}$  for GA,  $GA_2$ , and  $GA_3$ , and  $4 \text{ g} \cdot \text{l}^{-1}$  for PGA and pectin.  $0.1 \text{ mM CaCl}_2$  was added with PGA and pectin. PGA (grade II), pectin, GA, and saturated  $GA_2$  and  $GA_3$  were from Sigma. The mixture of oligogalacturonides, containing mainly unsaturated trimers to pentamers with longer oligomers, was obtained by digestion of PGA with the *E. chrysanthemi* pectate lyase *PelL*, according to Roy *et al.* (7). When required, antibiotics were added at the following concentrations: kanamycin (Km)  $50 \text{ mg} \cdot \text{l}^{-1}$ , ampicillin (Ap)  $50 \text{ mg} \cdot \text{l}^{-1}$ , and chloramphenicol  $20 \text{ mg} \cdot \text{l}^{-1}$ . For growth in conditions of oxygen limitation, fumarate was added at  $2 \text{ g} \cdot \text{l}^{-1}$ , and the culture was covered with paraffin oil. Media with high and low osmolarity were obtained by addition of  $0.3 \text{ M NaCl}$  and by 4-fold dilution of M63 medium, respectively.

**Purification of KdgM and Preparation of KdgM Antiserum**—KdgM was extracted from the *E. chrysanthemi kdgR* strain A837 after growth at  $30^\circ\text{C}$  in LB to an absorbance of 0.8 at 600 nm. The cells were harvested, disrupted in a French press in  $50 \text{ mM Tris-HCl}$ , pH 8.0,  $5 \text{ mM EDTA}$ . Unbroken cells were removed by centrifugation at  $10,000 \times g$  for 10 min, and the crude cell membrane fraction was sedimented by centrifugation at  $100,000 \times g$  for 2 h. The membranes were resuspended in  $50 \text{ mM Tris-HCl}$ , pH 8.0,  $2\%$  Triton X-100,  $5 \text{ mM MgCl}_2$ , incubated for 30 min with gentle agitation at room temperature, and centrifuged at  $100,000 \times g$  for 2 h. The supernatant was loaded onto a 15% preparative Tris glycine SDS-PAGE. After electrophoresis, the band corresponding to KdgM was cut out and crushed, and proteins were extracted by overnight incubation at  $4^\circ\text{C}$  with  $30 \text{ mM Tris-HCl}$ ,

pH 8.0,  $0.3\%$  SDS. The protein extract was used to immunize a rabbit to obtain the KdgM antiserum.

KdgM was also prepared from the recombinant *E. coli* strain PHL646/pKM2. Cells were grown at  $30^\circ\text{C}$  in LB with Ap to an absorbance of 1.0 at 600 nm. Isopropyl- $\beta$ -D-thiogalactopyranoside was added to  $1 \text{ mM}$ , and the culture was grown at  $30^\circ\text{C}$  for 4 h. The crude cell membrane fraction was prepared as above. The membranes were resuspended in  $10 \text{ mM Tris-HCl}$ , pH 8.0,  $0.5\%$  *N*-lauroylsarcosyl (Sigma), incubated for 1 h with gentle agitation at room temperature, and centrifuged at  $100,000 \times g$  for 2 h. The non-soluble fraction was resuspended in  $10 \text{ mM Tris-HCl}$ , pH 8.0,  $0.7\%$  *n*-octyl- $\beta$ -D-glucoside, incubated for 1 h with gentle agitation at room temperature, and centrifuged at  $100,000 \times g$  for 2 h. The same treatment was performed with the resulting non-soluble fraction resuspended in  $10 \text{ mM Tris-HCl}$ , pH 8.0,  $0.5\%$  SDS. Supernatant was loaded onto a  $20 \times 20\text{-cm}$  10% Tris-Tricine SDS-PAGE. After electrophoresis, the band containing KdgM was cut out and crushed, and proteins were extracted by overnight incubation at  $4^\circ\text{C}$  with  $10 \text{ mM Tris-HCl}$ , pH 8.0,  $0.5\%$  SDS. KdgM was further purified by extraction after migration from a  $20 \times 20\text{-cm}$  15% Tris glycine SDS-PAGE. These extracts were used for electrophysiological experiments.

**Gel Electrophoresis and Immunoblotting**—Tris glycine SDS-PAGE was usually performed according to Laemmli (26) with 15 and  $0.4\%$  of acrylamide and bisacrylamide, respectively. Protocols for electrophoresis, protein staining with Coomassie G-250, semi-dry transfer onto nitrocellulose membrane, Western blotting, and immunological screening of colonies with KdgM antiserum were performed according to Sambrook *et al.* (27). Tris-Tricine SDS-PAGE was adapted from Schägger and von Jagow (28) using 10 and  $0.27\%$  acrylamide and bisacrylamide, respectively. For immunodetection, the KdgM antiserum was diluted 1:5000 and purified by incubation for 1 h, at room temperature, with NM522 and A3573 cellular lysates. The secondary antibody, an anti-rabbit immunoglobulin G peroxidase conjugate (Sigma), was revealed with an ECL kit (Amersham Biosciences) according to the manufacturer's protocol.

**Subcellular Fractionation**—Subcellular fractionation was performed using *E. chrysanthemi kdgR* cells grown to early stationary phase after disruption with a French press. Membrane fractionation was carried out by sucrose density gradient centrifugation in a flotation gradient (29). Fractions collected from the bottom of the gradient were assayed according to Osborn *et al.* (30).

**N-terminal Amino Acid Sequence Analysis**—A crude cell membrane fraction of the *E. chrysanthemi kdgR* strain A837 was prepared as described above, separated by Tris glycine SDS-PAGE, and electroblotted onto a polyvinylidene difluoride membrane in CAPS buffer according to Matsudaira (31). The membrane was stained with  $0.5\%$  Ponceau S, and the KdgM band was cut out and subjected to N-terminal analysis by Edman degradation at Institut de Biologie et Chimie des Protéines (Lyon, France).

**Cross-linking**—*E. chrysanthemi* strains were grown at  $30^\circ\text{C}$  in LB and LB + PGA to an absorbance of 1.0 at 600 nm. Cells were washed with  $10 \text{ mM phosphate buffer}$ , pH 7.0, and incubated in  $10 \text{ mM phosphate buffer}$ , pH 7.0,  $1\%$  formaldehyde for 5 or 30 min at room temperature. After a second washing, extracts were either incubated for 10 min at  $30^\circ\text{C}$  or boiled for 20 min, separated by Tris glycine SDS-PAGE, and submitted to immunodetection. The KdgR protein was used as a control of dimerization (32, 33).

**Reconstitution of KdgM in Liposomes**—The purified protein KdgM ( $500 \text{ ng}$ ) was added to  $2 \text{ ml}$  of  $500 \text{ mM KCl}$ ,  $10 \text{ mM Hepes-KOH}$ , pH 7.4, and  $33 \text{ mM n-octyl-}\beta$ -D-glucoside buffer containing  $1 \text{ mg}$  of sonicated lipids (asolectin from soybean, type IV-S). After incubation for 15 min at room temperature,  $160 \text{ mg}$  wet weight of Bio-Beads SM-2 (Bio-Rad) were added to the suspension to remove the detergent. Incubation was carried out for 4 h at room temperature. The Bio Beads were discarded, and the suspension was centrifuged for 30 min at  $344,000 \times g$  at  $4^\circ\text{C}$ . The proteoliposomes were resuspended in  $0.1 \text{ ml}$  of  $500 \text{ mM KCl}$ ,  $10 \text{ mM Hepes-KOH}$ , pH 7.4.

