Catabolism of Raffinose, Sucrose, and Melibiose in *Erwinia chrysanthemi* 3937[∇]

Nicole Hugouvieux-Cotte-Pattat* and Sana Charaoui-Boukerzaza

Université de Lyon, Université Lyon 1, INSA Lyon, Microbiologie Adaptation et Pathogénie CNRS UMR5240, Domaine Scientifique de la Doua, 69622 Villeurbanne Cedex, France

Received 7 May 2009/Accepted 28 August 2009

Erwinia chrysanthemi (*Dickeya dadantii*) is a plant pathogenic bacterium that has a large capacity to degrade the plant cell wall polysaccharides. The present study reports the metabolic pathways used by *E. chrysanthemi* to assimilate the oligosaccharides sucrose and raffinose, which are particularly abundant plant sugars. *E. chrysanthemi* is able to use sucrose, raffinose, or melibiose as a sole carbon source for growth. The two gene clusters *scrKYABR* and *rafRBA* are necessary for their catabolism. The phenotypic analysis of *scr* and *raf* mutants revealed cross-links between the assimilation pathways of these oligosaccharides. Sucrose catabolism is mediated by the genes *scrKYAB*. While the *raf* cluster is sufficient to catabolize melibiose, it is incomplete for raffinose catabolism, which needs two additional steps that are provided by *scrY* and *scrB*. The *scr* and *raf* clusters are controlled by specific repressors, *ScrR* and *RafR*, respectively. Both clusters are controlled by the global activator of carbohydrate catabolism, the cyclic AMP receptor protein (CRP). *E. chrysanthemi* growth with lactose is possible only for mutants with a derepressed nonspecific lactose transport system, which was identified as *RafB*. *RafR* inactivation allows the bacteria to assimilate the novel substrates lactose, lactulose, stachyose, and melibiononic acid. The *raf* genes also are involved in the assimilation of α - and β -methyl-D-galactosides. Mutations in the *raf* or *scr* genes did not significantly affect *E. chrysanthemi* virulence. This could be explained by the large variety of carbon sources available in the plant tissue macerated by *E. chrysanthemi*.

Pectinolytic erwiniae are enterobacteria that cause disease in a wide range of plants, including many crops of economic importance (23). The soft-rot symptom produced by *Erwinia chrysanthemi* (syn. *Dickeya dadantii*) results from the degradation of polysaccharides involved in the cohesion of the plant cell wall. The plant tissue maceration is concomitant with a large increase in the bacterial population (13). To ensure this multiplication, the bacteria assimilate various oligosaccharides released in the macerated tissue, which provide carbon and energy sources.

E. chrysanthemi is known to use several carbon sources for growth, including sugars ranging from monosaccharides to polysaccharides. The completion of the *E. chrysanthemi* strain 3937 genome provides a genome-scale view into its potential catabolic capacities. A substantial part of the *E. chrysanthemi* genome is dedicated to genes involved in carbohydrate catabolism. In plant tissues, the most abundant soluble carbohydrates are the two oligosaccharides sucrose and raffinose (32). The trisaccharide raffinose [α -D-Galp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 2) β -D-Fruf] and the related disaccharides sucrose [α -D-Glcp-(1 $\rightarrow$ 2) β -D-Fruf] and melibiose [α -D-Galp-(1 $\rightarrow$ 6)-D-Glcp] are used as carbon sources for *E. chrysanthemi* growth. Previous studies suggested links between the transport of lactose and that of raffinose and melibiose (15). The *E. chrysanthemi* wild-

type strain 3937 does not use lactose [β -D-Galp-(1 $\rightarrow$ 4)-D-Glcp] as a carbon source for growth. This is due to the lack of a specific lactose transport system. However, spontaneous mutants able to assimilate lactose (designated Lac⁺) are easily obtained; they show a deregulation of the transport system *LmrT*, which is able to mediate lactose, melibiose, and raffinose transport (15). Despite our current knowledge of the strain 3937 genome sequence, no open reading frame (ORF) could be assigned to the *LmrT* gene, the identity of which remains unknown. We analyzed the *E. chrysanthemi* genome for the presence of potential genes involved in the catabolism of α -galactosides or α -glucosides. It contains a complete *scrKYABR* gene cluster that is involved in sucrose catabolism in various enterobacteria and a truncated *rafRBA* locus that is involved in raffinose catabolism. The growth with raffinose, despite the presence of an incomplete *raf* cluster, suggests that the missing functions are provided by other genes. Moreover, while *E. chrysanthemi* can catabolize melibiose, its genome does not contain homologues of the *Escherichia coli* *melABR* genes (30). Thus, to assimilate melibiose, *E. chrysanthemi* exploits other genes, which have yet to be identified. The present study mainly reports the role of the *E. chrysanthemi* gene clusters *scr* and *raf* in the catabolism of the oligosaccharides sucrose, raffinose, melibiose, and lactose. The importance of such catabolic pathways for bacterial multiplication in the plant tissues also was assessed during the infection process.

* Corresponding author. Mailing address: Microbiologie Adaptation et Pathogénie UMR5240, bâtiment Lwoff, 10 rue Dubois, Domaine Scientifique de la Doua, 69622 Villeurbanne Cedex, France. Phone: 33-472-43-1553. Fax: 33-472-43-1584. E-mail: Nicole.Cotte-Pattat@insa-lyon.fr.

