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Thierry Franza, Isabelle Michaud-Soret, Pierrette Piquerel, Dominique Expert. Coupling of iron assimilation and pectinolysis in *Erwinia chrysanthemi* 3937.. *Molecular Plant-Microbe Interactions*, 2002, 15 (11), pp.1181-91. 10.1094/MPMI.2002.15.11.1181 . hal-00936392

HAL Id: hal-00936392

<https://hal.science/hal-00936392v1>

Submitted on 31 May 2020

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Coupling of Iron Assimilation and Pectinolysis in *Erwinia chrysanthemi* 3937

Thierry Franza,¹ Isabelle Michaud-Soret,² Pierrette Piqueret,¹ and Dominique Expert¹

¹Laboratoire de Pathologie Végétale UMR 217 INRA/INA-PG/Université Paris 6, 16 Rue Claude Bernard, 75231 Paris cedex 05, France; ²Service de Chimie Inorganique et Biologique, UMR 5046 CNRS/CEA/UJF, DRFMC, CEA Grenoble, 17 Rue des Martyrs, 38054 Grenoble cedex 9, France

Submitted 15 April 2002. Accepted 19 July 2002.

Two major virulence determinants of the plant-pathogenic enterobacterium *Erwinia chrysanthemi* strain 3937 are the production of pectate lyase enzymes that degrade plant cell walls and expression of two high-affinity iron uptake systems mediated by two structurally unrelated siderophores, chrysochitin and achromobactin. Low iron availability is a signal that triggers transcription of the genes encoding pectate lyases PelD and PelE as well as that of genes involved in iron transport. This metalloregulation is mediated by the transcriptional repressor Fur. In this study, we analyzed the molecular mechanisms of this control. We purified the *Erwinia chrysanthemi* Fur protein. Band shift assays showed that Fur specifically binds *in vitro* to the regulatory regions of the genes encoding the ferrichrysochitin outer membrane receptor Fct and the pectate lyases PelD and PelE. We identified the Fur-binding sites of these promoter regions by performing DNase I footprinting experiments. From these data, we propose that Fur could inhibit the activation of the *pelD* and *pelE* genes by the cAMP receptor protein CRP according to an anti-activation mechanism. To identify other possible effectors involved in this control, we screened a bank of insertion mutants for an increase in transcriptional activity of *pelD* and *fct* genes in response to iron limitation. We isolated a mutant affected in the *kdgK* gene encoding the 2-keto-3-deoxygluconate (KDG) kinase, an enzyme involved in pectin catabolism. The growth of this mutant in the presence of pectic compounds led to a constitutive expression of iron transport genes as well as complete derepression of the pectinolysis genes. This effect was caused by intracellular accumulation of KDG. However, the derepression of iron transport genes by KDG does not involve the KdgR regulator of pectinolysis genes, which uses KDG as inducer. Thus, in *Erwinia chrysanthemi*, iron depletion or presence of KDG induces transcription of the genes involved in iron assimilation and pectinolysis. These important pathogenicity functions are coregulated by responding to common signals encountered in planta.

The pectinolytic enterobacterium *Erwinia chrysanthemi* (*Pectobacterium chrysanthemi*) causes soft-rot disease in a great variety of plants. The soft-rot symptom is associated with the secretion of a large set of cell wall-degrading enzymes, including pectin methylesterases, pectin acetyl esterases, pectate lyases, pectin lyases, and polygalacturonases (Hugouvieux-Cotte-Pattat et al. 1996; Nasser et al. 1999; Shevchik and

Hugouvieux-Cotte-Pattat, 1997; Shevchik et al. 1997, 1999a, 1999b). These enzymes enable the dissemination of bacteria throughout the plant, via the intercellular spaces of the leaf parenchyma and petiole cortex (Murdoch et al. 1999). They also provide bacteria with carbon sources derived from the degraded pectin. Among those enzymes, the pectate lyases PelA to PelE are the most active and play a major role in pathogenesis (Beaulieu et al. 1993; Barras et al. 1994; Boccara et al. 1988; Collmer and Keen 1986). Bacterial spreading (i.e., full virulence) also depends on the expression of two high-affinity iron transport systems involving chrysochitin and achromobactin, a catechol and a citrate/carboxylate siderophore, respectively (Enard et al. 1988; Expert 1999; Müzinger et al. 2000; Persmark et al. 1989). Indeed, mutants affected in the *fct**chsCEBA* operon, which encodes the ferrichrysochitin outer membrane receptor Fct and the CbsA-E enzymes involved in biosynthesis of the catechol moiety of chrysochitin, failed to incite a systemic disease in the African violet (*Saintpaulia ionantha*) (Franza and Expert 1991). Similarly, mutants altered in the achromobactin biosynthetic genes *acsA* and *acsC* (achromobactin synthesis) cause only localized symptoms. These genes belong to the recently characterized *yhcAacsCBA* operon (T. Franza, unpublished). Successful infection by *Erwinia chrysanthemi* cells depends on a fine control coordinating the expression of the iron transport genes and the *pel* genes encoding pectate lyase enzymes. Three regulatory loci *kdgR*, *pecS*, and *pecT* have been identified that negatively control the expression of pectinolysis genes (Nasser et al. 1992; Praillet et al. 1996, 1997; Reverchon et al. 1991; Surgey et al. 1996). Among these regulators, the KdgR protein is the main effector and acts as a transcriptional repressor for all the genes involved in the pectin degradation pathway. In the absence of pectic inducers, the KdgR protein binds to a specific 17-bp sequence, the KdgR box, which is conserved in the promoter region of the different pectinolysis genes (Nasser et al. 1994). Pectinase production also is positively regulated. The cAMP receptor protein, CRP, activates the transcription of the pectinolysis genes (Nasser et al. 1997; Reverchon et al. 1997; Rouanet et al. 1999). Furthermore, low iron availability is a signal that turns on the transcription of the genes encoding the major pectate lyases PelB, PelC, PelD, and PelE (Masclaux and Expert 1995; Masclaux et al. 1996; Sauvage and Expert 1994) as well as that of the genes belonging to the chrysochitin and achromobactin iron uptake systems.

