Iron Regulation and Pathogenicity in *Erwinia chrysanthemi* 3937: Role of the Fur Repressor Protein

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Low iron availability is a triggering signal for coordinated expression of the genes encoding pectate lyases PelB, PelC, PelD, and PelE, and chrysobactin iron transport functions, which are two main determinants of phytopathogenicity of the *Erwinia chrysanthemi* strain 3937. The possible implication of the ferric uptake regulation (Fur) protein in this process was investigated. The *E. chrysanthemi fur* gene was cloned by functional complementation of an *Escherichia coli fur* mutant and sequenced. The 444-bp open reading frame identified was found to code for a protein highly similar to the *E. coli* Fur regulator. An *E. chrysanthemi fur* null mutant was constructed by reverse genetics. This mutant showed altered growth capacity and reduced pathogenicity on African violets. In a *fur* background, transcriptional *lacZ* fusions to genes belonging to the *E. chrysanthemi* high affinity iron transport systems were constitutively expressed. Transcription of the *pelA*, *pelD*, and *pelE* genes was analyzed, using fusions to the *uidA* reporter gene. Iron availability and a *fur* mutation did not influence the expression of *pelA*. In the presence of iron, *pelD* and *pelE* transcription levels were higher in the *fur* mutant than in the parental strain. Furthermore, iron deficiency stimulated the expression of both fusions in the *fur* mutant. These findings indicate that, in *E. chrysanthemi* 3937, (i) Fur negatively controls iron transport and genes encoding PelD and PelE, and (ii) additional factor(s) mediate iron regulation of the *pel* genes.

Bacterial pathogens possess a multitude of methods allowing them to survive and multiply in host tissues. Two major determinants of the pathogenicity of the enterobacterium *Erwinia chrysanthemi* (strain 3937) on African violets are the production of pectinolytic enzymes and the expression of a siderophore-dependent iron uptake system (for reviews see Barras et al. 1994; Collmer and Keen 1986; Expert et al. 1996). Synthesis and secretion of various plant cell wall-degrading enzymes enable *E. chrysanthemi* cells to spread out from the plant extracellular compartments and macerate the leaf parenchymatous tissue. To overcome low iron availability

from the host, bacteria use a high-affinity iron transport system mediated by chrysobactin (Enard et al. 1988; Neema et al. 1993). Strain 3937 synthesizes multiple isoforms of pectinases, including two pectin methylesterases, encoded by the *pemA* and *pemB* genes (Laurent et al. 1993; Shevchik et al. 1996), and five major pectate lyases (Pel), encoded by the *pelA*, *pelB*, *pelC*, *pelD*, and *pelE* genes (Hugouvieux-Cotte-Pattat et al. 1996), that cleave pectin into unsaturated oligogalacturonates. These substrates are used by the bacteria as carbon and energy sources. However, Pel activities can generate catabolic products that are also able to act as elicitors for plant defense (Messiaen and Van Custem 1993, 1994). Thus, the pathogenesis process depends on a fine tuning of the genes encoding this complex enzymatic equipment.

Genetic experiments have led to the identification of three regulatory loci that negatively control the expression of pectinolysis genes: *kdgR*, *pecS*, and *pecT* (Praillet et al. 1996, 1997; Surgery et al. 1996). The KdgR protein is the main effector and acts as a transcriptional repressor for all the genes involved in the pectin degradation pathway (Reverchon et al. 1991; Nasser et al. 1992). In the absence of pectic inducers, the KdgR protein binds to a specific 17-bp sequence, called the KdgR box, that is conserved in the promoter region of the different pectinolysis genes (Nasser et al. 1994). Pectinase production is also subject to positive regulation. Previous studies have shown that the cyclic AMP (cAMP) receptor protein, CRP, activates the transcription of the pectinolysis genes (Reverchon et al. 1997; Nasser et al. 1997). This complex regulatory network confers on bacteria the ability to modulate their pectinolytic activity in accordance with a wide range of environmental signals (Hugouvieux-Cotte-Pattat et al. 1992). In addition, iron availability differentially controls *pel* gene expression: moderate iron limitation induces *pelB*, *pelC*, and *pelE* transcription, whereas higher iron deficiency is necessary to upregulate the *pelD* gene (Sauvage and Expert 1994; Masclaux et al. 1996).

In response to iron starvation, *E. chrysanthemi* synthesizes two siderophores, chrysobactin and achromobactin (Persmark et al. 1989; Mahé et al. 1995), that are low-molecular-weight molecules, chelating the ferric ion with high and medium affinity, respectively. Their cognate transport machinery is also induced under conditions of iron deficiency. However, induction of the achromobactin-dependent uptake system requires weaker iron limitation than that necessary for derepression of chrysobactin-mediated iron transport (Expert et al. 1992;

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Masclaux and Expert 1995; Mahé et al. 1995). This regulation was investigated in detail for the *ftcb*sCEBA operon encoding the ferrichrysobactin outer membrane receptor Fct and the enzymes leading to the catechol moiety in chrysobactin biosynthesis (Cbs) (Franza and Expert 1991). A Fur operator consensus sequence identified in the promoter region of this operon was shown to be functional in *Escherichia coli*, suggesting the occurrence of a *fur*-like gene in *E. chrysanthemi* (Sauvage et al. 1996). In many bacteria, iron regulation is achieved by the Fur protein, which acts as a transcriptional repressor with the ferrous ion as a co-repressor (Bagg and Neilands 1987; Escobar et al. 1997). In the presence of Fe(II), Fur binds to a conserved DNA sequence, the Fur box, shown to be present in the promoter region of a large number of iron-regulated genes (de Lorenzo et al. 1988a, 1988b; Calderwood and Mekalanos 1988; Chen et al. 1993; Stojiljkovic et al. 1994; Crosa 1997; Escobar et al. 1998). The regulator would be active after dimerization of the Fur monomer (Coy and Neilands 1991; Stojiljkovic and Hantke 1995).