[∇] Published ahead of print on 4 September 2009.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *E. chrysanthemi* strains and the plasmids used in this study are listed in Table 1. The ϕ EC2 generalized transducing phage was used for transduction (25). *E. chrysanthemi* cells were grown at

TABLE 1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid, or primer	Genotype/phenotype or sequence	Reference or source
<i>E. chrysanthemi</i> strains		
3937	Wild type	Laboratory collection
A350 ^a	<i>rafR ganB</i>	15
A4415	<i>pelD::uidA-Km</i>	14
A4167	<i>crp::Cm</i>	26
A4440	<i>scrR::Cm</i>	This work
A4441	<i>rafR::Cm</i>	This work
A4659	<i>scrB::uidA-Km</i>	This work
A4660	<i>rafA::uidA-Km</i>	This work
A4661	<i>rafB::uidA-Km</i>	This work
A4694	<i>scrY::uidA-Km</i>	This work
Plasmids		
pGEM-T	Vector for direct cloning of PCR product, Ap ^r	Promega
pBS Cm	pBluescript KS+, Cm ^r	Stratagene
pBS Ap	pBluescript KS+, Ap ^r	Stratagene
pI3126	pGEMt derivative with a 0.95-kb PCR fragment, ' <i>scrR</i> '	This work
pI3130	pGEMt derivative with a 1.15-kb PCR fragment, ' <i>rafR</i> '	This work
pI3178	pGEMt derivative with a 1.15-kb PCR fragment, ' <i>rafB</i> '	This work
pI3181	pGEMt derivative with a 2.2-kb PCR fragment, ' <i>rafA</i> '	This work
pI3183	pGEMt derivative with a 1.1-kb PCR fragment, ' <i>scrB</i> '	This work
pI3251	pGEMt derivative with a 1-kb PCR fragment, ' <i>scrY</i> '	This work
Primers ^b		
<i>rafA</i>		
Forward	GCAGATCTCATCACCCATCATTCAG	
Reverse	GCTCTAGACCGTATTACAGACGTTC	
<i>rafB</i>		
Forward	GCGGATCCTGTTTTCGTTTC	
Reverse	GCTCTAGACAGCGTAGTGCGATATAC	
<i>rafR</i>		
Forward	GCGGATCCTTCATGGAAATGGTGGC	
Reverse	GCTCTAGAAAGGCATCGAGACCGTTG	
<i>scrB</i>		
Forward	GCTCTAGAACCACGTC AACATGACG	
Reverse	GGTATGGATCCAGCCGTGACG	
<i>scrR</i>		
Forward	GCGGATCCGGAAATGACCAACTACG	
Reverse	GCTCTAGAGCTGTTGGGTGA AACG	
<i>scrY</i>		
Forward	GCGGATCCGCGTCATAGCCTTTCAG	
Reverse	GCTCTAGATAAGTTGCTTTTCCAGCC	

^a This study shows that the correct genotype of the strain A350 (also named L2) is *rafR* (instead of *Lac*⁺ or *lmrTc*). A previous study showed that the mutation first named *lacZ2* affects the gene *ganB* (8).

^b Restriction sites are underlined.

30°C in M63 medium (20). Carbon sources were added at 2 g · liter⁻¹. *Escherichia coli* cells were grown at 37°C in Luria-Bertani (LB) medium (20). The media were solidified with agar (15 g · liter⁻¹). When required, antibiotics were added at the following concentrations: kanamycin (Km), 20 µg · ml⁻¹; ampicillin (Ap), 50 µg · ml⁻¹; and chloramphenicol (Cm), 20 µg · ml⁻¹.

Strains were tested on Biolog Phenotype Microarray plates PM1 and PM2 using standard protocols (Biolog Corporation, Hayward CA). This system reports the bacterial utilization of various carbon sources in 96-well plates by detecting a color change due to the reduction of a tetrazolium dye in response to cellular respiration (4).

Recombinant DNA techniques. The preparation of plasmid or chromosomal DNA, restriction digestions, ligations, DNA electrophoresis, and transformations were carried out as previously described (28).

Sequence data were obtained from the genome-sequencing project for *E. chrysanthemi* strain 3937 (<http://asap.ahabs.wisc.edu/asap/ASAP1.htm>).

PCR primers (22 to 26 mers) were designed to amplify 0.95 to 2.2 kb of *E. chrysanthemi* chromosomal DNA (Table 1). Restriction sites were added to each primer to facilitate the determination of the DNA orientation in the vector (usually BamHI at the 5' end and XbaI at the 3' end). The PCR products were purified (QIAquick PCR purification kit; Qiagen) and ligated to the pGEM-T vector (Promega), which has a protruding T nucleotide at each 3' end.

Genetic fusions were constructed on the cloned genes by the insertion of the nonpolar *uidA*-Km cassettes (3) into a restriction site situated inside the corresponding ORF, namely the EcoRI site of *rafA*, the SmaI site of *rafB*, the EcoRI site of *scrB*, and the EcoRI site of *scrY*. The orientation of the *uidA*-Km cassette was determined by restriction analysis. Only plasmids in which *uidA* and the mutated gene have the same transcriptional direction were retained. The regulatory genes were inactivated by the insertion of the nonpolar CKC15 Cm^r cassette (18) into the SalI and Eco47III sites situated in *rafR* and *scrR*, respectively. Plasmids bearing the *uidA*-Km^r or Cm^r insertions then were introduced into *E. chrysanthemi* cells by electroporation. The insertions were integrated into the *E. chrysanthemi* chromosome by marker exchange recombination after successive cultures in low-phosphate medium in the presence of the appropriate antibiotic (27). The verification of the correct recombination of the insertions was performed by PCR.

Enzyme and uptake assays. For α-galactosidase and β-glucuronidase assays, strains were grown in minimal medium at 30°C to late exponential growth phase. Toluene was added to partially disrupt the cell membranes. To measure the α-galactosidase activity, the formation of *p*-nitrophenol, after the cleavage of *p*-nitrophenyl-α-D-galactoside (pNPGal), was monitored at 405 nm. The reactions were performed with 1 mM substrate, and potential inhibitors were used at a final concentration of 10 mM. To estimate the expression of the gene fusions, β-glucuronidase activity was measured by the degradation of *p*-nitrophenyl-β-D-glucuronide (3.2 mM) into *p*-nitrophenol at 405 nm. The specific activities of α-galactosidase and β-glucuronidase are expressed as nanomoles of product liberated per minute per milligram of bacterial dry weight.

To assay the lactose transport, the bacterial cultures were grown to mid-log phase. The uptake of [¹⁴C]lactose (1 mM) by whole cells was measured at room temperature by the Millipore filtration technique (15). Inhibitions were tested in the presence of a 10-fold excess of each unlabeled competitor.

Pathogenicity tests and competition assays. Chicory leaves were inoculated as previously described (12, 34). Leaves were slightly wounded with pipette tips prior to inoculation. Ten leaves were infected for each strain, using 10⁶ bacteria per inoculation site. After incubation in a dew chamber for 24 h, the length of rotted tissue was measured to estimate disease severity. The decayed tissue was recovered. β-Glucuronidase assays and bacterial cell enumeration were performed on the macerated tissues to estimate the expression of gene fusions during plant infection (19).