**Electrophysiological Recording**—Bilayers were formed from a solution of asolectin lipids dissolved in *n*-decane ( $30 \text{ mg} \cdot \text{ml}^{-1}$ ) across a  $250\text{-}\mu\text{m}$  diameter hole. Proteoliposomes ( $3 \text{ ng}$  of protein per ml, final concentration) were added to the *cis* compartment. Fusion was induced by imposing a salt gradient between the two chambers as follows:  $800 \text{ mM KCl}$ ,  $10 \text{ mM Hepes-KOH}$ , pH 7.4, in the *cis* compartment versus  $100 \text{ mM KCl}$ ,  $10 \text{ mM Hepes-KOH}$ , pH 7.4, in the *trans* compartment. The bilayer set up was connected to the external circuit through salt bridges with Ag/AgCl electrodes. Unitary currents were recorded using an Axon 200B patch clamp amplifier and stored on digital audio tape (Biologic DTR 1200 recorder). Recordings were filtered at  $1 \text{ kHz}$  through a 4-pole

TABLE I  
Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype	Origin
<i>E. chrysanthemi</i> 3937 derivatives		
A71 (L37)	<i>lmr</i> <sup>Tc</sup> <i>lacZ exuR</i>	17
A350 (L2)	<i>lmr</i> <sup>Tc</sup> <i>lacZ</i>	17
A576	<i>lmr</i> <sup>Tc</sup> <i>lacZ argG kdgK</i>	18
A837	<i>lmr</i> <sup>Tc</sup> <i>lacZ kdgR</i>	19
A1077	<i>lmr</i> <sup>Tc</sup> <i>lacZ kdgR::Cm</i>	19
A1524	<i>lmr</i> <sup>Tc</sup> <i>lacZ pecS::Cm</i>	20
A1851	<i>lmr</i> <sup>Tc</sup> <i>lacZ outD::Cm</i>	Laboratory collection
A2174	<i>lmr</i> <sup>Tc</sup> <i>lacZ pecT::Cm</i>	21
A2507	<i>lmr</i> <sup>Tc</sup> <i>lacZ crp::Cm</i>	22
A3009	<i>lmr</i> <sup>Tc</sup> <i>lacZ expR::Cm</i>	23
A3573	<i>lmr</i> <sup>Tc</sup> <i>lacZ kdgM::uidA-Km</i>	This work
A3576	<i>lmr</i> <sup>Tc</sup> <i>lacZ kdgR::Cm kdgM::uidA-Km</i>	This work
A3577	<i>lmr</i> <sup>Tc</sup> <i>lacZ outD::Cm kdgM::uidA-Km</i>	This work
A3724	<i>lacZ exuR kdgM::uidA-Km</i>	This work
A3725	<i>lmr</i> <sup>Tc</sup> <i>lacZ pecS::Cm kdgM::uidA-Km</i>	This work
A3726	<i>lmr</i> <sup>Tc</sup> <i>lacZ pecT::Cm kdgM::uidA-Km</i>	This work
A3727	<i>lmr</i> <sup>Tc</sup> <i>lacZ expR::Cm kdgM::uidA-Km</i>	This work
A3728	<i>lmr</i> <sup>Tc</sup> <i>lacZ crp::Cm kdgM::uidA-Km</i>	This work
A4040	<i>lmr</i> <sup>Tc</sup> <i>lacZ kdgR::Cm pecS::Ap kdgM::uidA-Km</i>	This work
A4041	<i>lmr</i> <sup>Tc</sup> <i>lacZ kdgR::Cm exuR::Ap kdgM::uidA-Km</i>	This work
Other <i>Erwinia</i> strains		
EC16	<i>E. chrysanthemi</i>	Laboratory collection
1891	<i>E. chrysanthemi</i>	Laboratory collection
SCRI449	<i>E. amylovora</i>	Laboratory collection
SCRI31	<i>E. carotovora</i> subsp. <i>atroseptica</i>	Laboratory collection
SCRI52-476	<i>E. carotovora</i> subsp. <i>betavasculorum</i>	Laboratory collection
SCRI193	<i>E. carotovora</i> subsp. <i>carotovora</i>	Laboratory collection
SCRI56-487	<i>E. carotovora</i> subsp. <i>odorifera</i>	Laboratory collection
SCRI51-440	<i>E. cypripedii</i>	Laboratory collection
<i>E. coli</i> strains		
MC4100	<i>araD139 Δ(argF-lac)U169 rspL150 relA1 flbB5301 deoC1 ptsF25 rsbR</i>	Laboratory collection
PHL646	MC4100 <i>ompR::Tn10</i>	Laboratory collection
NM522	<i>supE, thi-1 Δ(lac-proAB) Δ(hsdSM-mcrB)5 (r<sub>k</sub><sup>-</sup>, m<sub>k</sub><sup>-</sup>) [F' proAB lacIqZΔM15]</i>	Laboratory collection
Plasmids		
pROU2	<i>ogl, kdgR, kduI, kduD, pelW, togMNAB, kdgM... on a 30-kb fragment in pULB113, Ap<sup>R</sup>, Tc<sup>R</sup>, Km<sup>R</sup></i>	24
pUC18	Ap <sup>R</sup>	Stratagene
pKM	<i>kdgM</i> on a 7.5-kb <i>Sau3A</i> fragment in pUC18	This work
pKM2	<i>Bss</i> HII- <i>Sal</i> I deletion of pKM	This work

bessel filter and digitized off line at 2 kHz. The membrane potential refers to the potential of the *cis* side minus the potential of the *trans* side.

**Plate Tests and Enzyme Assays**—Plate assays for detection of secreted enzymes were performed as described previously (34). Pectate lyase activity was determined by monitoring the appearance of unsaturated oligomers at 230 nm (35). The  $\beta$ -glucuronidase activity was measured by monitoring the cleavage of *p*-nitrophenyl- $\beta$ -D-glucuronide at 405 nm (36). The alginate lyase activity was determined by monitoring the appearance of unsaturated oligomers at 230 nm according to Preiss and Ashwell (37). The assays were performed on toluenized extracts of cells grown to early stationary phase.

**Pathogenicity Test**—Chicory leaves were slightly wounded with a pipette tip prior to inoculation. At least 15 leaves were infected using 10<sup>6</sup> bacteria per inoculation site. After incubation in a dew chamber for 24 h at 30 °C, the length of rotted tissue was measured.

**Molecular Biology and Genetic Techniques**—Transduction with the generalized transducing phage phi-EC2 was performed according to Résibois *et al.* (38). Preparation of plasmid or chromosomal DNA or RNA, restriction digestions, ligations, transformations, and DNA or RNA electrophoresis were all carried out as described previously (27). DNA purification kits were from Qiagen (QIAquick Gel Extraction Kit) and Prolabo (DNA Purification Kit). Cloning of *kdgM* gene was performed by screening an *E. chrysanthemi* gene library constructed in pUC18 (23). Sequencing was performed by Genome Express SA (Grenoble, France). The *kdgM* nucleotide sequence accession number is AJ320226 in the EMBL, GenBank<sup>TM</sup>, and DDBJ nucleotide sequence data bases.

The *kdgM::uidA* transcriptional fusion was constructed by introduction of the *uidA*-Km cassette, which carries the  $\beta$ -glucuronidase gene, into the *PinAI* site of the *kdgM* coding region. The fusion was introduced into the *E. chrysanthemi* chromosome by marker exchange re-

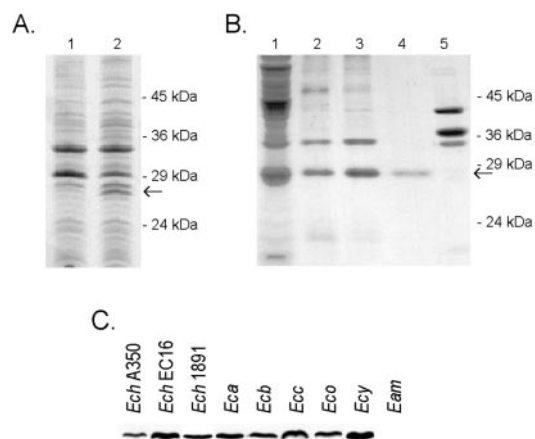
combination after growth in low phosphate concentration medium containing Km, as described by Roeder and Collmer (39).

Total RNA was extracted from the *E. coli* strain NM522/pROU2 (24). Cells were grown at 30 °C in LB, with Ap and GA, to an absorbance of 0.8 at 600 nm. pROU2 RNA was used as the matrix for primer extension experiments (AMV-RT Primer Extension Kit from Stratagene). The primer GCATTGACGCTAACAGAGATGCAACAGCC located 20 nucleotides downstream from the initiation codon was radiolabeled with [ $\gamma$ -<sup>32</sup>P]dATP.

