Isolation of *kdgK*–*lac* and *kdgA*–*lac* Gene Fusions in the Phytopathogenic Bacterium *Erwinia chrysanthemi*

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Erwinia chrysanthemi is a species of enterobacteria whose phytopathogenicity is mainly due to its pectinolytic activity. Pectin degradation leads to the formation of 2-keto-3-deoxygluconate which is catabolized via the *kdgA* and *kdgK* gene products. Mutants of *Er. chrysanthemi* containing genetic fusions of the *kdgK* or *kdgA* genes to the *lacZ* gene of *Escherichia coli* were isolated by infection of a *lacZ* mutant of *Er. chrysanthemi* with the phage Mu d(Ap *lac*). In these fusion strains, the absence of growth on galacturonate, glucuronate and polygalacturonate, and also β -galactosidase expression, are caused by a single Mu d(Ap *lac*) insertion. Synthesis of β -galactosidase in these strains was induced in the presence of galacturonate, glucuronate, polygalacturonate or some intermediates of the catabolism of these sugars in the culture medium; synthesis of β -galactosidase was not sensitive to glucose repression.

INTRODUCTION

Erwinia chrysanthemi is a phytopathogenic species of enterobacteria responsible for soft-rot disease of many plant species. Its pathogenicity is chiefly due to its pectinolysis of plant cell walls (Starr & Chatterjee, 1972; Chatterjee & Starr, 1980). Pectins are degraded according to the pathway in Fig. 1, being first demethoxylated into polygalacturonate (PGA) which is cleaved to give galacturonate (GalUA) and 5-keto-4-deoxyuronate (DKI). The catabolism of these two sugars leads to the formation of 2-keto-3-deoxygluconate (KDG) which is phosphorylated by enzyme V (Fig. 1) and then split by the specific aldolase VI (Fig. 1) into pyruvate and triose phosphate. In *Escherichia coli*, as in some *Erwinia* strains, the catabolism of another hexuronate, glucuronate (GlcUA) occurs by a pathway parallel to the GalUA degradation route which leads to KDG formation (Fig. 1).

In *E. coli* K12 regulation of the degradation of GalUA, GlcUA or KDG has been extensively analysed (Pouyssegur & Stoeber, 1974; Portalier *et al.*, 1980; Robert-Baudouy *et al.*, 1981; Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1981, 1983). In *Erwinia carotovora* this pathway, first described by Kilgore & Starr (1959), seems very similar to that in *E. coli*. Recently the genes of *Er. chrysanthemi* involved in GalUA, GlcUA and KDG catabolism, including the *kdgK* and *kdgA* genes, have been cloned on RP4::miniMu plasmids by complementation of *E. coli* mutations (Van Gijsegem & Toussaint, 1983), and mutants of *Er. chrysanthemi* blocked in different steps of GalUA and KDG catabolism have been isolated and analysed (Van Gijsegem *et al.*, 1985). The genes *kdgK* and *kdgA* appeared to be unlinked to the other genes involved in GalUA catabolism, *exuT*, *uxaC*, *uxaB* and *uxaA*, which map together near the *thy* marker on the *Er. chrysanthemi* genetic map. The *kdgK* and *kdgA* genes map near *ile* and *trp* respectively.

Abbreviations: GalUA, D-galacturonate; GlcUA, D-glucuronate; PGA, polygalacturonate; KDG, 2-keto-3-deoxy-D-gluconate; KDGP, 2-keto-3-deoxy-6-phospho-D-gluconate; DKI, 5-keto-4-deoxy-D-uronate (or 4-deoxy-L-threo-5-hexosulose uronate); DKII, 2,5-diketo-3-deoxy-D-gluconate (or 3-deoxy-D-glycero-2,5-hexodiulosonate); ONPG, *o*-nitrophenyl- β -D-thiogalactoside.

In this study we used the Mu d(Ap *lac*) phage (Casadaban & Cohen, 1979) to construct *lac* gene fusions in order to analyse the regulation of pectin degradation in *Er. chrysanthemi*. The *Er. chrysanthemi* strain we used is sensitive to phage Mu, which is not the case for all the strains of this species (Faelen *et al.*, 1981). Furthermore, wild-type strains of *Er. chrysanthemi* possess β -galactosidase activity although they are unable to use lactose as carbon source. In order to isolate *Er. chrysanthemi lacZ* mutants, we first obtained lactose-fermenting derivatives (Lac⁺) which have acquired a constitutive transport system for lactose; these Lac⁺ strains were then mutagenized to obtain Lac⁻ mutants, some of which lacked β -galactosidase activity (Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1985). The isolation of *Er. chrysanthemi lacZ* mutants permitted us to construct *lac* gene fusions. We report here that the Mu d(Ap *lac*) phage is useful for constructing *lac* operon fusions in *Er. chrysanthemi* analogous to those in *E. coli*. We have isolated *kdgK-lac* and *kdgA-lac* fusions showing β -galactosidase expression inducible by GalUA, GlcUA or PGA, the three substrates leading to KDG formation.