Mutants resistant to kanamycin (Km^r) were used to analyze their competitive capacity when mixed with the wild-type strain during infection. Chicory leaves were inoculated with a mixture of the two strains at a ratio of 50/50. Macerated tissues were recovered 24 h after infection. After the serial dilution of the macerated tissue, the replica plating of the appropriate dilutions on medium containing kanamycin allowed us to determine the ratio between mutant (Km^r) and wild-type clones. The expected ratio after infection, if no competition occurred, was 50%.

RESULTS AND DISCUSSION

Genome analysis reveals two potential clusters for catabolism of sucrose and raffinose. The strain 3937 genome was analyzed for the presence of genes potentially involved in the

FIG. 1. Genetic organization of the *scr* and *raf* clusters in *E. chrysanthemi* strain 3937. *E. chrysanthemi* contains a cluster of *scr* genes (ID 16549, 16548, 16547, 16546, and 16544) probably organized in three contiguous operons, *scrK-scrYAB-scrR*, and coding for a porin, the enzyme II Scr of the sucrose permease, a sucrose hydrolase, a fructose kinase, and a regulator, respectively. The *E. chrysanthemi* genome includes an incomplete *raf* cluster (ID 19140, 19139, and 19138) probably organized in two contiguous operons, *rafR-rafAB*, and encoding a regulator, an α -galactosidase, and a raffinose permease, respectively. The flags indicate the positions of the *uidA*-Km insertions resulting in gene fusions (triangles) and the Cm insertions in the regulatory genes (circles). The name of the corresponding mutant is given above each insertion. The small drawings indicate the position of sequences homologous to the binding sites of ScrR, RafR, and CRP.

catabolism of sucrose, melibiose, and raffinose. It contains a group of genes homologous to those involved in sucrose assimilation in other enterobacteria. This cluster includes five adjacent genes transcribed in the same direction, *scrKYABR* (Fig. 1). Its products are homologous (55 to 81% identity) to proteins encoded by the chromosome of *Erwinia amylovora* and *Klebsiella pneumoniae* but also by enterobacterial mobile genetic elements, the plasmid pUR400 of *E. coli* or *Salmonella* spp., and the transposon Tn2555 of *E. coli* (2, 6, 9, 11, 17, 31). The genetic organization of these sucrose utilization loci is well conserved (24), having the same gene order as that observed in *E. chrysanthemi* (Fig. 1). In *E. amylovora*, these genes are organized in three transcriptional units, *scrK*, *scrYAB*, and *scrR* (6). This cluster encodes a fructose kinase (ScrK), an outer membrane porin (ScrY), the sucrose-specific enzyme II of a phosphoenolpyruvate-dependent phosphotransferase system (ScrA), a sucrose hydrolase (ScrB), and a regulator of the LacI family (ScrR).

The *E. chrysanthemi* genome also contains a group of three contiguous genes (Fig. 1) whose products share 55 to 73% identity with proteins involved in raffinose catabolism and are encoded by the plasmid pSRD2, which is found in a few *E. coli* strains (1). These plasmidic genes are organized in two adjacent operons, *rafR* and *rafABDY*. The gene *rafR* encodes a regulator of the LacI family, *rafA* encodes an α -galactosidase that cleaves raffinose into galactose and sucrose, *rafB* encodes a transport system of the LacY family, *rafD* encodes a sucrose hydrolase, and *rafY* encodes a porin (1, 33). The *E. chrysanthemi* cluster *raf* includes only the first three genes: *rafR*, *rafA*, and *rafB* (Fig. 1). Thus, in *E. chrysanthemi*, RafD and RafY have to be functionally replaced by another hydrolase and porin, respectively.

Assimilation of sucrose, melibiose, and raffinose by *E. chrysanthemi*. The growth of the *E. chrysanthemi* wild-type strain 3937 was tested in minimal medium supplemented with various sugars as the sole carbon and energy source. Strain 3937 is able to use sucrose, melibiose, and raffinose as carbon sources for growth. In contrast, lactose was unable to support the growth of the wild-type strain. The growth rate was determined in the presence of these oligosaccharides and also with their constitutive monosaccharides, namely glucose, galactose, and fructose (Table 2). Doubling times shorter than 2 h were observed with fructose, glucose, and sucrose, which thus are readily utilizable carbon sources. Melibiose, galactose, and raffinose gave doubling times ranging between 2 and 3 h (Table 2).

Effect of *raf* and *scr* inactivation on oligosaccharide assimilation. The structural genes *rafA*, *rafB*, *scrB*, and *scrY* were inactivated by nonpolar insertions (Fig. 1). The growth phenotypes of the mutants were compared to that of the wild-type strain (Table 2). The *RafA*[−] and *RafB*[−] mutants became unable to grow with either raffinose or melibiose. The *ScrB*[−] mutant was incapable of growth with either sucrose or raffinose. The *ScrY*[−] mutant showed reduced growth with either sucrose or raffinose. The doubling time of the *ScrY*[−] mutant is about threefold longer than that of the wild-type strain in the presence of either sucrose or raffinose (Table 2). These growth data confirmed that the *E. chrysanthemi* genes *raf* and *scr* are involved in the catabolism of raffinose and sucrose, respectively. In addition, they reveal novel roles of these genes. First, the *rafA* and *rafB* genes are responsible for the catabolism of

TABLE 2. Growth phenotype of *E. chrysanthemi* wild-type and mutant strains

Carbon source ^a	Strain ^b							
	3937 (wild type)	A4660 (RafA [−])	A4661 (RafB [−])	A4441 (ScrB [−])	A4659 (ScrY [−])	A4694 (RafR [−])	A4440 (ScrR [−])	A4167 (CRP [−])
Fructose	+ (90 min)	+	+	+	+	+	+	+
Glucose	+ (100 min)	+	+	+	+	+	+	+
Sucrose	+ (110 min)	+	+	−	+/- (300 min)	+	+	+/-
Melibiose	+ (140 min)	−	−	+	+	+	+	−
Galactose	+ (150 min)	+	+	+	+	+	+	−
Raffinose	+ (180 min)	−	−	−	+/- (510 min)	+	+	−
Lactose	−	−	−	−	−	+/- (360 min)	−	−

^a The growth phenotype of each strain, observed after 24 h of growth at 30°C on solid minimal medium containing carbohydrates at 2 g · liter^{−1}, is indicated by plus or minus signs; a plus/minus sign indicates an intermediate phenotype, i.e., reduced growth.