**Band Shift Experiments**—The *kdgM* regulatory region was amplified by PCR using the primers CTTCCAGCGTCAAGCTTACCGTATTCTG-AAGCGCG and CCAGAGCATGACGCTATCTAGAGATGAACAGCC that carry sequence modifications (underlined letters) to include *Hind*-III and *Xba*I restriction sites. The 356-nucleotide fragment was digested with *Hind*III, labeled by incorporating [ $\gamma$ -<sup>32</sup>P]dATP (3000 Ci·mmol<sup>-1</sup>) with the Klenow fragment of DNA polymerase, and purified using the QIAquick gel extraction kit (Qiagen). Band shift assays were performed with the KdgR-, CRP-, and PecS-purified proteins (32, 33, 40). The labeled DNA fragment (50,000 cpm) and 5–100 nM of the purified regulator were incubated for 30 min at 30 °C in 20  $\mu$ l of binding buffer, and the reaction mixtures were submitted to electrophoresis on a 4% non-denaturing polyacrylamide gel, as described previously (33, 40). Bands were detected by autoradiography.

## RESULTS

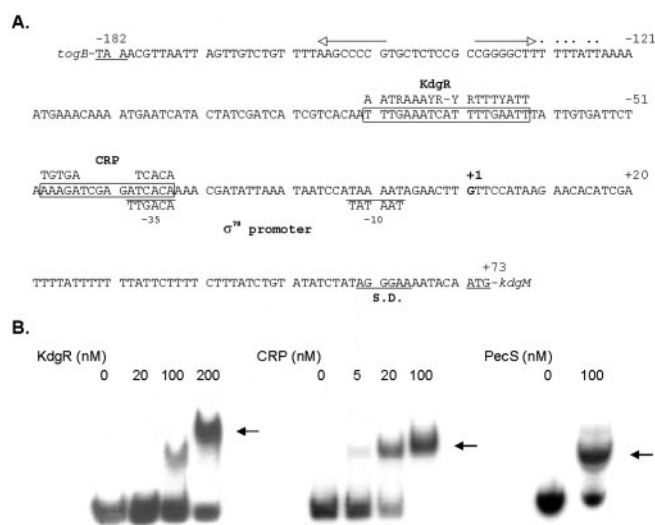
**KdgM Is a Major Outer Membrane Protein of Pectinolytic *Erwinia***—The KdgR repressor controls the synthesis of all the genes of the pectic degradation pathway (41). Analysis by Tris-Tricine SDS-PAGE of the membrane proteins of wild type and *kdgR* strains of *E. chrysanthemi* showed a protein strongly



**FIG. 2. Observation and purification of the KdgM protein.** A, Tris-Tricine SDS-PAGE of the membrane proteins of *E. chrysanthemi*. Lane 1, wild type strain A350. Lane 2, *kdgR* strain A837. KdgM is indicated by an arrow. B, Tris glycine SDS-PAGE of various extracts during the purification of KdgM. The *E. coli ompR* strain carrying the pKM2 plasmid was used. This mutant was used to decrease the content of general OmpF and OmpC porins. Lane 1, total membrane fraction. Lane 2, non-soluble fraction after *N*-lauroylsarcosine extraction. Lane 3, soluble fraction after SDS solubilization. Lane 4, KdgM purified after the two successive SDS-PAGE excisions. Lane 5, the same treatments were performed using the MC4100 *E. coli* strain as a control. C, immunodetection of KdgM homologues in cellular extracts of various pectinolytic *Erwinia* wild type strains as follows: *E. carotovora* subsp. *atroseptica* (Eca), *E. carotovora* subsp. *betavascularum* (Ecb), *E. carotovora* subsp. *carotovora* (Ecc), *E. carotovora* subsp. *odorifera* (Eco), *E. cypripedii* (Ecy), and non-pectinolytic *E. amylovora* (Eam).

expressed in the *kdgR* mutant (Fig. 2A). Synthesis of this protein, named KdgM, is also strongly induced when bacteria are grown in the presence of pectin or pectin derivatives, such as PGA or GA (data not shown). To localize KdgM more precisely, outer and inner membranes of the *kdgR* and the wild type strains were separated by centrifugation on a sucrose density gradient. KdgM was observed only in the outer membrane protein fraction (data not shown). A KdgM antiserum was obtained and used with extracts of various bacteria. A cross-reacting protein was detected in some pectinolytic *Erwiniae*, such as *Erwinia cypripedii* or *Erwinia carotovora* subspecies, but not in the non-pectinolytic *Erwinia amylovora* (Fig. 2C).

**Identification and Analysis of the *kdgM* Gene**—Screening of an *E. chrysanthemi* gene library with the KdgM antiserum allowed for the isolation of a plasmid, pKM, which carries a 10-kb DNA fragment encoding KdgM. The region containing the *kdgM* gene was reduced to a 1.3-kb *Bss*HIII-*Eco*47III fragment in plasmid pKM2. The nucleotide sequence of the *Bss*HIII-*Eco*47III DNA fragment was determined. A single complete open reading frame was identified. It encodes a 236-amino acid protein with a calculated molecular mass of 26,726 Da. A potential Shine-Dalgarno sequence (AGGGAA) lies 7 nucleotides upstream from the initiation codon (Fig. 3A). The *kdgM* transcription start was determined, by primer extension experiments (data not shown), to be 70 nucleotides upstream from the initiation codon. It is preceded by a  $-10$  Pribnow box (TAAAAT) and a  $-35$  region (ATCACA) that have 5/6 and 4/6 nucleotides of the  $\sigma^{70}$  promoter consensus. Farther upstream is located a short GC-rich inverted repeat, with a calculated free energy of formation of  $-67 \text{ kJ}\cdot\text{mol}^{-1}$ , followed by a run of T residues, which is typical for a rho-independent transcription termination site (Fig. 3A). Analysis of the DNA sequences situated on each side of *kdgM* enabled us to localize this gene



**FIG. 3. Binding of the KdgR, CRP, and PecS regulators to the promoter region of the *kdgM* gene.** A, sequence of the *togB-kdgM* intergenic region. The underlined sequences correspond to the *togB* translation stop codon, the *kdgM* start codon, the Shine Dalgarno sequence (S.D.) and to the  $-10$  and  $-35$  promoter sites. The transcription start is in boldface (position +1). The arrows show the inverted repeat of the potential *togB* rho-independent transcription terminator. Potential KdgR- and CRP-binding sites are boxed. The consensus of these binding sites and that of  $\sigma^{70}$  promoter are given above or below the sequence ( $R = G$  or  $A$  and  $Y = C$  or  $T$ ). B, gel shift assays with the purified KdgR, CRP, and PecS regulators. The amounts of purified protein used are indicated. The arrows indicate the DNA-protein complexes.

downstream from the *pelW-togMNAB* operon (12) and upstream from the *paeX* gene (Fig. 4), encoding an esterase.<sup>2</sup>

The presence of a signal sequence, suggested by a N-terminal hydrophobic region of 20 amino acids followed by the sequence Ala-Leu-Ala, which is a potential cleavage site for the LepB peptidase (5), was confirmed by the N-terminal sequencing of the protein purified from *E. chrysanthemi* membranes. KdgM C-terminal primary structure, with its Phe terminal residue, is characteristic of outer membrane proteins (42). The mature KdgM protein has a calculated molecular mass of 24,688 Da. The KdgM sequence was compared with other amino acid sequences deposited in the data banks and against translated complete and incomplete bacterial genomes. The best homologues are two *Yersinia pestis* proteins, which we named KdgM and KdgN, both with 64% identity to KdgM (Table II). *E. carotovora*, *Klebsiella pneumoniae*, *Pseudomonas syringae* pv. *tomato*, *S. typhimurium*, *Salmonella paratyphimurium*, *Vibrio haliotocoli*, and *E. coli* possess proteins that have less similarity with KdgM (Table II). Among these proteins only one has been assigned a putative activity, the alginate lyase AlyVGIII from *V. haliotocoli*.<sup>3</sup> The YshA proteins from *S. typhimurium* and *E. coli* present no strong homology with KdgM but seem to belong to the same family because they present significant homology with other proteins of this family.