In *Erwinia chrysanthemi*, this metalloregulation is achieved by the ferric uptake regulation (Fur) protein (Franza et al. 1999). In many gram-negative bacteria, Fur acts as a transcriptional repressor using the ferrous ion as corepressor. In the presence of Fe(II), Fur binds to a conserved DNA sequence,

Corresponding author: T. Franza, Telephone: 33 1 44 08 72 30; Fax: 33 1 44 08 16 31; E-mail: franza@inapg.inra.fr

the Fur box, present in the promoter region of a large number of iron-regulated genes (Bagg and Neilands 1987; de Lorenzo 1987, 1988a, 1988b; Escolar et al. 1999, 2000; Panina et al. 2001). The *Escherichia coli* Fur protein recently has been analyzed by several spectrometric methods. Fur is a homodimer (148 amino acids per subunit), that contains two metal binding sites per monomer. The iron atom is penta- or hexacoordinated with nitrogen or oxygen ligands, but not with sulfur ligands (Adrait et al. 1999). In addition to this metallic site, Fur also possesses one zinc site per monomer (Jacquemet et al. 1998). Gonzalez de Peredo and associates (1999) demonstrated that the zinc ligands are the two cysteines Cys-92 and Cys-95. Interestingly, these two cysteine residues were found to be essential for the activity of the Fur protein (Coy et al. 1994). A structural model describing the conformational changes of the Fur protein upon metal activation and DNA binding has recently been proposed by Gonzalez de Peredo and collaborators (2001). The *Erwinia chrysanthemi* Fur protein is highly similar to the *Escherichia coli* Fur regulator (89% identity). It negatively controls iron transport genes and the genes encoding PelD and PelE enzymes (Franza et al. 1999). In the present study, we further characterized the mechanisms of this regulation. Furthermore, using a screen to select mutants affected in

control of iron homeostasis, we identified other effectors involved in this coordinate metalloregulation.

RESULTS

Overproduction and purification of the *Erwinia chrysanthemi* Fur protein.

The *Erwinia chrysanthemi fur* gene could not be cloned into multicopy plasmids (Franza et al. 1999), therefore, we removed the promoter region of the *fur* gene as described in experimental procedures. The recombinant plasmid pTF30, pT7.5 containing the *fur* coding sequence, was introduced into the *Escherichia coli* strain BL21 to overproduce the Fur polypeptide. The *Erwinia chrysanthemi* Fur protein was purified as previously described by Wee and collaborators (1988), with the modifications indicated in experimental procedures. After chromatography on a chelating Zn-iminoacetate column, a second purification step was performed by gel filtration on a superdex 75 column. A mixture of two species corresponding to the Fur monomer and dimer was obtained. The molecular mass determined by mass spectrometry under denaturing conditions for both species indicated no additional covalent modification (data not shown). All the studies were performed with the dimeric form of the apoFur protein.

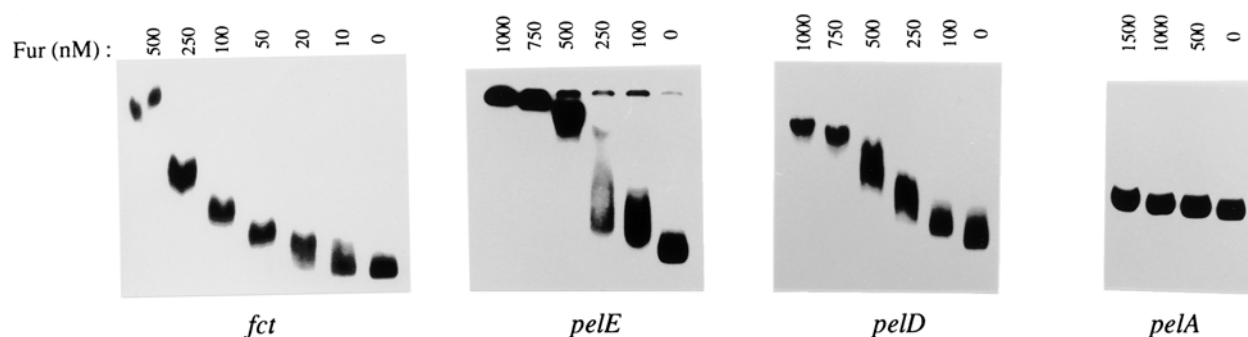


Fig. 1. Analysis of the interaction between Fur and the *fct*, *pelE*, *pelD*, and *pelA* promoter regions by electrophoretic mobility shift assay. Restriction fragments containing the *fct*, *pelE*, *pelD*, and *pelA* promoter regions, respectively, were incubated with increasing concentrations of purified Fur (monomer), as indicated.

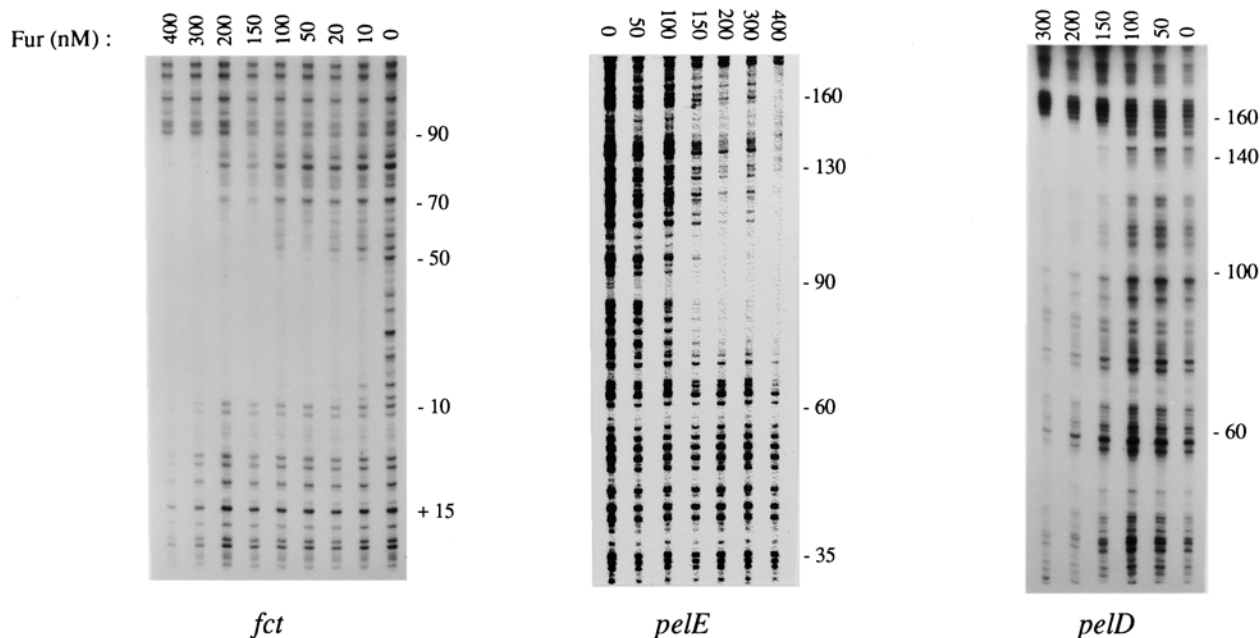


Fig. 2. DNase I footprinting of the binding sites for Fur in the regulatory region of the *fct*, *pelE*, and *pelD* genes. Purified Fur was added to the final concentration (monomer) indicated above each line. The sequences are numbered with respect to the transcriptional start (+1) identified for each gene.

curred (Fig. 2). Such an extensive protection likely was caused by polymerization of the Fur protein around the DNA, as visualized before by Le Cam and associates (1994) by electron microscopy. As observed in the electrophoretic mobility shift assay, Fur binds the *pelD* and *pelE* gene promoters with lower affinity than that observed for the promoter of the *fmt* gene. For the *pelE* promoter, protection occurred only for Fur concentrations superior to 100 nM (Fig. 2). The sequence protected by Fur included a part of the binding site of the cAMP-CRP protein (Nasser et al. 1997). Two overlapping degenerated Fur boxes, containing 13 and 11 out of 19 conserved positions, respectively, were identified in this protected region (Fig. 3). For the *pelD* regulatory region, the Fur-binding sequence was found to include the putative UP element of the promoter (Rouanet et al. 1999). Only one

degenerated Fur box, matching the consensus sequence at 9 out of 19 positions, was detected in this region, which is very rich in A and T nucleotides (Fig. 3). As observed for the *fmt* promoter, extensive protection by Fur of the regulatory regions of the *pelD* and *pelE* genes occurred for Fur concentrations above 200 nM (Fig. 2).