^b The doubling time is indicated next to the plus sign for the wild-type strain and below the plus/minus sign for the *ScrY*[−] and *RafR*[−] mutants. To determine the doubling time, cells were grown aerobically at 30°C in liquid minimal medium containing carbohydrates at 2 g · liter^{−1}.

FIG. 2. Role of the Raf and Scr proteins in the assimilation of sucrose, raffinose, melibiose, and lactose in *E. chrysanthemi*. The structure of the oligosaccharides is given. Scr, sucrose; Raf, raffinose; Mel, melibiose; Lac, lactose; Glc, glucose; Gal, galactose; and Fru, fructose. The addition of 1P or 6P to a sugar abbreviation indicates that it is phosphorylated. ScrY, sucrose and raffinose porin; RafB, raffinose and melibiose permease; ScrA, enzyme II Scr of the sucrose permease; PTS, other components of the phosphoenolpyruvate-dependent phosphotransferase system; RafA, α -galactosidase; ScrB, sucrose hydrolase; and ScrK, fructose kinase.

melibiose. Second, the genes *scrB* and *scrY* are involved in raffinose catabolism.

Even if their name does not reflect their double function, the *raf* genes appear to contribute equally to the catabolism of melibiose and raffinose in *E. chrysanthemi* (Fig. 2). The transporter and the α -galactosidase encoded by *rafBA* are sufficient for melibiose catabolism. Raffinose catabolism needs *rafA*, *rafB*, and two additional steps provided by *scrY* and *scrB*. The porin ScrY facilitates the diffusion of both sucrose and raffinose across the outer membrane. The enzyme ScrB hydrolyzes both sucrose and sucrose-6-phosphate (Fig. 2). Thus, ScrY and ScrB functionally replace the RafY and RafD proteins encoded by the plasmidic raffinose clusters. Previous studies reported functional homologies between Scr and Raf proteins. The substrate range of the plasmid-encoded porins ScrY and RafY were shown to be similar, and since they are not strictly specific, both are able to facilitate the diffusion of sucrose and raffinose (33). Similarly, it was noticed that the hydrolase ScrB resembles RafD in terms of substrate specificity (29). Thus, the genes *rafY* and *rafD* are dispensable when the genome contains the genes *scrY* and *scrB*. This explains the functionality of the incomplete *raf* cluster in *E. chrysanthemi*.

To test a large range of carbon sources, the mutants were compared to the wild-type strain on Biolog Phenotype Microarray plates for carbon catabolism (4). The *RafB*[−] and *ScrB*[−] mutants were affected only for the phenotypes reported in Table 2. The *ScrY*[−] mutant had no visible phenotype. The *RafA*[−] mutant appeared unable to assimilate α -methyl-D-galactoside in addition to raffinose and melibiose. Thus, the

α -galactosidase *RafA* also is necessary for the cleavage of α -methyl-D-galactoside.

Effect of regulator mutations on oligosaccharide assimilation. Since the cyclic AMP receptor protein (CRP) is involved in the global control of carbohydrate catabolism, the assimilation of several sugars is abolished in a CRP[−] mutant. An *E. chrysanthemi* CRP[−] mutant was shown to be able to use glucose but was unable to grow with either glycerol, gluconate, galacturonate, or polygalacturonate as the sole carbon source (26). We tested its growth phenotype with other sugars (Table 2). The CRP[−] mutant is unable to use raffinose, melibiose, and galactose. However, it remains able to assimilate fructose, showing normal growth, or sucrose, with decreased growth. Thus, the global regulator CRP is necessary for the activation of the catabolism of both raffinose and melibiose. CRP also is involved in sucrose catabolism, but with a weaker effect.

The potential repressor genes associated with each cluster, *scrR* and *rafR*, were inactivated by the insertion of a nonpolar Cm^r cassette (Fig. 1). The *RafR*[−] and *ScrR*[−] mutants showed normal growth with sucrose, melibiose, raffinose, fructose, glucose, or galactose (Table 2). In contrast, the *RafR*[−] mutant has acquired the capacity to use lactose as the sole carbon source for growth. This observation suggested a direct role of the *raf* cluster in lactose assimilation.

Using the Biolog Phenotype Microarray plates for carbon catabolism (4), the *ScrR*[−] mutant had no visible modified phenotype. The *RafR*[−] mutant became able to assimilate lactose and also lactulose [β -D-Galp-(1 $\rightarrow$ 4)-D-Fruf], melibionidic acid [α -D-Galp-(1 $\rightarrow$ 6)-D-GlcA], and stachyose [α -D-Galp-(1 $\rightarrow$ 6)- α -

TABLE 3. α -Galactosidase activity in *E. chrysanthemi* cells grown in the presence of different carbon sources

Carbon source	Sp act (nmol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹) of strain ^a :		
	3937 (wild type)	A4441 (RafR ⁻)	A350 (Lac ⁺)
Glycerol	2	1,514	1,520
Raffinose	225	1,205	1,179
Melibiose	519	1,115	1,206
Galactose	43	1,647	1,448

^a Strains were grown to late exponential growth phase in minimal medium containing carbohydrates at 2 g $\cdot$ liter⁻¹. The results shown are the averages from at least three independent experiments with standard deviations corresponding to less than 20%.

D-Galp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 2) β -D-Fruc]. The RafR⁻ mutant also showed increased growth with β -methyl-D-galactoside. Thus, the derepression of the *raf* genes could trigger the assimilation of various oligosaccharides related to their substrates, melibiose and raffinose. The *raf* cluster facilitates either the uptake of these compounds (lactose, lactulose, and β -methyl-D-galactoside), their cleavage (α -methyl-D-galactoside), or both activities (melibiononic acid and stachyose).