**Genetic Organization of *kdgM* Homologues**—*kdgM* is localized in a pathogenicity island of the *E. chrysanthemi* chromosome that contains several genes involved in pectin catabolism (Fig. 4) (24, 43). The localization of the *kdgM* gene is very similar in *Y. pestis*; the gene lies downstream from the *pelW-ogtABCD* operon, which encodes the pectate lyase PelW, and a potential Ogt ABC transporter that is highly homologous to the TogMNAB transporter (12). In *K. pneumoniae*, the *kdgM* ho-

<sup>2</sup> N. Hugouvieux-Cotte-Pattat and V. E. Shevchik, personal communication.

<sup>3</sup> I. Sugimura, T. Sawabe, and Y. Ezura, unpublished observations.

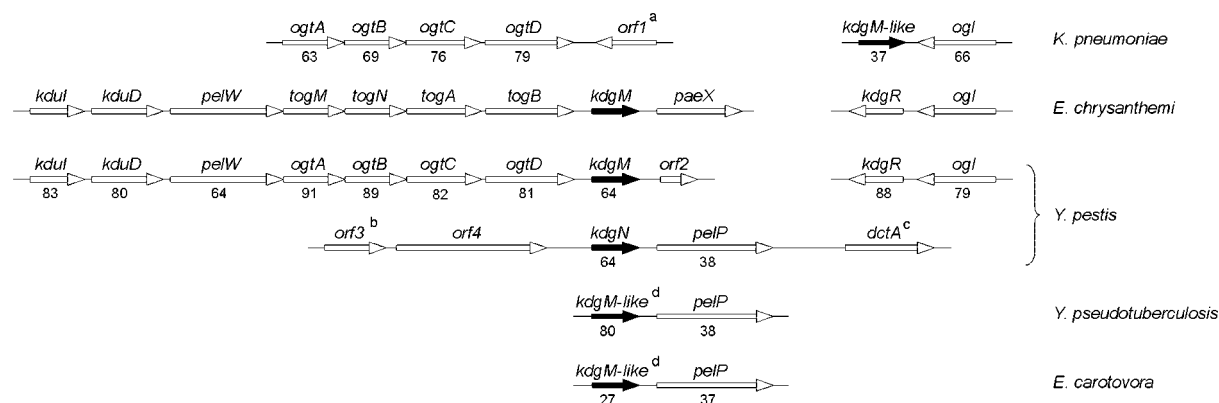


FIG. 4. Genetic organization of genes encoding KdgM homologues. Numbers indicate the percentages of identity of the proteins with their homologous protein in *E. chrysanthemi* (PelW in the case of PelP). The *kdgM* gene is located at the vicinity of genes encoding ABC transporters (the *ogtABCD* genes are homologous with the *togMNAB* genes) or pectinases (oligogalacturonate lyase Ogl, pectate lyases PelP). <sup>a</sup> indicates *orf1* encodes a protein with 58% identity with the RafY glycoporin of *E. coli*. <sup>b</sup> indicates *orf3* encodes a protein with 29% identity with the CelY cellulase of *E. chrysanthemi*. <sup>c</sup> indicates *dctA* encodes a protein with 86% identity with the DctA C4-dicarboxylate transporter of *E. coli*. <sup>d</sup> indicates only the C-terminal parts of the putative KdgM-like proteins of *E. carotovora* and *Y. pseudotuberculosis* are known, and the identity values are approximate.

TABLE II  
Characteristics of proteins of the KdgM family

The KdgM sequence was compared with amino acid and translated nucleic sequences deposited in data banks. Identity between mature proteins with mature KdgM is indicated. The C + G content of *kdgM* homologues is compared with that of the corresponding genome.

Organism	Protein (accession number) <sup>a</sup>	Residue number <sup>b</sup>	% identity with KdgM	Genome G + C	Gene G + C
			%	%	%
<i>E. chrysanthemi</i> 3937	KdgM	236		52 <sup>c</sup>	42.9
<i>E. carotovora</i> subsp. <i>carotovora</i> SCRI193		ND <sup>d</sup>	(27) <sup>d</sup>		
<i>Y. pestis</i> CO-92 Biovar	KdgM	236	64	47.6	40.8
	KdgN	234	64		39.3
<i>Y. pseudotuberculosis</i> TCPB3821		ND <sup>d</sup>	(80) <sup>d</sup>		
<i>K. pneumoniae</i> MGH78578		231	37		
<i>P. syringae</i> pv. <i>tomato</i> DC3000		240	31		
<i>S. typhimurium</i> LT2	OrfM	230	37	53.1	36.1
	YiiY (P43022)	227	39		42.1
	YshA	230			39.2
<i>S. paratyphi</i> A ATCC9150	OrfM	230	37		
	YiiY	227	39		
<i>V. haliotocoli</i> IAM14596T	AlyVGIII (AAF22513)	235	30		
<i>E. coli</i> K-12 MG1655	Yjha (P39372)	238	23	50.8	43.0
	YshA (P76773)	230			40.8

<sup>a</sup> Protein accession number is indicated except when homologues were found in translated bacterial genomes.

<sup>b</sup> The number indicates the number of residues of the native proteins.

<sup>c</sup> The value is approximate, the genome sequence remaining incomplete.

<sup>d</sup> ND, not determined; the *kdgM* homologous gene has not been completely sequenced, and the value of identity with KdgM is approximate.

homologue is adjacent to the *ogI* gene, encoding the oligogalacturonate lyase, a site where the regulator *kdgR* is found in *E. chrysanthemi* and *Y. pestis* (Fig. 4). A gene encoding a putative glycoporin (*orf1*) is downstream from the ABC transporter *ogt* genes of *K. pneumoniae*. A second set of genes encoding a KdgM homologue is found in front of the *pelP* gene, encoding a pectate lyase in *Yersinia pseudotuberculosis*, *E. carotovora* subsp. *carotovora* (44, 45), and *Y. pestis* (*kdgN*) (Fig. 4). All these genes are found in clusters containing genes involved in pectin catabolism or transport, suggesting that they may encode proteins involved in related functions.

Other genes of the *kdgM* family are found in the vicinity of putative sugar transporters and permeases. The gene encoding the KdgM homologous protein in *P. syringae* pv. *tomato* is situated downstream from genes encoding an ABC transporter. The *orfM* and *yiiY* genes of *S. typhimurium* and *S. paratyphi-murium* are located upstream from genes encoding a putative metabolite transporter and the rhamnose-H<sup>+</sup> symporter, RhaT (46), respectively. Similarly, the *yshA* genes from *S. typhimurium* and *E. coli* are found upstream from genes encoding putative sugar transporters.

The G + C content of the *kdgM* coding region is 42.9%. It

strongly contrasts with the value usually found in *E. chrysanthemi* genes, which is about 52%. For example, the *pelW-togM-NAB* operon is 51.1% G + C-rich. The same phenomenon was observed with the genes encoding KdgM homologues in *Y. pestis*, *S. typhimurium*, and *E. coli* (Table II). It appears that genes of the *kdgM* family are characterized by an AT-rich coding region.

**Regulation of the *kdgM* Expression**—The transcription of each gene involved in pectinolysis is tightly controlled by several transcriptional regulators. To study *kdgM* regulation, an *uidA*-Km cassette was introduced into the gene, and the *kdgM::uidA* fusion was recombined into the *E. chrysanthemi* chromosome. Expression of  $\beta$ -glucuronidase was measured in various environmental conditions and in mutants inactivated for transcriptional regulators known to be involved in the regulation of pectinolytic genes. The expression of the fusion is induced at about 14-fold in the presence of PGA and pectin and 4-fold in the presence of GA. In a wild type background, the *kdgM* expression is dependent on environmental conditions such as pH, osmolarity, temperature, and oxygen availability (Table III). The maximum expression was observed at pH 7.7, at 260 mosm and at 30 °C, which is the optimum temperature

TABLE III  
Regulation of *kdgM* expression

*kdgM::uidA* expression was analyzed in various growth conditions and in various regulatory backgrounds. All values correspond to at least four independent assays. Cells were grown on supplemented M63 medium. The temperature, pH, and osmolarity values are 30 °C, 6.9, and 260 mosM, respectively, except where indicated.