Mutagenesis and isolation of mutants altered in iron regulation of *pelD* and *fmt* genes.

To investigate the possibility that additional effectors mediate iron regulation of the *pel* genes, we tried to isolate mutants changed in their response to iron. As an iron-regulated reporter gene, we chose the *pelD* gene, because only severe iron starvation induces its transcription. Indeed, in Tris medium, the *pelD* gene was found to be derepressed only in a chrysoactin-deficient strain (Masclaux et al. 1996). In a *cbsE* mutant, that is altered in chrysoactin biosynthesis, the sensitivity to iron availability of a transcriptional *pelD::uidA* fusion is increased. By random insertion of the miniMu element MudII PR13, we mutagenized a *cbsE*-negative strain harboring a chromosomal transcriptional *pelD::uidA* fusion. Differences in expression of the *pelD::uidA* fusion were scored on L-agar plates containing the X-gal indicator and weak amount of the iron chelator EDDHA. Under these conditions, the parental strain (L37 *cbsE pelD::uidA*), which is chrysoactin deficient, could still grow. It gave rise to bluish colonies, because the *pelD* gene was not fully derepressed. Approximately 4,000 chloramphenicol-resistant clones were screened for increased expression of the *pelD::uidA* fusion. Fifteen dark blue clones were selected and purified. To confirm the observed phenotype, we transduced the corresponding *Cm^r* mutations back into the strain L37 *cbsE pelD::uidA*. Then, the mutations conferring a dark blue phenotype were transduced into a strain carrying a chromosomal fusion to the ferrichrysoactin receptor gene (L37 *fmt::lacZ*). *Cm^r* transductants were streaked on L-agar plates containing X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) as indicator. Out of the 15 initial clones, 3 gave rise to dark-blue colonies under these conditions. Increased *fmt* gene transcription was confirmed by assessing β -galactosidase activity in liquid cultures (data not shown). These three mutations conferring increased expression of the *fmt* and *pelD* genes, were named *hel* (higher expression level).

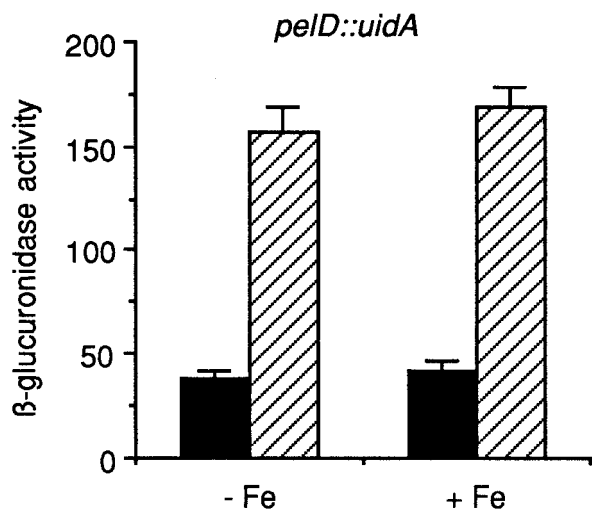


Fig. 4. Expression of chromosomal *pelD::uidA* fusion in *kdgK⁺* (solid bar) and *kdgK⁻* (hatched bar) cells of *Erwinia chrysanthemi* L37. Strains were grown in Tris medium with (20 μ M FeCl_3) or without iron with glycerol and polygalacturonate as carbon sources. Samples were collected when cultures reached an optical density at 600 nm of 0.8. Experiments were performed three times and standard deviations are shown. β -Glucuronidase activity is expressed in nanomoles of paranitrophenol liberated per minute and per unit of optical density at 600 nm.

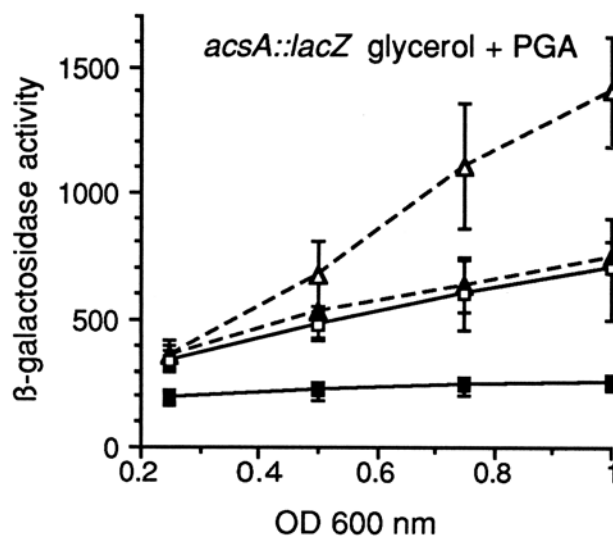
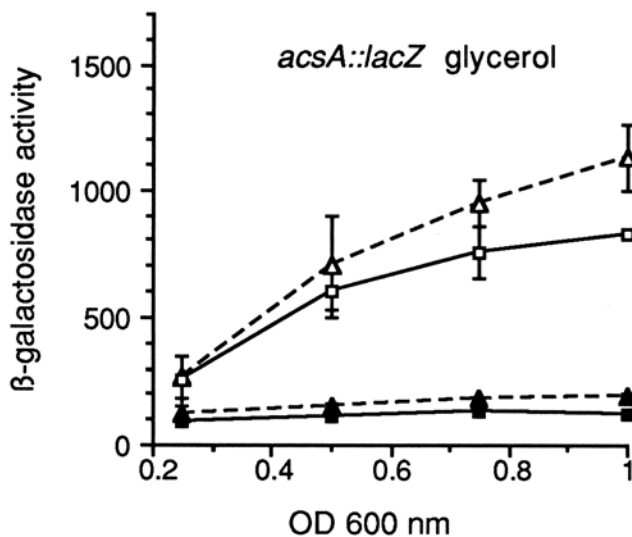


Fig. 5. Expression of a chromosomal *acsA::lacZ* gene fusion in *kdgK⁺* (square) and *kdgK⁻* (triangle and dotted line) cells of *Erwinia chrysanthemi* L37. Strains were grown in iron-rich (20 μ M FeCl_3) (solid symbols) or iron-poor (open symbols) conditions with the indicated carbon sources. Bacterial growth was assessed by measuring optical density (OD) at 600 nm. Experiments were performed three times and standard deviations are shown. β -Galactosidase activity is expressed in Miller units.