METHODS

Strains. The bacterial strains and bacteriophage used are listed in Table 1. All the *Er. chrysanthemi* strains originated from the wild-type strain 3937j (Kotoujansky *et al.*, 1982). This strain is unable to use glucuronate (GlcUA) as carbon source for growth; mutants utilizing this sugar (Gur⁺), such as A40, were obtained spontaneously at a frequency of about 10⁻⁵ after a few days. A40 expressed constitutively the enzymes II, III, IV (Fig. 1) and the hexuronate transport system H (Hugouvieux-Cotte-Pattat *et al.*, 1983) which permits the entry of GlcUA into the cells. A40 is also a spontaneous mutant able to grow on lactose (Lac⁺), but the Gur⁺ and Lac⁺ phenotypes were obtained in two independent steps.

Media and growth conditions. Cells were usually grown at 30 °C in complete medium L (Miller, 1972) or in synthetic medium M63 (Miller, 1972) supplemented with a carbon source (0.2%) and, when necessary, amino acids (40 µg ml⁻¹), bases (2–50 µg ml⁻¹) or biotin (1 µg ml⁻¹).

Enzyme assays. β -Galactosidase was assayed in exponentially growing cells after toluene treatment as described by Miller (1972) except that *o*-nitrophenyl- β -D-thiogalactoside (ONPG) cleavage was followed spectrometrically at 420 nm. Enzymes V (2-keto-3-deoxygluconate kinase) and VI (2-keto-3-deoxy-6-phosphogluconate aldolase) were measured in French press extracts according to previously published methods (Pouyssegur & Stoeber, 1971).

Chemicals. 2-Keto-3-deoxygluconate (KDG) and 2-keto-3-deoxy-6-phosphogluconate (KDGP), necessary for assays of enzyme V and VI, were prepared from glucuronate according to the procedures described by Pouyssegur (1973).

Preparation of Mu d(Ap *lac*) phage lysates. Mu d(Ap *lac*) lysates were prepared by heat induction of the dilysonic *E. coli* strain MAL103 (Casadaban & Cohen, 1979). Only fresh lysates, titrating more than 5 × 10⁸ plaque forming units ml⁻¹ on *E. coli* strain MC4100, were used for infection.

Table 1. *Bacterial strains, bacteriophages and plasmids*

Strain	Genotype or phenotype	Origin
<i>Er. chrysanthemi</i>		
3937j	Wild-type	Kotoujansky <i>et al.</i> (1982)
A40	Lac2 ⁺ Gur2 ⁺	Spontaneous mutant of 3937j
L37	as A40 but <i>lacZ</i> 37	Mutants of A40
L50	as A40 but <i>lacZ</i> 50	
A233	as L37 but <i>kdgK</i> ::Mu d(Ap <i>lac</i>)	This work
A236	as L50 but <i>kdgK</i> ::Mu d(Ap <i>lac</i>)	
A237	as L50 but <i>kdgK</i> ::Mu d(Ap <i>lac</i>)	
A242	as L37 but <i>kdgK</i> ::Mu d(Ap <i>lac</i>)	
A238	as L37 but <i>kdgA</i> ::Mu d(Ap <i>lac</i>)	
<i>E. coli</i>		
MC4100	F ⁻ , <i>araD</i> 139 <i>lacU</i> 169 <i>rpsL</i> Thi ⁻	Casadaban & Cohen (1979)
MAL103	F ⁻ , Δ (<i>pro-lac</i>)XIII <i>rpsL</i> Thi ⁻ (Mu d(Ap <i>lac</i>)/Mu cts)	
Phages		
Mu d(Ap <i>lac</i>)	<i>cts</i> d1(Ap ^r <i>lac</i>)	Casadaban & Cohen (1979)
phiEC2		Resibois <i>et al.</i> (1984)
Plasmids		
pULB113	<i>tra</i> ⁺ <i>bla</i> ⁺ (TEM-2) Tn1 <i>ter</i> ⁺ <i>aphA</i> ⁺ (Mu 3A)	Van Gijsegem & Toussaint (1983)
pULB118	pULB113 carrying <i>exuT</i> <i>uxaC</i> <i>uxaB</i> <i>uxaA</i>	
pULB119	pULB113 carrying <i>kdgK</i>	
pULB120	pULB113 carrying <i>kdgA</i>