Role of RafB in lactose assimilation. Lac⁺ derivatives of strain 3937 results from the deregulation of a nonspecific transport system previously named LmrT (15). The ability of the RafR⁻ mutant to grow with lactose raises two hypotheses: (i) the Lac⁺ derivatives arise from RafR inactivation, and (ii) LmrT corresponds to RafB.

To verify the first hypothesis, 12 spontaneous Lac⁺ derivatives of the strain 3937 were analyzed. The strain A350 (also named L2), which often has been used to construct *lacZ* gene fusions, was included in this analysis. This strain is a spontaneous Lac⁺ mutant (*lmrTc*) that then was submitted to mutagenesis to inactivate the main β -galactosidase activity (16). Considering their genetic organization, the genes *rafA* and *rafB* probably are cotranscribed from a RafR-regulated promoter situated upstream of *rafA* (Fig. 1). The *rafA* expression was tested by a direct α -galactosidase assay. In the RafA⁻ mutant, no α -galactosidase activity could be detected, confirming that this activity is due entirely to RafA. In the strain 3937, the α -galactosidase activity was very low in the presence of glycerol as the carbon source, but it increased in the presence of raffinose, melibiose, or galactose (Table 3). In the RafR⁻ mutant, α -galactosidase was produced at a high level in all growth conditions used (Table 3). In the 12 Lac⁺ derivatives tested, the α -galactosidase activity also was highly derepressed (Table 3). Thus, the Lac⁺ phenotype is correlated with the derepression of *rafA*. Most of the spontaneous Lac⁺ mutants are RafR⁻ mutants.

The RafB⁻ mutant was unable to provide Lac⁺ derivatives, while they were obtained at the frequency of 10⁻⁷ with the wild-type strain. While the RafR⁻ mutant is able to assimilate lactose, the RafR⁻ RafB⁻ double mutant has lost this capacity. Thus, an intact *rafB* gene is required for lactose uptake. The transport system previously named LmrT is, in fact, RafB. This also is consistent with biochemical studies showing that LmrT is more specific to melibiose and raffinose than to lactose (10, 15). Lactose cannot induce the *raf* genes but will be taken up through RafB when expressed, as in the RafR⁻ mutant. In a previous report, it was shown that the β -galactosidase activity

TABLE 4. Inhibition of RafB and RafA activities by different sugars

Assay and inhibitor ^a	Relative activity ^b (%)
[¹⁴ C]lactose uptake ^c	
None	100
Raffinose	65
Melibiose	5
Sucrose	100
Galactose	10
Glucose	100
pNP α Gal cleavage ^d	
None	100
Raffinose	86
Melibiose	43
Sucrose	100
Galactose	77
Glucose	85

^a Each inhibitor was added at a final concentration of 10 mM immediately before the start of the reaction.

^b The data reported are the averages from three repetitions, with standard deviations corresponding to less than 20% for the uptake assay and less than 5% for the enzyme assay.

^c The uptake of [¹⁴C]lactose (1 mM), reflecting the RafB activity, was measured on whole cells of the RafR⁻ mutant grown in glycerol minimal medium.

^d The α -galactosidase activity (RafA) was assayed on extracts of the RafR⁻ mutant grown in glycerol minimal medium, using 1 mM pNP α Gal as the substrate. One hundred percent activity corresponded to about 1,500 nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹.

of *E. chrysanthemi* is due mostly to GanB, an enzyme involved in galactane degradation (8). Thus, in this enterobacterium adapted to a plant host, lactose assimilation results from the secondary activities of proteins involved in the catabolism of plant oligosaccharides. The truncated lactose operon found in the *E. chrysanthemi* genome (*lacI-lacZ*) has minor functional significance.

Specificity of RafB and RafA for raffinose and melibiose. Since RafB is responsible for lactose transport in *E. chrysanthemi*, its activity was measured by the assay of [¹⁴C]lactose uptake in whole cells of a RafR⁻ mutant grown in glycerol minimal medium. To assess the RafB specificity, [¹⁴C]lactose uptake was assayed in the presence of a 10-fold excess of unlabeled oligosaccharides used as competitors (Table 4). Melibiose was a strong inhibitor of lactose uptake. Raffinose inhibited lactose uptake to a lesser extent. Sucrose did not exhibit any effect. This inhibition pattern suggests that RafB has a higher affinity for the disaccharide melibiose than for the trisaccharide raffinose.

To analyze the affinity of RafA for melibiose and raffinose, inhibition experiments were performed using the α -galactosidase assay based on the cleavage of a chromogenic substrate (Table 4). This reaction was strongly inhibited by melibiose, and it was weakly inhibited by raffinose, glucose, and galactose. Sucrose had no effect. RafA thus is inhibited by its substrates and by its reaction products, glucose and galactose. The comparison of melibiose and raffinose effects suggests that RafA has more affinity for the disaccharide than for the trisaccharide.

These biochemical data indicate that, despite their names, the *rafA* and *rafB* genes of *E. chrysanthemi* are better adapted to melibiose catabolism than to raffinose catabolism. This also

TABLE 5. Expression of *raf* transcriptional fusions in *Erwinia chrysanthemi*

Mutation and growth condition	Sp act (nmol · min ⁻¹ · mg ⁻¹) of strain ^a :	
	<i>rafA::uidA</i>	<i>rafB::uidA</i>
None		
Glycerol	49	20
Glycerol + raffinose	4,369	40
Glycerol + melibiose	4,019	88
Galactose	2,662	42
Glucose	5	12
Glucose + raffinose	535	15
RafR ⁻		
Glycerol	9,426	3,019
Glycerol + raffinose	9,918	3,413
CRP ⁻		
Glucose	4	12
Glucose + raffinose	17	18

^a The results reported for β-glucuronidase activities are the averages from at least three independent experiments with standard deviations corresponding to less than 20%, except for very low activities (<10 nmol · min⁻¹ · mg⁻¹), where the standard deviations were up to 40%.

could be true in other bacteria in which the *raf* genes have been identified previously and in which melibiose catabolism was not analyzed simultaneously. In *Enterobacter cloacae*, the RafB homologue (77% identity) was named MelY (22), since it was identified as being a melibiose transporter, but its role in raffinose assimilation has not been tested.