Culture medium	Additional mutation	$\beta$ -Glucuronidase-specific activity <sup>a</sup>
Glycerol		135 ± 22
Glycerol GA		534 ± 62
Glycerol PGA		1981 ± 343
Glycerol pectin		1809 ± 251
Glycerol 25 °C		49 ± 7
Glycerol 37 °C		89 ± 17
Glycerol fumarate <sup>b</sup>		454 ± 25
Glycerol, pH 5.6		40 ± 18
Glycerol, pH 7.0		170 ± 19
Glycerol, pH 7.7		332 ± 79
Glycerol, 80 mosM		47 ± 13
Glycerol, 770 mosM		43 ± 4
Glucose		51 ± 8
Glycerol	<i>kdgR</i>	1295 ± 255
Glycerol GA	<i>kdgR</i>	2838 ± 479
Glycerol	<i>pecS</i>	1017 ± 192
Glycerol	<i>kdgR pecS</i>	1350 ± 94
Glycerol	<i>exuR</i>	518 ± 107
Glycerol	<i>kdgR exuR</i>	1307 ± 98
Glycerol	<i>pecT</i>	223 ± 14
Glycerol	<i>expR</i>	142 ± 38
Glucose	<i>crp</i>	57 ± 10

<sup>a</sup>  $\beta$ -Glucuronidase specific activity is expressed in nanomoles of *para*-nitrophenol liberated per min per mg of bacterial dry weight.

<sup>b</sup> The fumarate permits oxygen limitation conditions of growth.

for *E. chrysanthemi* growth, and also in conditions of oxygen limitation.

Induction in the presence of pectin derivatives mainly results from the interaction of the KdgR repressor with intracellular pectic catabolites such as and 5-keto-4-deoxyuronate or 2-keto-3-deoxygluconate (2). A 10-fold increase in the fusion expression in a *kdgR* background (Table III) confirmed that the *kdgM* expression is controlled by the main regulator of genes involved in pectin degradation, KdgR (2, 41, 47). However, the expression of the fusion in a *kdgR* mutant is still induced by GA. Transcriptional regulation of genes involved in pectin and GA degradation pathways is independent. GA catabolism is specifically controlled by the ExuR repressor (18). *kdgM* expression increased 4-fold in an *exuR* background, indicating that it also belongs to the ExuR regulon. The additive effect of the *kdgR* and *exuR* mutations (Table III) suggests that KdgR and ExuR control the *kdgM* expression independently. CRP is a transcriptional activator of many genes involved in sugar catabolism, in response to the cAMP intracellular level (22). The expression of *kdgM* is 3-fold repressed with the presence of glucose in the medium or in a *crp* mutant, indicating that *kdgM* is submitted to the catabolite repression via the CRP activator. A 7-fold increase in *kdgM* expression was observed in a *pecS* mutant (40, 20). This regulation by PecS seems to occur essentially during the late exponential or early stationary phase of growth (data not shown). The additive effect of the *kdgR* and *pecS* mutations (Table III) suggests that KdgR and PecS control the *kdgM* expression independently. In contrast, the regulators ExpR, which is involved in the cell density-dependent regulation (23), and PecT (21) do not control the expression of *kdgM*.

Gel shift experiments were performed to test the direct interaction of KdgR, CRP, and PecS regulators with the *kdgM* promoter region. A 356-nucleotide fragment (position -228 to +128, relative to the transcription start) overlapping the *togB-kdgM* intergenic region was used as a probe. These experiments showed that KdgR, CRP, and PecS interact directly with the *kdgM* promoter region, giving rise to protein-DNA com-

TABLE IV  
Effect of a *kdgM* mutation on the pectate lyase activity

Pectate lyase synthesis of wide type strain A350 and *kdgM* strain A3573 was analyzed in the presence of various inducers. The assays were performed on toluenized extracts of bacterial cultures.

Growth medium	Pectate lyase-specific activity <sup>a</sup>	
	Strain A350	Strain A3573
Glycerol	0.04 ± 0.01	0.03 ± 0.01
Glycerol GA	0.34 ± 0.10	0.28 ± 0.02
Glycerol PGA	1.81 ± 0.30	1.33 ± 0.25
Glycerol pectin	2.81 ± 0.17	1.76 ± 0.33
GA	0.69 ± 0.13	0.57 ± 0.09
PGA	10.09 ± 1.78	5.80 ± 0.95
Pectin	9.10 ± 2.24	6.62 ± 0.68

<sup>a</sup> The pectate lyase-specific activity is expressed in micromoles of unsaturated oligogalacturonides liberated in 1 min per mg of bacterial dry weight.

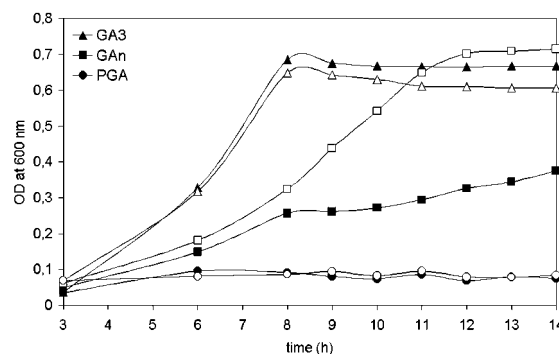


FIG. 5. Growth of a *kdgM* mutant with various oligogalacturonides. The *outD* strain A1851 (open symbols) and the *outD kdgM* strains A3577 (closed symbols) were grown in the presence of GA<sub>3</sub> (triangles), GA<sub>n</sub> (squares), and PGA (circles). The growth curves with GA and GA<sub>2</sub> are identical to those of GA<sub>3</sub> and are not represented.

plexes (Fig. 3B). Analysis of the sequence revealed a putative CRP-binding site overlapping the -35 region of the  $\sigma^{70}$  promoter, centered at position -41.5, and a putative KdgR-binding site centered at position -72 versus the transcription start (Fig. 3A). Until now, no consensus for the PecS-binding site has been characterized.

**A *kdgM* Mutation Affects Pectinolysis**—The pathogenicity of a *kdgM* mutant was studied in *planta* to observe the effects of a *kdgM* mutation on *E. chrysanthemi* pathogenesis. Chicory leaves were infected with 10<sup>6</sup> bacteria of wild type strain or *kdgM* mutant. The length of soft rotted tissue is indicative of the virulence of the strain. The length of rotted tissue measured after 24 h of incubation in a dew chamber at 30 °C was 14 ± 7 mm for the *kdgM* strain A3573, whereas it was 35 ± 9 mm for the wild type strain A350, indicating that KdgM is necessary for the full virulence of *E. chrysanthemi*. The effect of the *kdgM* mutation on pectate lyase synthesis was studied by assay of the pectate lyase activity on toluenized extracts of cells grown without inducer or with PGA and GA as inducer (Table IV). The mutation does not affect pectate lyase synthesis of cells grown without inducer or in the presence of GA. In contrast, the pectate lyase activity of the *kdgM* mutant is reduced when PGA is used as an inducer or as the sole carbon source for growth. Considering the putative alginate lyase activity of the KdgM homologue, AlyVGIII, we tested whether the purified KdgM protein possesses any pectate lyase or alginate lyase activity. No activity was observed in any tested conditions (data not shown). Such data could suggest that KdgM is involved in pectate lyase secretion. The cellulase secretion, which occurs through the same Out machinery as pectate lyases (48), is not modified in a *kdgM* mutant, and the pectate lyase activity of the total toluenized extracts and the supernatants is