Fig. 1. Degradative pathway of pectin and polygalacturonate in *Erwinia chrysanthemi*. The different steps are catalysed by the following enzymes: A, pectin methylsterase (EC 3.1.1.11); B, polygalacturonase (EC 3.2.1.15); C and E, polygalacturonate and pectin transeliminase (EC 4.2.2.2); D, oligouronide transeliminase (EC 4.2.2.6); F, 5-keto-4-deoxyuronate isomerase (EC 5.3.1.17); G, 2-keto-3-deoxygluconate oxidoreductase (EC 1.1.1.127); H, hexuronate transport system; II, uronate isomerase (EC 5.3.1.12); III, mannonate oxidoreductase (EC 1.1.1.57); IV, mannonate hydrolyase (EC 4.2.1.8); III', altronate oxidoreductase (EC 1.1.1.58); IV', altronate hydrolyase (EC 4.2.1.7); V, 2-keto-3-deoxygluconate kinase (EC 2.7.1.45); VI, 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14). The gene symbols to the left of the arrows denote the corresponding structural genes.

Isolation of Mu d(Ap lac) lysogens. A fresh saturated culture of the recipient strain was infected with Mu d(Ap lac) lysates for 20 min at 30 °C (Casadaban & Cohen, 1979). CaCl₂ (5 mM) and MgSO₄ (10 mM) were usually added. The multiplicity of infection was 1 by calculation from plaque forming units of the lysate on *E. coli* MC4100. To allow the expression of ampicillin resistance, the mixture was diluted tenfold in L broth and incubated with shaking for 30 min at 30 °C. Ampicillin resistant lysogens were selected by plating the appropriate dilution on L agar plates containing ampicillin, at 25 µg ml⁻¹ for *E. coli*, and at 10 µg ml⁻¹ for *Er. chrysanthemi*, which is inhibited by higher concentrations.

Transductions and matings. Transductions with the *Er. chrysanthemi* generalized transducing phage phi-EC2 were carried out according to Resibois *et al.* (1984). Matings for plasmid transfer were made according to Van Gijssel & Toussaint (1983).

RESULTS AND DISCUSSION

Isolation of Mu d(Ap lac) lysogens

After infection with Mu d(Ap lac), ampicillin resistant lysogens were found at a frequency 100 times lower for *Er. chrysanthemi* than for *E. coli*. Titration of Mu lysates for plaque forming units gave the same difference between *E. coli* and *Er. chrysanthemi*. The presence of CaCl₂ (5 mM) or MgSO₄ (10 mM) during infection slightly increased the frequency of lysogenization.

About 7500 lysogens of *Er. chrysanthemi* L37 or L50, from ten independent experiments, were selected on L agar plates containing ampicillin. About 40% of these lysogens expressed β-galactosidase and showed a wide range of Lac phenotypes. Presumably the other 60%, which did not express β-galactosidase, have the phage inserted either in the opposite direction or in genes not expressed under these growth conditions. A similar proportion of Lac⁺ lysogens was found for *E. coli* MC4100 and *Salmonella typhimurium* by Rosenfeld & Brenchley (1980). Thus lac gene fusions can be isolated in *Er. chrysanthemi* as in *E. coli*. Of the 7500 lysogens, 96 (1.3%) could not grow on glucose minimal medium and were scored for auxotrophy. For 77 clones, the supplement required for growth was determined among the amino-acids, bases and vitamins

Table 2. *Analysis of Mu d(Ap lac) lysogens mutated in the PGA or GalUA catabolic pathway*

Ampicillin resistant *Mu d(Ap lac)* lysogens were selected on L agar plates containing ampicillin; 7500 were scored for their growth on PGA, GalUA and GlcUA.

Class	No. of mutants	Growth with:				Step probably mutated
		PGA	GalUA	GlcUA	Glycerol + GalUA, GlcUA or PGA	
1	13	+	—	+	+	III or IV
2	4	+	—	—	+	H or II
3	4	—	—	—	+	V
4	1	—	—	—	—	VI
5	5	—	+	+	+	Pectinases (B, C, D) or secretion

tested; 14 different requirements were found. For bacteriophage *Mu* it is known that 1–2% of insertions in the chromosome of *E. coli* or *S. typhimurium* result in auxotrophy. The high frequency of certain auxotrophs (25% of our auxotrophs were *Arg*[−]) may reflect the multiple genes involved in the synthesis of these amino acids. The fact that with chemical (nitrosoguanidine) mutagenic treatment, the frequency of *Arg*[−] mutants reached 10–15% of the auxotrophs (unpublished results) indicates that the high frequency of *Arg*[−] mutants was not due to preferential insertion of *Mu* at particular sites in the *Er. chrysanthemi* chromosome.