Expression of the *rafA* and *rafB* genes. The insertion of *uidA*-Km cassettes in the *rafA* and *rafB* genes allowed us to inactivate these genes and also to obtain transcriptional fusions (3). The expression of these fusions was monitored in various conditions (Table 5). The transcription of the *rafA* fusion strongly increased in the presence of raffinose, melibiose, and galactose (Table 5). The direct assay of α-galactosidase also indicated that RafA synthesis is induced in the presence of these sugars (Table 3). Since the RafA⁻ mutant is unable to metabolize raffinose and melibiose (Table 2), this induction indicates that the oligosaccharides are inducers without further cleavage by RafA (Fig. 2). The low induction of the *rafB* fusion in the presence of the *rafA* inducers (Table 5) could be explained by the absence of the specific uptake of the sugars when the RafB transporter is inactivated. Sucrose, lactose, or fructose did not induce the *rafA* or *rafB* fusions (data not shown).

To analyze the potential effect of the regulators, the *rafA* and *rafB* fusions were transferred to RafR⁻ and CRP⁻ mutants using transduction with the φEC2 phage. When the RafR repressor was inactivated, the expression of the *rafA* and *rafB* fusions highly increased in the absence of inducers (Table 5). The decreased *rafA* and *rafB* expression in the presence of glucose and in the CRP⁻ mutant (Table 5) indicated that these genes are submitted to catabolite repression and that CRP is necessary for their activation. This is consistent with the fact that a CRP⁻ mutant is unable to use raffinose or melibiose as its sole carbon and energy source (Table 2). The examination of the *raf* cluster for conserved regulatory sequences revealed the presence of potential CRP and RafR binding sites in the 5'

TABLE 6. Expression of *scr* transcriptional fusions in *Erwinia chrysanthemi*

Mutation and growth condition	Sp act (nmol · min ⁻¹ · mg ⁻¹) of strain ^a :	
	<i>scrB::uidA</i>	<i>scrY::uidA</i>
None		
Glycerol	56	6
Glycerol + raffinose	69	1,175
Glycerol + sucrose	68	3,330
Fructose	81	46
Glucose	31	1
Glucose + raffinose	43	352
Glucose + sucrose	36	1,483
ScrR ⁻		
Glycerol	7,528	11,722
Glycerol + raffinose	3,533	9,821
Glycerol + sucrose	2,478	8,443
CRP ⁻		
Glucose	28	1
Glucose + raffinose	33	3
Glucose + sucrose	40	112

^a The results reported for β-glucuronidase activities are the averages from at least three independent experiments with standard deviations corresponding to less than 20%, except for very low activities (<10 nmol · min⁻¹ · mg⁻¹), where the standard deviations were up to 40%.

noncoding end of *rafA* (Fig. 1). The nucleotide sequence upstream of *rafA* contains two repeated 14-bp sequences (CCG AAACGTTTCGG) included in the imperfect palindrome identified as the RafR binding site (1). This suggests that regulation by RafR in *E. chrysanthemi* results from a mechanism similar to that previously characterized in *E. coli* (21). The absence of a potential CRP or RafR binding site in the 5' noncoding end of *rafB*, despite its *in vivo* regulation by CRP and RafR (Table 5), is a strong indication that *rafB* is cotranscribed with *rafA* and that these regulation events occur at the level of the *rafA* promoter.

Expression of the *scrB* and *scrY* genes. Transcriptional fusions in the *scrB* and *scrY* genes also were obtained by the insertion of *uidA*-Km cassettes. The transcription of the *scrY* fusion was highly stimulated in the presence of sucrose and raffinose (Table 6). Since the inactivation of the porin ScrY reduces the growth rate with each of these oligosaccharides (Table 1), it decreases their uptake without preventing induction. The transcription of the *scrB* fusion was not significantly affected by the addition of sucrose or raffinose (Table 6). This absence of induction suggests that ScrB is necessary for the formation of the intracellular inducer, with such formation being prevented in the ScrB⁻ mutant. The sucrose hydrolase ScrB cleaves sucrose-6-phosphate to give glucose-6-phosphate and fructose (Fig. 2). In *E. amylovora*, the inducer of the *scr* cluster is supposed to be fructose (6). In *E. chrysanthemi*, the addition of fructose to the culture medium provoked only a low induction of the *scrY* fusion (Table 6). Extracellular fructose is transported into the cells by a specific phosphoenolpyruvate-dependent phosphotransferase system; this transport is concomitant with substrate phosphorylation, resulting in the intracellular formation of fructose-1-phosphate (Fig. 2). Thus, fructose-1-phosphate is not a good inducer of the *scrY* gene. In *E. chrysanthemi*, the true *scr* inducers are formed after the ScrB

FIG. 3. Infection of chicory leaves with the *Raf*⁻ and *Scr*⁻ mutants. (A) After a 24-h infection with each mutant and the wild-type strain (WT), the length of rotted tissue was measured to estimate the disease severity. The mean values and the standard deviations reported correspond to 12 leaves inoculated with each strain. (B) After infection, the macerated tissue was recovered in order to perform a β -glucuronidase (GUS) assay and bacterial cell numeration to estimate the expression of the *uidA* fusions. Specific activity is expressed as nanomoles of product liberated per minute per 10^9 bacteria. The results reported are the mean values from 12 leaves with the corresponding standard deviations. (C) To analyze the potential competition between a mutant (*Km*⁻) and the wild-type strain, chicory leaves were inoculated with a mixture of the two strains at a ratio of 50/50. Macerated tissues were recovered after 24 h, and the ratio of mutant to wild-type strain was determined after serial dilutions on LB medium and replica plating on LB *Km* medium. The data given (percent mutant) are the means obtained with four leaves and the corresponding standard deviations.

step; they are either fructose, fructose-6-phosphate (formed by *ScrK*), or both compounds (Fig. 2). The addition of glucose decreased both *scrB* and *scrY* expression (Table 6), indicating that these genes are submitted to catabolite repression.