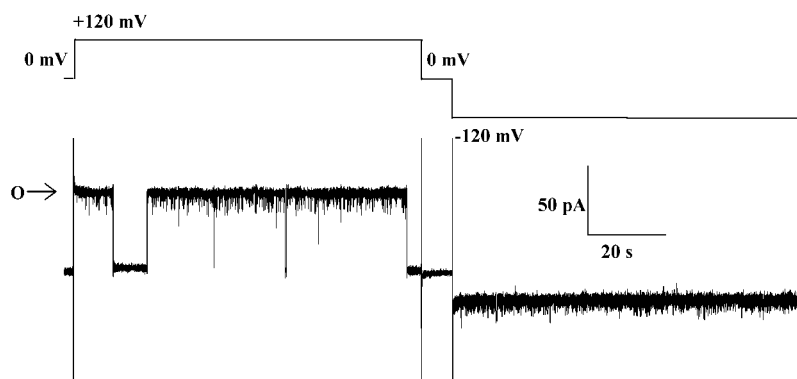


FIG. 6. **Channel activity of KdgM at two opposite potentials.** Proteoliposomes reconstituted with purified KdgM were added to the *cis* compartment of a bilayer chamber ( $3 \text{ ng}\cdot\text{ml}^{-1}$ , final protein concentration). Fusion to the bilayer was induced by imposing a salt gradient between the two chambers. After insertion of one channel in the planar bilayer, symmetrical media ( $800 \text{ mM KCl}$ ,  $10 \text{ mM Hepes-KOH}$ ,  $\text{pH } 7.4$ ) were established in the two chambers and the activity recorded. Voltage steps were applied as indicated. Application of a high positive potential ( $+120 \text{ mV}$ ) induced the closure of the channel, which could reopen at  $0 \text{ mV}$ . Application of a negative potential ( $-120 \text{ mV}$ ) did not induce closure of the channel. O, full open level of the channel.

identical (data not shown), showing that the reduced amount of pectate lyases does not result from an impaired secretion. Thus, KdgM seems to be essential for the conversion of PGA, but not GA, into inducing molecules. Its localization in the outer membrane suggests a role in the entry of the  $\text{GA}_n$  into the bacteria.

To assess this role of KdgM, the wild type and *kdgM* *E. chrysanthemi* strains were grown in media that contain, as the sole carbon source, GA,  $\text{GA}_2$ ,  $\text{GA}_3$ , or a mixture of  $\text{GA}_n$  obtained by digestion of PGA by the pectate lyase PeL. To prevent extracellular degradation of  $\text{GA}_n$  by the secreted pectinases, an *out* mutation was added to the strains. No growth was observed with PGA because of the absence of secreted pectinases that prevents its degradation (Fig. 5). Growth of both strains was identical with GA,  $\text{GA}_2$  and  $\text{GA}_3$ . Growth of the *outD* strain A1851 was slower with  $\text{GA}_n$  than with GA but reached the same final OD. In contrast, growth of the *outD kdgM* mutant A3577 with  $\text{GA}_n$  was very limited and stopped quickly (Fig. 5). These data suggest that this strain probably metabolized the limited amount of short oligomers present in the mixture but could not use the longer  $\text{GA}_n$ . Thus, KdgM could be a porin required for the entry of  $\text{GA}_n$  into the bacteria, explaining the reduced virulence and the reduced induction of pectate lyase synthesis in the *kdgM* mutant.

**Purification and Analysis of the KdgM Protein**—KdgM was purified for further electrophysiological assays on a lipid bilayer. Many detergents commonly used in porin purification were inefficient for KdgM extraction. KdgM appears to be particularly hard to solubilize in neutral detergents. Two consecutive extractions with  $0.5\% N$ -lauroylsarcosyl and  $0.7\% n$ -octyl- $\beta$ -D-glucoside were finally performed to extract most of the outer membrane proteins, and KdgM was solubilized with  $0.5\% \text{ SDS}$ . KdgM was further purified by two consecutive Tris-Tricine and Tris glycine SDS-PAGEs (Fig. 2B). The identity of the protein and the purity of the preparation were checked by mass spectroscopy.

Various biochemical characteristics of porins were tested with KdgM. Like many porin subunits, the KdgM protein migrates abnormally because its apparent molecular mass is about  $28 \text{ kDa}$ , whereas its calculated molecular mass is  $24.688 \text{ kDa}$ . Most, but not all, porins are homotrimers. These trimers can be observed in SDS-PAGE, because they are stable in the presence of SDS unless heated at  $100^\circ\text{C}$ . In the case of KdgM, migration in SDS-PAGE was the same whether the samples were boiled or not. Furthermore, formaldehyde cross-linking did not show any multimerization of KdgM, whereas dimers were observed for the control protein KdGR (data not shown). The same result was observed when bacteria were grown in the

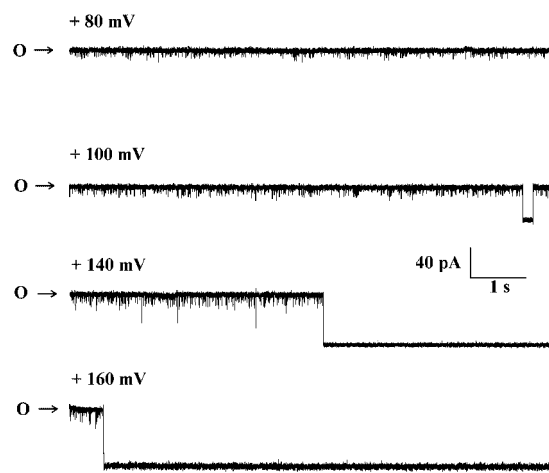


FIG. 7. **Voltage dependence of the KdgM channel.** Recordings of the channel are at different positive membrane potentials. The bilayer was subjected to various positive potentials as indicated. Between pulses, the bilayer was held at  $0 \text{ mV}$  for several seconds. The beginning of each trace corresponds to the onset of each pulse. The ionic conditions are the same as in Fig. 6.

absence or in the presence of PGA. Thus, KdgM appears to have a monomeric organization.

**Porin Activity of KdgM in Planar Lipid Bilayers**—The purified KdgM protein was introduced into liposomes. Addition of these proteoliposomes to the *cis* compartment of a bilayer chamber, at low protein concentration ( $3 \text{ ng}\cdot\text{ml}^{-1}$ , final concentration), resulted in the rapid insertion in the bilayer of large conductance channels. These channels were open at  $0 \text{ mV}$  and at low, positive or negative, potentials. By termination of the mixing in the *cis* compartment after the insertion of the first channel, we could record the activity of a single channel. In most of the cases, application of a high positive membrane potential ( $100 \text{ mV}$  and above) resulted in the closure of the channel, whereas highly negative membrane potentials had no effect (Fig. 6). Even the application of a very highly negative potential, down to  $-200 \text{ mV}$ , was ineffective. In some cases, the opposite behavior was observed (*i.e.* closure at negative potentials and no effect at high positive potentials) suggesting that the channel had been inserted with an opposite orientation in the bilayer. Closure at positive and negative potentials could also be observed following the insertion of several channels, presumably inserted with opposite polarities. Fig. 7 illustrates the effect of voltage pulses of increasing magnitude, at positive potential, on the closure of the channel. Between pulses the



membrane potential was held at 0 mV, which resulted in the reopening of the channel. Although fast gating could be observed, closure followed the pattern of slow kinetics documented for porin channels. The higher the membrane potential, the faster the channel could be closed.

Most of the porins that have been studied electrophysiologically are trimeric proteins. In these cases, several reports have shown that voltage-dependent closure occurs in three steps, the conductance of each step corresponding to one-third of that of the inserted trimers. It was proposed that these steps correspond to the closure of individual monomers (49, 50), later shown by structural studies to correspond to individual channels. It is noteworthy that, in this study, we could repeatedly record channels that closed in only one step corresponding to the inserted conductance (Figs. 6 and 7). This observation is consistent with the biochemical data suggesting that KdgM is a monomer.

In contrast to other porins for which multiple conductance steps are usually observed, one predominant conductance was observed for KdgM. The I-V curve was markedly non-linear (Fig. 8). At a positive potential the conductance of the channel was 450 pS in symmetrical 800 mM KCl media. Under asymmetrical conditions (800 mM KCl versus 100 mM KCl), the reversal potential was  $18.5 \pm 8.2$  mV (S.D.,  $n = 16$ ), corresponding to a 2.8 preference for chloride over potassium, as calculated from the Goldman-Hodgkin-Katz equation.