To identify the loci corresponding to the mutations *hel-29*, *hel-43*, and *hel-53*, we first isolated the host DNA sequence flanking the left terminal part of the MudII PR13 prophage responsible for each mutation. Total DNAs from these mutants were hydrolyzed with *Sall* and ligated into the pUC18 vector linearized with the same enzyme. After electroporation of *Escherichia coli* DH5 α cells, transformants were selected on agar plates containing ampicillin and chloramphenicol. The three corresponding plasmids pPP29, pPP43, and pPP53 were found to contain a DNA insert of 4.5, 14, and 3.5 kb, respectively. Using a primer complementary to the left end of the Mu DNA, we determined the nucleotide sequence of the host DNA flanking regions. No significant homology was detected for the DNA from plasmid pPP29. The Mu prophage responsible for the *hel-43* mutation was found to be inserted into a region sharing some homology with the DNA from the *Escherichia coli* retronphage phiR73. A search with the program BlastP confirmed that the Mu DNA is integrated into a putative open reading frame of 2,700 nucleotides encoding a polypeptide displaying 33% identity (44% similarity) with the DNA primase of this retronphage phiR73. This phage belongs to the CP4 family and its DNA sequence often is associated with pathogenicity islands found in gram-negative bacteria (Hacker et al. 1997; Moss et al. 1999). This boundary region, which codes for potential mobility function and may be responsible for genetic instability, was not characterized further. The locus containing the mutation *hel-53* was found to be the *kdgK* gene encoding the 2-keto-3-deoxygluconate (KDG) kinase (Hugouvieux-Cotte-Pattat et al. 1994). In *Erwinia chrysanthemi*, pectin and galacturonate are degraded into metabolites leading to the production of KDG (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1987). KDG is a final catabolite which then is phosphorylated by the KdgK enzyme, before its cleavage into 3-phosphoglyceraldehyde and pyruvate by the KdgA aldolase (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1994). It was not surprising to find that a *kdgK* mutation conferred a higher expression of the *pelD* gene, because KDG is an inducer of the genes belonging to the *kdgR* regulon (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1987; Hugouvieux-Cotte-Pattat et al. 1986; Nasser et al. 1992, 1994). There is a fourfold increase of the *pelD* gene transcription level in the presence of polygalacturonate (PGA) com-

pared with the level observed for the wild-type strain in the same condition (Fig. 4). Thus, in a *kdgK* mutant growing in L-agar medium, the basal level of KDG can lead to a partial derepression of the *pelD* gene transcription. However, the link between the presence of this molecule and the increased expression of the *fct* gene remained to be elucidated.

Effect of the *kdgK* mutation on the regulation of genes involved in siderophore-mediated iron assimilation.

To study the effect of a *kdgK* mutation on expression of the achromobactin system, we transduced this mutation into a strain carrying a chromosomal *acsA::lacZ* fusion (L37 *acsA::lacZ*). β -galactosidase activity was monitored over time in *kdgK*⁺ and *kdgK* cells grown in Tris medium with or without iron supplementation. Glycerol or glycerol and PGA were used as carbon sources. In the presence of PGA, *kdgK* cells accumulate KDG intracellularly. When glycerol was used as a sole carbon source, we observed a slight increase in the activity of the *acsA::lacZ* fusion in *kdgK* cells relative to the wild-type level, mostly in the absence of iron (Fig. 5). The addition of PGA caused a stimulation in the expression of the fusion in *kdgK* cells only. However, this fusion remained iron regulated (Fig. 5).

Expression of the *fct::lacZ* fusion was assessed under the same growth conditions. In the presence of glycerol, we observed a higher expression of the *fct::lacZ* fusion in *kdgK* cells relative to the wild-type level, regardless the iron concentration (Fig. 6). When PGA was added to the medium, the *fct::lacZ* fusion in *kdgK* cells was fully derepressed (Fig. 6). In *kdgK*⁺ cells, expression of the fusion was maintained at a low constitutive level. Similarly, we found that, in *kdgK* cells, chrysoactin was produced constitutively (data not shown). These results indicated that, during pectinolysis, *Erwinia chrysanthemi* cells produce and accumulate pectic catabolites and that transcription of the *acsA* and *fct* genes is induced regardless the iron concentration.

The KDG molecule, but not the KdgR repressor, mediates regulation of the *fct* gene.

In order to determine which pectic catabolite is involved in the induction of iron transport genes, we first examined the expression of the *fct::lacZ* fusion in *kdgK*⁺ and *kdgK* cells con-

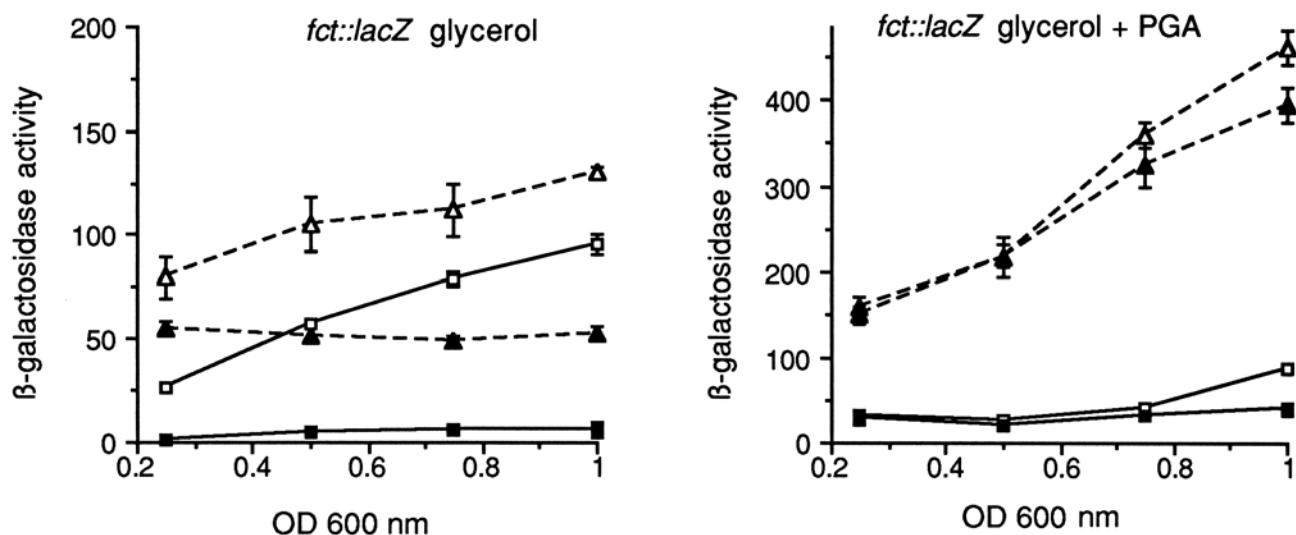


Fig. 6. Expression of a chromosomal *fct::lacZ* gene fusion in *kdgK*⁺ (square) and *kdgK* (triangle and dotted line) cells of *Erwinia chrysanthemi* L37. Strains were grown in iron-rich (20 μ M FeCl₃) (solid symbols) or iron-poor (open symbols) conditions with the indicated carbon sources. Bacterial growth was assessed by measuring optical density (OD) at 600 nm. Experiments were performed three times and standard deviations are shown. β -Galactosidase activity is expressed in Miller units.