To verify the functionality of the *ScrR* repressor, the fusions were transferred by transduction into the *ScrR*⁻ mutant. The expression of the *scrB* and *scrY* fusions strongly increased in the *ScrR*⁻ mutant, surpassing the levels observed in the wild-type background (Table 6). The expression of *scrB* or *scrY* decreased in the *CRP*⁻ mutant (Table 6), indicating that *CRP* is involved in their activation. The examination of the *scr* cluster revealed a sequence homologous to the consensus of the *E. coli* *CRP* binding site (7) in the 5' noncoding end of *scrY* and revealed a sequence homologous to the palindrome involved in *ScrR* binding (6, 17) upstream of *scrB* and *scrY* (Fig. 1). The absence of this palindrome upstream of *scrB* or *scrA* suggests that these two genes are cotranscribed with *scrY*.

The analysis of the *scrB* and *scrY* fusions in the *RafR*⁻ mutant indicated that *RafR* is not involved in the control of these genes. Similarly, the analysis of the *rafA* and *rafB* fusions in the *ScrR*⁻ mutant indicated that *ScrR* is not involved in the control of these genes either (data not shown). Despite common steps in the catabolism of raffinose and sucrose, *scr* and *raf* genes are independently controlled by *ScrR* and *RafR*, respectively.

Virulence of the *Raf*⁻ and *Scr*⁻ mutants and expression of the fusions during plant infection. Chicory leaves were infected with the *Raf*⁻ and *Scr*⁻ mutants to analyze the effect of

the mutations on virulence. We observed no significant difference in the degree of maceration caused by the *RafA*⁻, *RafB*⁻, *ScrB*⁻, or *ScrY*⁻ mutant and the parental strain 3937 (Fig. 3A). To determine whether the genes are expressed during infection, the expression of *rafA*, *rafB*, *scrB*, and *scrY* transcriptional fusions was assayed in the macerated tissues. A fusion in the highly inducible pectate lyase gene *pelD* (14) was used as the internal control. While no *rafB* expression was detected, the *rafA*, *scrB*, and *scrY* fusions all were expressed in the macerated tissue (Fig. 3B). As shown previously, the absence of the uptake of the substrates in the *RafB*⁻ mutant prevents the induction of the fusion. The induction of *rafA* during infection shows that at least one of the substrates, raffinose or melibiose, is present in the macerated tissue. Similarly, the induction of the *scrY* fusion indicates that sucrose and/or raffinose is available in the macerated tissue. These oligosaccharides may provide nutritional resources for bacterial growth in planta. Bacterial numerations were performed 24 h after infection. No significant difference was observed between the cell densities of the mutants and the wild-type strain; the bacterial populations reached a level of about 4×10^9 bacteria per g of macerated tissue with each strain. The absence of an effect of the *Raf*⁻ or *Scr*⁻ mutation on bacterial multiplication is quite surprising, considering that sucrose and raffinose are available in the plant tissues. To highlight any fine differences between the mutants and the wild-type strain, we analyzed the ability of each mutant to compete with the wild-type strain during infection. The *KdgM*⁻ mutant, which is affected in the uptake of

pectic oligomers (5), was used as the control. The *RafA*[−], *RafB*[−], or *PeiD*[−] mutant was able to multiply as well as the wild-type strain during infection (Fig. 3C). In contrast, the *KdgM*[−] and *ScrY*[−] mutants were significantly impaired in their ability to compete with the wild-type strain. The decrease in the competitive ability of the *KdgM*[−] mutant reflects the fact that oligogalacturonides are used by *E. chrysanthemi* for its multiplication in planta. Similarly, the decreased competitive capacity of the *ScrY*[−] mutant suggests that the entry of sucrose and/or raffinose favors *E. chrysanthemi* growth in plant tissues. The inactivation of a catabolic pathway becomes apparent when the mutant has to compete with the wild-type strain. The variety of carbon sources available in the macerated tissues conceals the effect of a single mutation on the bacterial population.

ACKNOWLEDGMENTS

We acknowledge members of the International *Erwinia* Consortium for the exchange of unpublished data concerning the *E. chrysanthemi* strain 3937 genome sequence. We thank Geraldine Effantin for her assistance with some experiments. We thank the ParMic Platform of UMR 5557 for the use of the Biolog equipment.

This work was supported by grants from the Centre National de la Recherche Scientifique and from the Ministère de l'Éducation Nationale et de la Recherche.