The effect of GA<sub>3</sub> on ion channel conduction through the KdgM channel was examined. Addition of relatively high concentrations of GA<sub>3</sub> to the *trans* compartment resulted, at positive potential, in an apparent decrease of the single channel conductance and in an increase in the channel noise (Fig. 9A). No effect was observed at negative potential. Conversely, addition of GA<sub>3</sub> to the *cis* compartment induced a similar inhibition at negative potential but not at positive potential. The behavior shown in Fig. 9 is that described for fast blockers whose residence time is too short to be distinctly resolved by

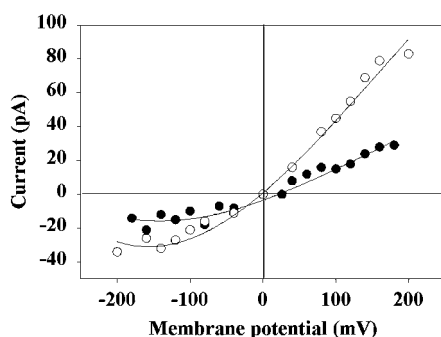


FIG. 8. Current-voltage relationship of the KdgM channel. Elementary currents recorded under symmetrical conditions (open circle, 800 mM KCl, 10 mM Hepes-KOH, pH 7.4) and asymmetrical conditions (closed circle, 800 mM KCl, 10 mM Hepes-KOH, pH 7.4, in the *cis* compartment versus 100 mM KCl, 10 mM Hepes-KOH, pH 7.4, in the *trans* compartment) were plotted against the membrane potential.

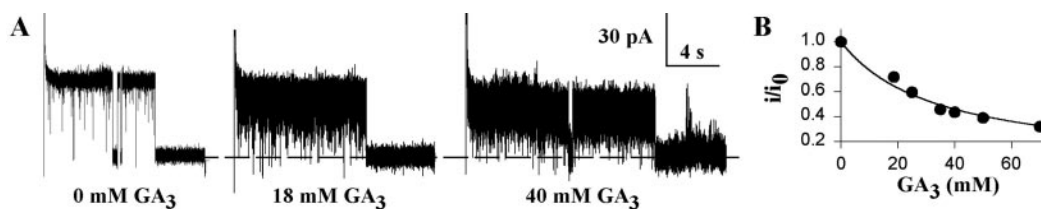


FIG. 9. Fast block of the KdgM channel by trisaccharide. A, effect of various concentrations of trisaccharide on the single channel conductance. The membrane bilayer contained one KdgM channel. The beginning of each trace corresponds to the onset of a voltage pulse to +100 mV to induce channel closure. Increasing concentrations of trisaccharide (GA<sub>3</sub>) were present in the *trans* chamber as indicated. The ionic conditions are the same as in Fig. 6. The dashed line corresponds to the closed level. B, ratio of the single channel current in the presence of trisaccharide to that in its absence,  $i/i_0$ , at +100 mV, versus trisaccharide (GA<sub>3</sub>) concentration. For the determination of  $i$ , records, such as that shown in A, were heavily filtered (20 Hz). The curve was fitted to the equation  $i/i_0 = 1/(1 + [GA_3]/K_D)$ , with  $K_D = 34$  mM.

single channel recording (51, 52). For a membrane potential of 100 mV, a  $K_D$  of 34 mM could be determined for GA<sub>3</sub> (Fig. 9B). Maltotriose up to 50 mM, used as a control, had no effect on the channel conductance. However, 50 mM ATP was also able to induce a voltage-dependent fast block of the channel.

## DISCUSSION

KdgM is one of the most abundant proteins of the outer membrane of *E. chrysanthemi* grown in the presence of pectin. The regulation of *kdgM* expression resembles the regulation of the pectinolytic genes, whose expression is controlled by the KdGR, PecS, and CRP regulators, and of the GA catabolism genes, whose expression is controlled by the ExuR repressor. Thus, the *kdgM* expression behaves like that of genes involved in pectin catabolism.

The pectin degradation pathway has been extensively studied in *E. chrysanthemi*. However, up to now, the means of translocation of GA<sub>n</sub> across the outer membrane remained an unanswered question. We showed that KdgM is a porin specific for GA<sub>n</sub>, allowing for their transport into the periplasm. *E. chrysanthemi* possesses aspecific porins similar to the OmpF and OmpC porins of *E. coli*, which are responsible for membrane permeability for molecules of <600 Da (14). Such porins could be responsible for GA<sub>2</sub> and GA<sub>3</sub> uptake (GA<sub>3</sub> is 546 Da) but not for the uptake of larger GA<sub>n</sub>. When grown in the presence of GA<sub>n</sub> (Fig. 5), the *kdgM* mutant can assimilate GA<sub>n</sub> smaller than tetramers, indicating that these molecules can pass through aspecific porins. However, *E. chrysanthemi* is able to grow on larger oligomers only when *kdgM* is efficient, suggesting that KdgM is probably the sole porin through which these molecules can enter the bacteria. The *kdgM* mutation decreases the virulence of *E. chrysanthemi*. This could be due to a lower formation of intracellular inducing compounds that, in turn, prevent the full induction of pectate lyase synthesis. This suggests that a large proportion of long GA<sub>n</sub> enters the bacteria, while pectinolysis is taking place, during the course of plant infection. *E. chrysanthemi* possesses, in the periplasm, several enzymes, such as PelX and PehX, that are able to cleave these oligomers (10, 53).

We tested whether KdgM presents the classical porin properties using electrophysiological experiments. After insertion in planar lipid bilayers, KdgM channels exhibited the following characteristics. The channels had a high conductance, were open at low membrane potential, and could be closed upon application of a high voltage. Voltage dependence was asymmetric. These characteristics constitute the electrophysiological fingerprints of porin channels. The channel exhibited a weak anion selectivity, consistent with its putative role in the translocation of GA<sub>n</sub> across the outer membrane. Similarly, the PhoE porin, which is induced in *E. coli* cells that are grown under phosphate limitation, is weakly anion-selective (50, 54, 55).

Most porins are trimeric (14). OmpA is an example of a protein of the outer membrane of *E. coli* that is monomeric. Its

N-terminal domain is integrated into the membrane in the form of a  $\beta$ -barrel of eight  $\beta$ -strands (56). Osmotic swelling experiments (57, 58), as well as planar bilayer experiments (59, 60), have been reported, indicating that OmpA forms pores. OprF from *Pseudomonas aeruginosa*, which is closely related to OmpA, has also been reported to form pores (61). However, whether OmpA is a *bona fide* porin remains controversial (56). Recently the *E. coli* porin OmpG has been characterized, and it has been suggested that it is a monomeric porin (62, 63). The biochemical data presented here indicate that KdgM is a monomer. In addition, its electrophysiological behavior, as compared with that of trimeric porins, is also consistent with a monomeric organization. KdgM is therefore one of the first clear examples of a monomeric porin.

Several specific porins can be blocked by their substrates in electrophysiology experiments, indicating the presence of binding sites (14). A well studied example is that of LamB (64, 65), which facilitates the diffusion of maltose and maltodextrins. Although in this case, a well resolved block of the channel, at the single-channel level, can be observed (66), in the case of KdgM the short lived blocked states could not be resolved. At the very least, these data indicate that GA<sub>3</sub> is driven through the pore and that it interacts with the pore. Electrophysiological evidence in favor of the conduction of longer polymers of defined length will first require the purification of the corresponding molecules.

Biochemical data indicate that KdgM defines a novel family of porins that have no sequence homology with characterized porins. In contrast, they present several specific characteristics and these data suggest that the KdgM homologues probably function as sugar porins too. The genes of the *kdgM* family are usually situated at the vicinity of sugar transport and sugar catabolism genes (Fig. 4), and most of them are characterized by an AT-rich coding region (Table II). Sequence conservation between the proteins of the KdgM family is very low, a property that is usually observed in the porin families. Considering the relatively small size of the KdgM homologues (between 205 and 216 amino acids for the mature form), it appears improbable that they possess 16 or 18 transmembrane segments like most of the characterized porins (15). Sequence homologies, as well as this relatively small size of KdgM and its monomeric state, are the clearest elements proving the characterization of a new porin family.