taining a *kdgT^C* mutation. This mutant constitutively expresses the inner membrane KDG transporter KdgT (Condemine and Robert-Baudouy 1987). Upon addition of KDG to the culture medium, we could analyze the effect of this compound on expression of the *fmt* gene. *kdgK⁺* and *kdgK* cells were grown in Tris medium supplemented with iron. Glycerol was the carbon source. When bacteria entered the exponential growth phase, KDG was added to the culture to a final concentration of 0.2%. In *kdgK⁺* bacteria, there was a transient increase in the *fmt* gene transcriptional activity 45 min after addition of KDG that lasted 2 h (Fig. 7). In *kdgK* cells, the *fmt* gene transcriptional activity was derepressed constantly (Fig. 7). Thus, intracellular accumulation of KDG resulted in increased *fmt* gene

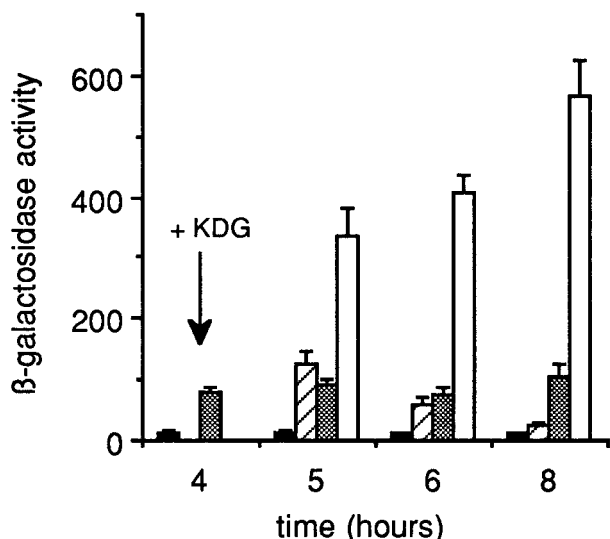


Fig. 7. Effect of 2-keto-3-deoxygluconate (KDG) on the expression of a chromosomal *fmt::lacZ* gene fusion. *kdgK⁺* and *kdgK* cells of *Erwinia chrysanthemi* L37, which constitutively transport KDG, were grown in Tris medium supplemented with iron (20 μ M), with glycerol as a carbon source. KDG was added to the culture at the time indicated by an arrow. β -Galactosidase activity was determined in samples collected every hour after addition of KDG. The different conditions are represented by columns: *kdgK⁺* cells, no KDG, solid bar; *kdgK⁺* cells, with KDG, hatched bar; *kdgK* cells, no KDG, shaded bar; *kdgK* cells, with KDG, open bar. Experiments were performed three times and standard deviations are shown. β -Galactosidase activity is expressed in Miller units.

transcription. This regulatory effect might be mediated by the regulator KdgR. Indeed, in the presence of KDG, the binding of the KdgR repressor to the operator sites from the pectinolytic genes is prevented and the KdgR-operator complex is dissociated (Nasser et al. 1992, 1994). Thus, we analyzed the transcription of the *fmt* and *acsA* genes in a *kdgR* mutant. Whatever the carbon source was, we detected no change in the *fmt* and *acsA* gene expression (Fig. 8). In this mutant, both genes remained totally regulated by iron and their expression levels were similar to those observed for the wild-type strain. This result indicates that the *kdgR* locus is not involved in the induction of iron transport genes by KDG.

DISCUSSION

Because iron is not readily available in host tissues, acquisition of this metal by pathogenic bacteria during pathogenesis is primordial (Ratledge and Dover 2000; Weinberg 1998;). Furthermore, iron homeostasis plays an important role in the regulation of virulence genes from pathogenic bacteria in animals (Crosa 1997; Litwin and Calderwood 1993; Panina et al. 2001). A similar control of pathogenicity genes takes place in the plant pathogenic enterobacterium *Erwinia chrysanthemi*. In strain 3937, iron depletion stimulates the transcription of the *pel* genes that code for pectate lyase enzymes, as well as that of the iron transport genes (Masclaux and Expert 1995; Masclaux et al. 1996; Sauvage and Expert 1994). We previously demonstrated that this metalloregulation is mediated by the Fur transcriptional repressor protein (Franza et al. 1999). In this study, we investigated the fine mechanisms of this regulatory control and identified other effectors involved in this process.

For this purpose, we purified the *Erwinia chrysanthemi* Fur protein and analyzed its binding to the promoter region of the *pelD* and *pelE* genes, as well as that of the ferrichrysoabactin receptor gene *fmt* in electrophoretic mobility shift assays. After metallation, the purified Fur protein could specifically bind with different affinity the promoter regions of the *fmt* gene and those encoding the PelD and PelE enzymes. The *Erwinia chrysanthemi* Fur repressor displays higher affinity for the *fmt* gene promoter than for the regulatory regions of the *pelD* and *pelE* genes. The Fur-binding site of these operators was determined by DNase I footprinting experiments. In contrast to the Fur-binding site determined for the *fmt* gene promoter region, which includes the -35 and -10 promoter elements, the regula-