Isolation of kdgK and kdgA mutants

Of the 7500 lysogens, 27 appeared from their growth phenotype to be mutated in the PGA or GalUA degradative pathway; they could be grouped into five classes (Table 2). Mutants of class 1 lacked one step specifically involved in GalUA catabolism (III or IV, Fig. 1). Mutants of class 2 were blocked in steps involved in GalUA and GlcUA utilization. Class 3 mutants lacked enzymes necessary for GalUA, GlcUA and PGA degradation; they are probably *kdgK* mutants. The class 4 mutant lacked also a step common to GalUA, GlcUA and PGA utilization, but this mutant died in the presence of these three compounds. In *E. coli*, this is characteristic of *kdgA* mutants, which accumulate KDGP, a compound toxic for the cells (Pouyssegur & Stoeber, 1974). Class 5 mutants could be mutated either in enzymes involved in PGA cleavage (steps B, C, D; Fig. 1) or in the excretion process of these enzymes.

Identification of kdgK and kdgA mutants

Four mutants, A233, A236, A237 and A242, showed a growth phenotype corresponding to *kdgK* mutations. One, A238, seemed to be a *kdgA* mutant. All five were assayed for the *kdgK* and *kdgA* gene products (enzymes V and VI) and for β -galactosidase, in glycerol minimal medium containing GalUA as inducer (Table 3). Complementation tests were carried out on GalUA minimal medium by introducing plasmids bearing other genes involved in GalUA catabolism (*kdgK* or *kdgA* or *exuT* *uxaC* *uxaB* *uxaA*; see Fig. 1). When complementation occurred, it was checked whether mutants complemented to the GalUA⁺ phenotype also became GlcUA⁺ and PGA⁺ (Table 3).

To verify that the *kdgK* and *kdgA* mutations really resulted from *Mu d(Ap lac)* insertion in these genes, we transduced A233, A236, A237 and A238 to the GalUA⁺ phenotype by a stock of the generalized transducing phage ϕ IEC2 made on the wild-type strain 3937j. For all strains the GalUA⁺ transductants were 100% cotransduced with the following characters: GlcUA⁺, PGA⁺, ampicillin sensitivity and absence of β -galactosidase activity. This shows that a single *Mu d(Ap lac)* insertion resulted in the GalUA[−] phenotype and in the β -galactosidase synthesis.

Inducibility of the kdgK-lac fusions

The β -galactosidase activity of the *kdgK-lac* fusion strains, A233, A236 and A242, was tested in glycerol minimal medium supplemented or not with GalUA, GlcUA or PGA (Table 4). All the fusion strains showed a significant basal level of activity. GalUA was the best inducer for all the strains, although GlcUA and PGA also induced significantly (Table 4).

Table 3. Analysis of *kdgK* and *kdgA* mutants

Strains, except A238, were grown in glycerol + galacturonate minimal medium to exponential phase and enzymes were assayed after disrupting the cells with a French press. Strain A238 was grown in glucose + galacturonate medium to avoid the strong inhibition of growth that occurred in the other medium. Specific activities of enzymes V and VI are expressed as $\mu\text{mol product min}^{-1}$ (mg bacterial dry wt) $^{-1}$. Specific activity of β -galactosidase is expressed as nmol of ONPG hydrolysed min^{-1} (mg bacterial dry wt) $^{-1}$. Complementation tests were performed on galacturonate minimal medium with the different plasmids carrying the genes shown in parentheses.

Strain	Specific activity of:			Complementation with:		
	β -Galactosidase	Enzyme V (<i>kdgK</i> product)	Enzyme VI (<i>kdgA</i> product)	pULB119 (<i>kdgK</i>)	pULB120 (<i>kdgA</i>)	pULB118 (<i>exuT uxaC uxaB uxaA</i>)
A233	205	2	33	+	—	—
A236	158	5	53	+	—	—
A237	1	10	57	+	—	—
A242	583	12	44	+	—	—
A238	267	56	3	—	+	—
L37	1	87	38			
3937j	35	92	49			

Table 4. Regulation of β -galactosidase in strains with a *kdgK* : *Mu d(Ap lac)* or a *kdgA* : *Mu d(Ap lac)* insertion

The strains were grown in minimal medium with a carbon source (0.2%) and the inducer (0.2%) to exponential phase. β -Galactosidase was assayed after toluene treatment. One unit of β -galactosidase activity is the amount of enzyme that hydrolyses 1 nmol ONPG min^{-1} (mg bacterial dry wt) $^{-1}$.