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#### REFERENCES

- Hauben, L., Moore, E. R., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L., and Swings, J. (1998) *Syst. Appl. Microbiol.* **21**, 384–397
- Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., and Reverchon, S. (1996) *Annu. Rev. Microbiol.* **50**, 213–257
- Barras, F., Van Gijsegem, F., and Chatterjee, A. K. (1994) *Annu. Rev. Phytopathol.* **32**, 201–234
- Condemine, G., Dorel, C., Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. (1992) *Mol. Microbiol.* **6**, 3199–3211
- Pugsley, A. P. (1993) *Microbiol. Rev.* **57**, 50–108
- Preston, J. F., Rice, J. D., Ingram, L. O., and Keen, N. T. (1992) *J. Bacteriol.* **174**, 2039–2042
- Roy, C., Kester, H., Visser, J., Shevchik, V., Hugouvieux-Cotte-Pattat, N., Robert-Baudouy, J., and Benen, J. (1999) *J. Bacteriol.* **181**, 3705–3709
- Collmer, A., and Bateman, D. F. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 3920–3924
- San Francisco, M. J. D., Xiang, Z., and Keeman, R. W. (1996) *Mol. Plant-Microbe Interact.* **9**, 144–147
- Shevchik, V. E., Kester, H. C., Benen, J. A., Visser, J., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. (1999) *J. Bacteriol.* **181**, 1652–1663
- Shevchik, V. E., Condemine, G., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. (1999) *J. Bacteriol.* **181**, 3912–3919
- Hugouvieux-Cotte-Pattat, N., Blot, N., and Reverchon, S. (2002) *Mol. Microbiol.* **41**, 1113–1123
- Hugouvieux-Cotte-Pattat, N., and Reverchon, S. (2002) *Mol. Microbiol.* **41**, 1125–1132
- Nikaido, H. (1994) *J. Biol. Chem.* **269**, 3905–3908
- Koebnik, R., Locher, K. P., and van Gelder, P. (2000) *Mol. Microbiol.* **37**, 239–253
- Boos, W., and Shuman, H. (1998) *Microbiol. Mol. Biol. Rev.* **62**, 204–229
- Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. (1985) *J. Bacteriol.* **162**, 248–255
- Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. (1987) *J. Bacteriol.* **169**, 1223–1231
- Condemine, G., Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. (1986) *J. Bacteriol.* **165**, 937–941
- Reverchon, S., Nasser, W., and Robert-Baudouy, J. (1994) *Mol. Microbiol.* **11**, 1127–1139
- Surgey, N., Robert-Baudouy, J., and Condemine, G. (1996) *J. Bacteriol.* **178**, 1593–1599
- Reverchon, S., Expert, D., Robert-Baudouy, J., and Nasser, W. (1997) *J. Bacteriol.* **179**, 3500–3508
- Nasser, W., Bouillant, M. L., Salmond, G., and Reverchon, S. (1998) *Mol. Microbiol.* **29**, 1391–1405
- Reverchon, S., Nasser, W., and Robert-Baudouy, J. (1991) *Mol. Microbiol.* **5**, 2203–2216
- Miller, J. H. (1972) *Experiments In Molecular Genetics*, pp. 431–435, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
- Possot, O., and Pugsley, A. P. (1994) *Mol. Microbiol.* **12**, 287–299
- Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) *J. Biol. Chem.* **247**, 3962–3972
- Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038
- Nasser, W., Reverchon, S., and Robert-Baudouy, J. (1992) *Mol. Microbiol.* **6**, 257–265
- Nasser, W., Robert-Baudouy, J., and Reverchon, S. (1997) *Mol. Microbiol.* **26**, 1071–1082
- Ji, J. W., Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. (1987) *J. Gen. Microbiol.* **133**, 793–802
- Tardy, F., Nasser, W., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. (1997) *J. Bacteriol.* **179**, 2503–2511
- Bardonnnet, N., and Blanco, C. (1992) *FEMS Microbiol. Lett.* **72**, 243–247
- Preiss, J., and Ashwell, G. (1962) *J. Biol. Chem.* **237**, 309–316
- Résibois, A., Colet, M., Faelen, M., Schoonejans, E., and Toussaint, A. (1984) *Virology* **137**, 102–112
- Roeder, D. L., and Collmer, A. (1985) *J. Bacteriol.* **164**, 51–56
- Praillet, T., Nasser, W., Robert-Baudouy, J., and Reverchon, S. (1996) *Mol. Microbiol.* **20**, 391–402
- Condemine, G., and Robert-Baudouy, J. (1987) *FEMS Microbiol. Lett.* **42**, 39–46
- Struyve, M., Moons, M., and Tommassen, J. (1991) *J. Mol. Biol.* **218**, 141–148
- Condemine, G., and Robert-Baudouy, J. (1991) *Mol. Microbiol.* **5**, 2191–2202
- Hinton, J. C., Sidebotham, J. M., Gill, D. R., and Salmond, G. P. (1989) *Mol. Microbiol.* **3**, 1785–1795
- Manulis, S., Kobayashi, D. Y., and Keen, N. T. (1988) *J. Bacteriol.* **170**, 1825–1830
- Tate, C. G., Muiry, J. A., and Henderson, P. J. (1992) *J. Biol. Chem.* **267**, 6923–6932
- Nasser, W., Reverchon, S., Condemine, G., and Robert-Baudouy, J. (1994) *J. Mol. Biol.* **236**, 427–440
- Andro, T., Chambost, J. P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., Van Gijsegem, F., and Coleno, A. (1984) *J. Bacteriol.* **160**, 1199–1203
- Schindler, H., and Rosenbusch, J. P. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 2302–2306
- Dargent, B., Hofmann, W., Pattus, F., and Rosenbusch, J. P. (1986) *EMBO J.* **5**, 773–778
- Yellen, G. (1984) *J. Gen. Physiol.* **84**, 157–186
- Moczydlowski, E. (1986) in *Ion Channel Reconstitution* (Miller, C., ed) pp. 75–113, Plenum Publishing Corp., New York
- Nasser, W., Shevchik, V. E., and Hugouvieux-Cotte-Pattat, N. (1999) *Mol. Microbiol.* **34**, 641–650
- Benz, R., Darveau, R. P., and Hancock, R. E. W. (1984) *Eur. J. Biochem.* **140**, 319–324
- Berrier, C., Besnard, M., and Ghazi, A. (1997) *J. Membr. Biol.* **156**, 105–115
- Pautsch, A., and Schulz, G. E. (1998) *Nat. Struct. Biol.* **5**, 1013–1017
- Sugarawa, E., and Nikaido, H. (1992) *J. Biol. Chem.* **267**, 2507–2511
- Sugarawa, E., and Nikaido, H. (1994) *J. Biol. Chem.* **269**, 17981–17987
- Saint, N., Dé, E., Julien, S., Orange, N., and Molle, G. (1993) *Biochim. Biophys. Acta* **1145**, 119–123
- Arora, A., Rinehart, D., Szabo, G., and Tamm, L. K. (2000) *J. Biol. Chem.* **275**, 1594–1600
- Nikaido, H., Nikaido, K., and Harayama, S. (1991) *J. Biol. Chem.* **266**, 770–779
- Fajardo, D. A., Cheung, J., Ito, C., Sugawara, E., Nikaido, H., and Misra, R. (1998) *J. Bacteriol.* **180**, 4452–4459
- Conlan, S., Zhang, Y., Cheley, S., and Bayley, H. (2000) *Biochemistry* **39**, 11845–11854
- Benz, R., Schmid, A., Nakae, T., and Vos-Scheperkeuter, G. H. (1986) *J. Bacteriol.* **165**, 978–986
- Dargent, B., Rosenbusch, J., and Pattus, F. (1987) *FEBS Lett.* **220**, 136–142
- Bezrukov, S. M., Kullman, L., and Winterhalter, M. (2000) *FEBS Lett.* **476**, 224–228