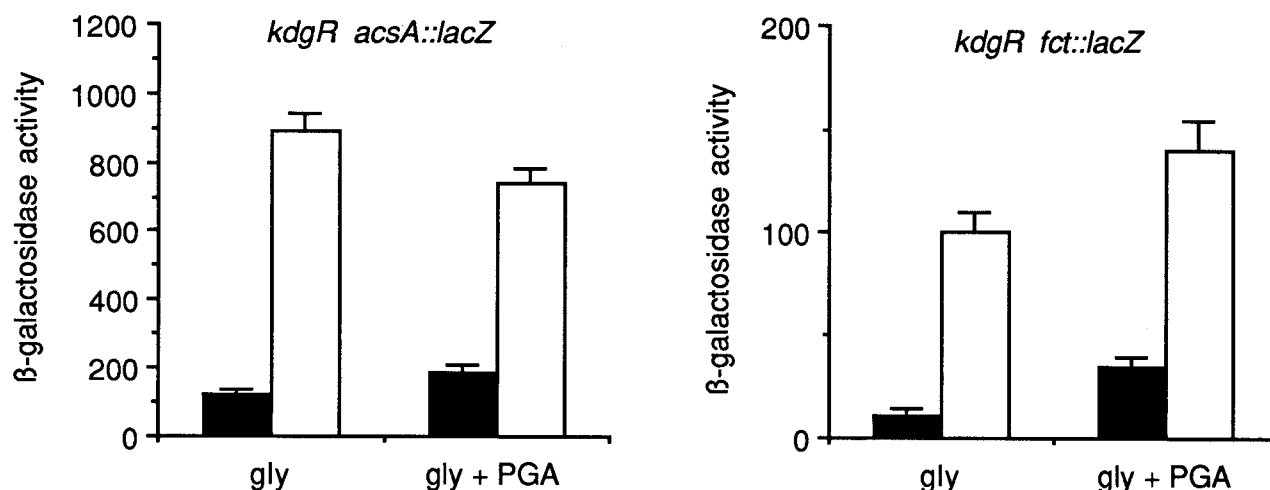


Fig. 8. Effect of the *kdgR* mutation on the expression of the *fmt* and *acsA* genes. *kdgR* cells were grown in iron-rich (20 μ M FeCl_3) (solid bars) or iron-poor (open bars) conditions with the indicated carbon sources. β -Galactosidase activity was determined in samples collected at the end of the exponential growth phase. Experiments were performed three times and standard deviations are shown. β -Galactosidase activity is expressed in Miller units.

tory regions of the *pelD* and *pelE* genes protected by Fur are located upstream from these promoter elements. Indeed, for the *pelE* promoter, the Fur-binding site extends from positions -119 to -74 and includes a part of the sequence recognized by the cAMP-CRP protein (Nasser et al. 1997). This result suggests that Fur could regulate the *pelE* gene by an antiactivation mechanism. In this case, Fur could hinder CRP binding and subsequent gene transcription activation. The *pelD* regulatory region recognized by Fur encompasses the positions -142 to -98 and is located in the putative UP element of this promoter (Rouanet et al. 1999). Interestingly, this Fur-binding site is also in the close vicinity of the cAMP-CRP operator site (Rouanet et al. 1999). The UP element of bacterial promoter is known to stimulate transcription by interacting with the C-terminal domain of the RNA polymerase (Aiyar et al. 1998; Estrem et al. 1998). The presence of Fur to this sequence would thus inhibit the synergistic binding of CRP and RNA polymerase and lead to a decrease of promoter activity.

The localization of the Fur recognition sites of the *pelD* and *pelE* promoters is unusual, because these binding sites are upstream of the -35 and -10 elements of both promoters. Indeed, many iron-regulated promoters appear to have multiple overlapping Fur boxes situated close to the -35 and -10 promoter elements (Escolar et al. 1999; Newman and Shapiro 1999). Escolar and collaborators (1997, 1998) showed that Fur metalloregulation involves a direct competition between the RNA polymerase and this repressor. Our results indicate that a similar process likely occurs for the control of the *fct* gene in *Erwinia chrysanthemi*. On the other hand, in the case of the *pelD* and *pelE* genes, it seems that Fur would rather act as an antiactivator of transcription by blocking the action of CRP. An interpretation of these data would be that, in the presence of pectic inducers and iron, the repressor KdgR is inactivated and Fur is active and can thus prevent activation of the *pelD* and *pelE* genes by CRP. However, the negative metalloregulation mediated by Fur is partially relieved during pectinolysis. A mutagenesis approach, using the miniMu element MudIIPR13, was performed to identify the effectors involved in this process. We searched for mutations conferring increased expres-

sion of both *pelD* and *fct* genes. The prophage responsible for the *hel-53* mutation was found to be inserted in the *kdgK* gene encoding the KDG kinase. The KDG phosphorylation is the last step of the pectin or galacturonate catabolic pathway, before this compound is converted into 3-phosphoglyceraldehyde and pyruvate. A mutation in the *kdgK* gene blocks this metabolic conversion and can lead to the intracellular accumulation of KDG (Hugouvieux-Cotte-Pattat et al. 1994). This catabolite is an inducer of the pectinolysis genes by inhibiting the binding of the transcriptional KdgR repressor to its operator sites and leading to the KdgR-operator complex dissociation (Nasser et al. 1994). Therefore, it was not surprising to find that this mutation caused an increased expression of the *pelD* gene. More intriguing was the role of this mutation on the regulation of the chrysoactin iron transport gene *fct*. Indeed, in this mutant, the *fct* gene was found to be constitutively expressed regardless the iron levels if PGA was added to the culture. The effect of the *kdgK* mutation on the regulation of the iron uptake system dependent on achromobactin also was examined. The presence of pectic compounds in the culture medium led to derepression of the *acsA* gene transcription that, however, remained iron regulated. The involvement of the KDG molecule in the modulation of the *fct* gene expression was shown by using a *kdgT^c* strain that constitutively transports KDG. Under iron-replete conditions, addition of KDG to exponentially growing cells induced the *fct* gene transcription after 45 min. This induction lasted 2 h, indicating that this inducer was metabolized by the bacteria. However in a *kdgK* mutant that accumulates KDG, the *fct* gene transcription appeared to be constantly derepressed during growth. Thus, in *Erwinia chrysanthemi*, the intracellular concentration of KDG modulates the expression of the *pel* genes as well as that of the iron transport genes. For the pectinolysis genes, this control mainly is mediated by the transcriptional repressor KdgR. However, we found that no derepression of the *fct* and *acsA* genes occurred in a *kdgR* mutant, indicating that this regulator is not involved in this control. One interpretation of these data might be that KDG controls the transcription of the *fur* gene. If that was case, the level of the Fur protein would decrease with