Fusion strain	Carbon source	Inducer	Enzyme units	Induction ratio
A233 (<i>kdgK</i>)	Glycerol	None	18	
		GalUA	198	8.6
		GlcUA	129	7.2
		PGA	141	6.1
	Glucose	None	15	
		GalUA	116	7.7
		GlcUA	119	7.9
		PGA	52	3.4
A236 (<i>kdgK</i>)	Glycerol	None	16	
		GalUA	146	9.3
		GlcUA	120	7.5
		PGA	143	8.6
A242 (<i>kdgK</i>)	Glycerol	None	72	
		GalUA	597	8.3
		GlcUA	571	7.9
		PGA	528	7.3
A238 (<i>kdgA</i>)	Glycerol	None	114	
		GalUA	245	2.1
		GlcUA	238	2.1
		PGA	207	1.8
	Glucose	None	103	
		GalUA	374	3.6
		GlcUA	289	2.8
		PGA	215	2.1

The β -galactosidase activity of strain A233 was also assayed in medium containing glucose rather than glycerol as carbon source, to assess catabolite repression (Table 4). Glucose grown cells showed an expression generally similar to glycerol grown cells, but there was a decrease of induction by PGA. This may be related to the finding that the enzymes involved in the cleavage of PGA are very sensitive to glucose repression (Tsumumu, 1979), so degradative products are synthesized less in the presence of glucose.

Table 5. *Induction of β -galactosidase in strain A233 by different intermediates of GalUA, GlcUA and PGA degradation*

Conditions and enzyme units were the same as described for Table 4.

Inducer	Concn (mM)	Enzyme units	Induction ratio
None		18	
Tagaturonate	5	171	9.5
Altronate	5	15	0.8
Fructuronate	5	189	10.5
Mannonate	5	162	9
DKI	1.5	198	11
DKII	1.5	204	11.3
KDG	5	177	9.8
Unsaturated digalacturonate	5	144	8

Other compounds, particularly intermediates of GalUA, GlcUA or PGA catabolism, were tested as inducers of β -galactosidase in strain A233 (Table 5). Tagaturonate, fructuronate, mannonate, DKI, DKII, KDG and unsaturated digalacturonate were all good inducers, while altronate showed no induction. From these data, we cannot identify which sugars are primary inducers of *kdgA* and *kdgK* genes. GalUA, GlcUA and PGA may only be secondary inducers because of their catabolism to pathway intermediates. Analysis of mutants blocked in each step of the GalUA, GlcUA, PGA catabolic pathway may lead to the identification of the true inducer. We can already note that KDG is probably a good inducer of the enzymes of PGA catabolism because in our *kdgK* mutants, accumulating KDG, we found higher polygalacturonate transeliminase (step C) activity than in wild-type strains, in induced (GalUA, GlcUA, PGA) conditions.

Inducibility of the kdgA-lac fusion

GalUA, GlcUA and PGA were also tested as inducer of the *kdgA-lac* fusion in strain A238 (Table 4), but they were toxic because of KDGP accumulation. This could explain the low inducibility ratios found, about 2–3. Glucose grown cells had β -galactosidase expression similar to that of glycerol grown cells, but the induction ratios for GalUA and GlcUA were higher with glucose as carbon source. The toxic effect of KDGP seemed to be decreased in the presence of glucose, as was found in *E. coli* (Pouyssegur & Stoeber, 1974).

Conclusion

Gene fusion has already proved to be a valuable tool for studying the regulation of metabolic systems in *E. coli* and other enterobacteria, such as *Klebsiella pneumoniae* (MacNeil *et al.*, 1981), *Salmonella typhimurium* (Rosenfeld & Brenchley, 1980) and *Erwinia carotovora* (Jayaswal *et al.*, 1984). We have shown that the method with Mu d(Ap *lac*) phage can be used to select *lac* gene fusions in *Er. chrysanthemi lacZ* mutants. These fusion strains should be useful for further studies of the regulation of the expression of genes involved in pectinolysis, cellulolysis or secretory mechanisms in *Er. chrysanthemi*, and for the selection of regulatory mutations.

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