REFERENCES

- Aslanidis, C., K. Schmid, and R. Schmitt. 1989. Nucleotide sequences and operon structure of plasmid-borne genes mediating uptake and utilization of raffinose in *Escherichia coli*. *J. Bacteriol.* **171**:6753–6763.
- Aulkemeyer, P., R. Ebner, G. Heilenmann, K. Jahreis, K. Schmid, S. Wrieden, and J. W. Lengeler. 1991. Molecular analysis of two fructokinases involved in sucrose metabolism of enteric bacteria. *Mol. Microbiol.* **5**:2913–2922.
- Bardonnet, N., and C. Blanco. 1992. *uidA* antibiotic resistance cassettes for insertion mutagenesis, gene fusion and genetic constructions. *FEMS Microbiol. Lett.* **93**:243–248.
- Bochner, B. R. 2009. Global phenotypic characterization of bacteria. *FEMS Microbiol. Rev.* **33**:191–205.
- Blot, N., C. Berrier, N. Hugouvieux-Cotte-Pattat, A. Ghazi, and G. Condemine. 2002. The oligogalacturonate-specific porin KdgM of *Erwinia chrysanthemi* 3937 belongs to a new porin family. *J. Biol. Chem.* **277**:7936–7944.
- Bogs, J., and K. Geider. 2000. Molecular analysis of sucrose metabolism of *Erwinia amylovora* and influence on bacterial virulence. *J. Bacteriol.* **182**:5351–5358.
- Chen, S., A. Gunasekera, X. Zhang, T. A. Kunkel, R. H. Ebricht, and H. M. Berman. 2001. Indirect readout of DNA sequence at the primary-kink site in the CAP-DNA complex: alteration of DNA binding specificity through alteration of DNA kinking. *J. Mol. Biol.* **314**:75–82.
- Delangle, A., A. F. Prouvest, V. Cogez, J. P. Bohin, J. M. Lacroix, and N. Hugouvieux-Cotte-Pattat. 2007. Characterization of the *Erwinia chrysanthemi* gan locus involved in galactan catabolism. *J. Bacteriol.* **189**:7053–7061.
- Doroshenko, V. G., and V. A. Livshits. 2004. Structure and mode of transposition of Tn2555 carrying sucrose utilization genes. *FEMS Microbiol. Lett.* **233**:353–359.
- Gray, J. S., W. A. Lindner, J. M. Brand, and J. P. Mildenhall. 1986. Lactose and melibiose metabolism in *Erwinia chrysanthemi*. *J. Bacteriol.* **168**:886–891.
- Hardesty, C., C. Ferran, and J. M. DiRienzo. 1991. Plasmid-mediated sucrose metabolism in *Escherichia coli*: characterization of *scrY*, the structural gene for a phosphoenolpyruvate-dependent sucrose phosphotransferase system outer membrane porin. *J. Bacteriol.* **173**:449–456.
- Hugouvieux-Cotte-Pattat, N. 2004. The RhaS activator controls the *Erwinia chrysanthemi* 3937 genes *rhiN*, *rhiT* and *rhiE* involved in rhamnogalacturonan catabolism. *Mol. Microbiol.* **51**:1361–1374.
- Hugouvieux-Cotte-Pattat, N., G. Condemine, W. Nasser, and S. Reverchon. 1996. Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annu. Rev. Microbiol.* **50**:213–257.
- Hugouvieux-Cotte-Pattat, N., H. Dominguez, and J. Robert-Baudouy. 1992. Environmental conditions affect the transcription of the pectinases gene of *Erwinia chrysanthemi* 3937. *J. Bacteriol.* **174**:7807–7818.
- Hugouvieux-Cotte-Pattat, N., and J. Robert-Baudouy. 1985. Lactose catabolism in *Erwinia chrysanthemi*. *J. Bacteriol.* **162**:248–255.
- Hugouvieux-Cotte-Pattat, N., and J. Robert-Baudouy. 1985. Isolation of *kdgK-lac* and *kdgA-lac* gene fusions in the phytopathogenic bacteria *Erwinia chrysanthemi*. *J. Gen. Microbiol.* **131**:1205–1211.
- Jahreis, K., and J. W. Lengeler. 1993. Molecular analysis of two ScrR repressors and of a ScrR-FruR hybrid repressor for sucrose and D-fructose specific regulons from enteric bacteria. *Mol. Microbiol.* **9**:195–209.
- Lebeau, A., S. Reverchon, S. Gaubert, Y. Kraepiel, E. Simon-Cote, W. Nasser, and F. Van Gijsegem. 2008. The GacA global regulator is required for the appropriate expression of *Erwinia chrysanthemi* 3937 pathogenicity genes during plant infection. *Environ. Microbiol.* **10**:545–559.
- Lojkowska, E., C. Dorel, P. Reignault, N. Hugouvieux-Cotte-Pattat, and J. Robert-Baudouy. 1993. Use of GUS fusions to study the expression of *Erwinia chrysanthemi* pectinase genes during infection of potato tubers. *Mol. Plant-Microbe Interact.* **6**:488–494.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Muiznieks, L., N. Rostoks, and R. Schmitt. 1999. Efficient control of *raf* gene expression by CAP and two Raf repressors that bend DNA in opposite directions. *Biol. Chem.* **380**:19–29.
- Okazaki, N., X. X. Jue, H. Miyake, M. Kuroda, T. Shimamoto, and T. Tsuchiya. 1997. A melibiose transporter and an operon containing its gene in *Enterobacter cloacae*. *J. Bacteriol.* **179**:4443–4445.
- Perombelon, M. C. M., and A. Kelman. 1980. Ecology of the soft rot erwinias. *Annu. Rev. Phytopathol.* **18**:361–387.
- Reid, S. J., and V. R. Abratt. 2005. Sucrose utilisation in bacteria: genetic organisation and regulation. *Appl. Microbiol. Biotechnol.* **67**:312–321.
- Resibois, A., M. Colet, M. Faalen, E. Schoonejans, and A. Toussaint. 1984. ϕ EC2, a new generalized transducing phage of *Erwinia chrysanthemi*. *Virology* **137**:102–112.
- Reverchon, S., D. Expert, J. Robert-Baudouy, and W. Nasser. 1997. The cyclic AMP receptor protein is the main activator of pectinolysis genes in *Erwinia chrysanthemi*. *J. Bacteriol.* **179**:3500–3508.
- Roeder, D. L., and A. Collmer. 1985. Marker-exchange mutagenesis of pectate lyase isozyme gene in *Erwinia chrysanthemi*. *J. Bacteriol.* **164**:51–56.
- Sambrook, J. E., F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schmid, K., M. Schupfner, and R. Schmitt. 1982. Plasmid-mediated uptake and metabolism of sucrose by *Escherichia coli* K-12. *J. Bacteriol.* **151**:68–76.
- Schmitt, R. 1968. Analysis of melibiose mutants deficient in alpha-galactosidase and thiomethylgalactoside permease II in *Escherichia coli* K-12. *J. Bacteriol.* **96**:462–471.
- Titgemeyer, F., K. Jahreis, R. Ebner, and J. W. Lengeler. 1996. Molecular analysis of the *scrA* and *scrB* genes from *Klebsiella pneumoniae* and plasmid pUR400, which encode the sucrose transport protein enzyme II Scr of the phosphotransferase system and a sucrose-6-phosphate invertase. *Mol. Genet.* **250**:197–206.
- Trugo, L. C., A. Farah, and L. Cabral. 1995. Oligosaccharide distribution in Brazilian soya bean cultivars. *Food Chem.* **52**:385–387.
- Ulmke, C., J. W. Lengeler, and K. Schmid. 1997. Identification of a new porin, RafY, encoded by raffinose plasmid pRSD2 of *Escherichia coli*. *J. Bacteriol.* **179**:5783–5788.
- Van Gijsegem, F., A. Włodarczyk, A. Cornu, S. Reverchon, and N. Hugouvieux-Cotte-Pattat. 2008. Analysis of the LacI family regulators of *Erwinia chrysanthemi* 3937, involvement in the bacterial phytopathogenicity. *Mol. Plant-Microbe Interact.* **21**:1471–1481.