Table 1. Bacterial strains and bacteriophages used in this work

Strain	Characteristics ^a	Reference or source
<i>Erwinia chrysanthemi</i>		
L37	Lac ⁻ derivative from 3937	from N. Hugouvieux-Cotte-Pattat
L37 <i>acsA::lacZ</i>	Acs ⁻ derivative from L37, Km ^c	Mahé et al. 1995
L37 <i>fct::lacZ34</i>	Fct ⁻ and Cbs ⁻ , Km ^f	Masclaux and Expert 1995
L37 <i>pelD::uidA</i>	PelD ⁻ , Km ^f	Masclaux et al. 1996
L37 <i>pelD::uidA cbsE</i>	PelD ⁻ and Cbs ⁻ , <i>cbsE::Ω</i> , Km ^f and Spec ^f	Masclaux et al. 1996.
L37 <i>hel-53</i>	KdgK ⁻ , MudIIPR13 lysogenic, Cm ^f	This study
L37 <i>hel-43</i>	MudIIPR13 lysogenic, Cm ^f	This study
L37 <i>hel-29</i>	MudIIPR13 lysogenic, Cm ^f	This study
L37 <i>hel-53 pelD::uidA</i>	KdgK ⁻ and PelD ⁻ , Cm ^f and Km ^f	This study
L37 <i>hel-53 fct::lacZ34</i>	KdgK ⁻ , Fct ⁻ , and Cbs ⁻ , Cm ^f and Km ^f	This study
L37 <i>hel-53 acsA::lacZ</i>	KdgK ⁻ and Acs ⁻ , Cm ^f and Km ^f	This study
A837	<i>lmrT^c lac⁻ kdgR</i>	Condemine and Robert-Baudouy 1987
A837 <i>fct::lacZ34</i>	Fct ⁻ , <i>lmrT^c lac⁻ kdgR</i> , Km ^f	This study
A837 <i>acsA::lacZ</i>	Acs ⁻ <i>lmrT^c lac⁻ kdgR</i> , Km ^f	This study
A410	<i>lmrT^c lac⁻ kdgT^c</i>	Condemine and Robert-Baudouy 1987
A410 <i>fct::lacZ34</i>	Fct ⁻ , <i>lmrT^c lac⁻ kdgT^c</i> , Km ^f	This study
A621	<i>lmrT^c lac⁻ kdgT^c kdgK</i>	Condemine and Robert-Baudouy 1987
A621 <i>fct::lacZ34</i>	Fct ⁻ , <i>lmrT^c lac⁻ kdgT^c kdgK</i> Km ^f	This study
<i>Escherichia coli</i>		
DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi relA1</i>	Sambrook et al. 1989
BL21	F ⁻ <i>ompT gal dcm lon hsdS_B (r_B⁻ m_B⁻)</i> with a λ prophage carrying the T7 RNA polymerase	Studier et al. 1990
Phages		
PhiEC2	General transducing phage of <i>Erwinia chrysanthemi</i>	Résibois et al. 1984
MudIIPR13	Mu <i>cts62 Δ(Mu AB) Cm^f Δ(lac ZYA)</i>	van Gijsegem et al. 1987

^a Amp = ampicillin, Cm = chloramphenicol, Km = kanamycin, Spec = spectinomycin, and Tc = tetracycline.

increasing intracellular concentrations of KDG. However, the fast effect of addition of KDG on the *fmt* gene expression favors the idea of a direct action of this compound on the Fur regulatory activity (i.e., inhibition of the binding of Fur to its target sequences). Indeed, Fuhrman and associates (1998) showed that an *Escherichia coli kdgA* mutant, lacking 2-keto-3-deoxy-6-phosphogluconate aldolase, can accumulate 10 mM KDG 10 min after addition of gluconate. A high intracellular concentration of KDG could inhibit the metalated Fur protein by scavenging its cofactors Fe²⁺ or Zn²⁺ ions. However, the affinity of KDG for these cations is predicted to be rather low. Another possibility would be that KDG prevents the binding of Fur to its target sequences in the same way it does for the KdgR repressor. Interestingly, we found that, in a *fur* mutant, presence of PGA or KDG has no effect on the expression of the *fmt* and *acsA* genes that are fully derepressed (data not shown). Unfortunately, we were not able to construct a *fur kdgK* mutant. We analyzed the effect of KDG on the Fur protein in electrophoretic mobility shift assays and DNase I footprinting experiments (data not shown). No reliable and conclusive data could be obtained. To investigate the influence of KDG on the structure and dynamics of the Fur protein, other approaches, such as spectroscopic studies, must be considered.

In conclusion, we found that, in *Erwinia chrysanthemi*, the expression of two functions essential for pathogenicity, iron acquisition and pectinolysis, is regulated in a coordinate manner by responding to common environmental signals, iron availability, and pectin. This metabolic coupling, that should confer an important advantage to the bacteria during the infection process is, in part, exerted by the Fur and KdgR proteins at different level of action, although we cannot exclude the existence of another unknown regulator involved in the response to KDG.

MATERIALS AND METHODS

Strains, phages and plasmids.

The bacterial strains, bacteriophages, and plasmids used in this work are described in Tables 1 and 2, respectively.

Culture conditions.

Nutrient-rich media were L-broth and L-agar (Miller 1972). Tris medium was used as low iron minimal medium, containing glycerol, PGA, or KDG at 2 g/l as a carbon source (Franza et al. 1991). For iron-rich conditions, FeCl₃ was added at a fi-

nal concentration of 20 μM. When necessary, the following antibiotics (Sigma, Saint Quentin Fallavier, France) were used at final concentrations as follows: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 30 μg/ml; or tetracycline, 25 μg/ml. TA7 was used for soft agar overlays (Franza et al. 1991). For recombinant plasmid selection, isopropyl and X-gal (Euromedex: Mundolsheim, France) were added at a final concentration of 250 and 40 μg/ml, respectively. To provide iron-limited conditions, ethylene-diamine-N,N'-bis[2-hydroxyphenyl]acetic acid (EDDHA), purchased from Sigma, was added to L-agar medium, at a final concentration of 10 μg/ml. The substrate for glucuronidase activity X-Gluc (Euromedex: Mundolsheim, France) was added at a final concentration of 50 μg/ml.

Mutagenesis and mutant phenotype analysis.

An *Erwinia chrysanthemi* strain L37 *chsE pelD::uidA* was mutagenized with the MudIIPR13 derivative as described by Enard and associates (1988). Chloramphenicol-resistant survivors were replicated on L-agar plates containing the indicator X-gluc and the iron chelator EDDHA. Dark-blue clones were selected. The corresponding mutations were transduced back into the L37 *chsE pelD::uidA* strain in order to confirm the phenotype. Phage phiEC2 transductions were performed as described by Franza and associates (1991). Mutations conferring increased *pelD* gene transcriptional activity then were transduced into strain L37 *fmt::lacZ*. β-Galactosidase and β-glucuronidase activities were determined in liquid cultures as described previously (Franza et al. 1999). Only mutations conferring increased expression of both genes were characterized further.

DNA methods.

DNA manipulations (plasmid isolation, cloning, and electrophoresis) were carried out as described by Sambrook and associates (1989). All cloning experiments were performed in the DH5α strain of *Escherichia coli*. DH5α cells were transformed by electroporation as described by Sambrook and associates (1989). To identify the gene affected in each mutant, the host DNA sequence flanking the left terminal part of the MudIIPR13 prophage was cloned as described above. When necessary, DNA/DNA hybridization analysis was performed by using the Denhardt's method described by Sambrook and associates (1989). DNA sequencing was performed on doublestrand plasmid by the dideoxynucleotide chain termination method with

Table 2. Plasmids used in this work

Plasmids	Characteristics ^a	Source or reference
pUC9	2.5-kb vector, Amp ^r	Sambrook et al. 1989
pUC18	2.7-kb vector, Amp ^r	Sambrook et al. 1989
pBC	3.4-kb vector derived from pUC19, Cm ^r	Stratagene
pWSK29	5.4-kb low copy number vector, Amp ^r , pSC101 derivative	Wang and Kushner 1991
pT7-5	2.4-kb surexpression vector, Amp ^r	Studier et al. 1990
pTF20	1.2-kb <i>PstI-BamHI</i> fragment containing the <i>Erwinia chrysanthemi fur</i> gene cloned in pWSK29, Amp ^r	Franza et al. 1999
pTF30	0.6-kb <i>EclXI/PvuII</i> fragment from pTF20 cloned in pBC, Cm ^r	This study
pTF31	0.6-kb <i>SacI/ClaI</i> fragment from pTF30 cloned in pT7-5, Amp ^r	This study
pTF32	520-bp <i>NsiI/EcoRV</i> fragment containing the <i>fmt</i> gene promoter cloned in pBC, Cm ^r	This study
pSR1321	375-bp <i>HpaI/MaeIII</i> fragment containing the <i>pelA</i> gene promoter cloned in the <i>EcoRV</i> site of pBluescript, Amp ^r	Nasser et al. 1994
pN1272	725-bp <i>HindIII/BglII</i> fragment containing the <i>pelD</i> gene promoter cloned in the <i>HindIII/BamHI</i> site of pBluescript, Amp ^r	Rouanet et al. 1999
pSR1175	275-bp <i>DraI/DpnI</i> fragment containing the <i>pelE</i> gene promoter cloned in the <i>EcoRV</i> site of pBluescript, Amp ^r	Nasser et al. 1994
pPP53	3.5-kb <i>SalI</i> fragment containing the left flanking region of the MudIIPR13 responsible for the mutation <i>hel-53 kdgK</i> , Amp ^r and Cm ^r	This study
pPP43	14-kb <i>SalI</i> fragment containing the left flanking region of the MudIIPR13 responsible for the <i>hel-43</i> mutation, Amp ^r and Cm ^r	This study
pPP29	4.5-kb <i>SalI</i> fragment containing the left flanking region of the MudIIPR13 responsible for the <i>hel-29</i> mutation, Amp ^r and Cm ^r	This study

^a Amp = ampicillin, Cm = chloramphenicol, Km = kanamycin, Spec = spectinomycin, and Tc = tetracycline.

the Sequenase version 2.0 and [α - 35 S] dATP (Amersham Biosciences: Orsay, France). When necessary, dimethyl sulfoxide (Sigma) was added in the annealing mixture (5% final concentration). Data were analyzed with the software package UW GCG provided by Bisance (Dessen et al. 1990).

Overproduction and purification of the *Erwinia chrysanthemi* Fur protein.

To delete the *fur* gene promoter, the 0.6-kb *Ecl*XI/*Pvu*II fragment of plasmid pTF20 was cloned into the pBC bluescript vector hydrolyzed with *Not*I and *Eco*RV. The recombinant plasmid was hydrolyzed with *Sac*I and *Cla*I. The 0.6-kb *Sac*I/*Cla*I fragment, containing the *fur* gene, then was cloned into the pT7.5 vector linearized with the same enzymes. The recombinant plasmid pTF30 was introduced into the *Escherichia coli* strain BL21 to overproduce the *Erwinia chrysanthemi* Fur protein by the T7 RNA polymerase/promoter system according to the procedure described by Studier and associates (1990). The Fur protein was purified as previously described by Wee and collaborators (1988), but with some modifications. The extraction buffer (0.1 M MOPS, pH 8, containing 10% [wt/vol] sucrose and 10% [v/v] glycerol) was supplemented with trypsin-chymotrypsin inhibitors at 10 mg/ml together with EDTA at 20 mM, phenylmethylsulfonyl fluoride at 1.4 mM, and pepstatin at 6 μ M. A treatment with 20 mM EDTA for 30 min at 4°C was carried out on the pooled fractions collected from the chelating column (Zn-iminodiacetate acetate column, Superose HR 16/5 from Amersham Biosciences). The treated fractions were precipitated overnight at 80% ammonium sulfate saturation, before solubilization of the protein in 2 ml of 0.1 M Tris/HCl, pH 8 (5°C), containing 0.1 M KCl with 10% (vol/vol) glycerol. We then performed a second step of purification with a gel filtration on superdex 75 (Amersham Biosciences) in 0.1 M Tris/HCl, pH 8 (5°C), containing 0.1 M KCl. Samples collected from the gel filtration were concentrated and 10% (vol/vol) glycerol was added. Samples were frozen in liquid nitrogen and stored at -80°C.

Preparation of operator fragments for binding studies.

The regulatory regions from the *pelA*, *pelD*, *pelE*, and *ftc* genes were cloned in bluescript vectors (Table 2). Promoter DNAs were hydrolyzed with appropriate restriction enzymes in order to create a unique 3' recessed terminus. Restriction fragments were purified after electrophoresis on low-melting agarose gels by using the QIAquick extraction kit from Qiagen (Courtaboeuf, France). These DNA fragments were end-labeled with the appropriate (α - 32 P)-dNTP by using the Klenow enzyme.

Gel retardation assays.

DNA fragments were diluted to a final concentration of 0.5 nM in binding buffer containing 10 mM Bis-Tris (pH 7.5), 1 mM MgCl₂, 40 mM KCl, bovine serum albumin (BSA) at 0.1 mg/ml, 5% glycerol, and salmon sperm DNA at 100 μ g/ml. MnCl₂ was added just prior the binding experiments at a final concentration of 150 μ M. Samples were preincubated in a final volume of 20 μ l with indicated amount of Fur protein for 10 min at 37°C. Samples were loaded on nondenaturing polyacrylamide gel (4 to 7%). DNA/protein complexes were separated by electrophoresis at 4°C for 1 h at 100 V in a buffer containing 40 mM BisTris-Borate (pH 7) and 100 μ M MnCl₂. The Mini-protean system from Bio-Rad was used for electrophoresis (Marnes-la-Coquette, France).

Footprinting with DNase I.

Labeled DNA fragments were diluted to 0.2 nM in a final volume of 100 μ l of 10 mM Tris (pH 8), 2 mM MgCl₂, 100

mM KCl, 1 mM CaCl₂, 0.1 mg/ml BSA, salmon sperm DNA at 15 μ g/ml, and 150 μ M MnCl₂. Samples were incubated for 5 min at 37°C with the indicated amount of the Fur protein, and 10⁻³ unit of DNase I (Roche, Meylan, France) was added to each sample. After 5 min at 37°C, reactions were stopped by the addition of 50 μ l of stop buffer (0.1 M EDTA, tRNA 200 μ g/ml). Proteins were removed by one phenol/chloroform extraction. Nucleic acids were precipitated with the addition of 50 μ l of 8 M ammonium acetate and 500 μ l of ethanol. Samples were loaded on 5% polyacrylamide sequencing gel with 7 M urea. A+G and C+T chemical sequencing reactions were carried out and loaded onto the gels with the treated samples (Maxam and Gilbert 1980).

ACKNOWLEDGMENTS

This work was supported by the Programme Physique et Chimie du Vivant 2000 from the CNRS and by grants from INRA. D. Expert and I. Michaud-Soret are researchers from CNRS. We thank D. Anker and N. Hugouvieux-Cotte-Pattat for the gift of KDG; W. Nasser and S. Reverchon for their constant interest in this work and for sending us plasmids pSR1321, pN1272, pSR1175, and the *Erwinia chrysanthemi* strains A410, A837, and A621; and we thank D. Touati for helpful and valuable discussions.

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