This work was aimed at cloning the *E. chrysanthemi fur* gene and defining its precise role in the control of iron transport and genes encoding Pel enzymes.

RESULTS

Cloning of the *E. chrysanthemi fur* homologue.

The *E. chrysanthemi fur*-like gene was isolated by complementation of a Fur⁻ *E. coli* strain. An *E. chrysanthemi* genomic library, constructed in the mobilizable cosmid pAL2917, was introduced by conjugation into *E. coli* H1618, which contains a point mutation in the *fur* gene and a transcriptional *lacZ* fusion to the Fur-regulated *fiu* gene (Hantke 1984). This fusion is expressed constitutively regardless of the iron concentration. Strain H1618 thus gives blue colonies (Lac⁺) on LB agar plates supplemented with iron and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Transconjugants were plated on selective medium containing streptomycin, tetracycline, X-Gal, and 20 μ M FeCl₃, and checked for iron regulation of the reporter fusion. All isolated white colonies were found to contain the same cosmid. The corresponding cosmid pCSF harbors an approximately 30-kb genomic insert. With the *E. coli fur* gene used as a probe, the *E. chrysanthemi fur* locus was localized on a 2.4-kb *Pst*I and a 2.7-kb *Eco*RI restriction fragment of pCSF (Fig. 1). It was not

possible to subclone the *Pst*I restriction fragment into the multicopy plasmid pUC19. However, the overlapping *Eco*RI restriction fragment was successfully cloned into pUC19. Sequence analysis indicated that the whole *fur* gene was not present on this fragment (Fig. 1). Thus, it was decided to shorten this region by subcloning, and to reassemble the *fur* gene by ligating the 0.7-kb *Pst*I-*Eco*RI fragment (from pTF22) and the 0.5-kb *Eco*RI-*Bam*HI fragment (from pTF23) into the mono- and low-copy number plasmids pWSK29 and pRK767, respectively, generating plasmids pTF20 and pTF21 (Fig. 1). Both recombinant plasmids were able to functionally complement the *E. coli* strain H1618 (data not shown). Thus, a *fur* gene does exist in *E. chrysanthemi* and can complement an *E. coli fur* mutant. However, it was not possible to clone this *Pst*I-*Bam*HI segment into a multicopy plasmid in *E. coli*.

Sequence analysis of the *E. chrysanthemi fur* locus.

Nucleotide composition of the *E. chrysanthemi fur* gene was determined by sequencing the 2.7-kb *Eco*RI and 0.5-kb *Eco*RI-*Bam*HI restriction fragments cloned into pUC19 (plasmids pTF24 and pTF23, respectively). An open reading frame (ORF) of 444 bp, extending from position 436 to 879, that showed similarity to the *E. coli fur* gene, was identified. The ATG start codon is preceded by a putative Shine-Dalgarno sequence AGGA. Computer analysis indicated the presence of a putative promoter located 86 bp upstream from the start codon. At the 3' untranslated region of the *fur* gene, an inverted repeat may form a 15-bp hairpin that could act as an rho-independent transcription terminator (Fig. 2).

This ORF was found to encode a 148 amino acid protein exhibiting high relatedness with the *E. coli* (89% amino acid identity), *Yersinia pestis* (86%), and *Klebsiella pneumoniae* (85%) Fur proteins (Schäffer et al 1985; Staggs and Perry 1992; Achenbach and Yang 1997). We also identified a partial ORF 78 bp downstream of the *fur* stop codon that is oriented divergently relative to the *fur* gene. This partial ORF of 318 bp displays 70% identity with the 3' end of the *E. coli glnS* gene encoding the glutaminyl-tRNA synthetase.

Construction of an *E. chrysanthemi fur* null mutant.

For this purpose, we attempted to introduce the interposon Ω , coding for spectinomycin resistance, into the chromosomal *fur* gene by reverse genetics. This strategy was unsuccessful. Failure to construct a *fur* null mutant has already been reported for other bacteria including *Pseudomonas aeruginosa*, *Vibrio anguillarum*, *Haemophilus ducreyi*, *Neisseria gonorrhoeae*, and a *Synechococcus* sp. (Prince et al. 1993; Tomalsky et al. 1994; Biegel Carson et al 1996; Thomas and Sparling 1996; Ghassemian and Straus 1996). Iron is a notoriously toxic element because of its capacity to catalyze in the presence of oxygen the formation of hydroxyl radicals (Halliwell and Gutteridge 1985). A *fur* mutant, which does not sense iron, tends to accumulate a toxic concentration of this element. Therefore, we decided to use a strain altered in the chrysobactin iron transport system for constructing such a *fur* null mutant. To screen for the Fur⁻ phenotype, a mutant harboring a transcriptional *lacZ* fusion in the *ftc* gene was used. After four passages in low phosphate medium, a total of 40 out of 2,000 clones were Spec^r Cm^s and were analyzed further for constitutive expression of the reporter fusion. Three clones showed an intermediate phenotype, i.e., partial derepression of

Fig. 1. Cloning strategy of the *fur* locus. Cloning restriction sites are indicated: B, *Bam*HI; E, *Eco*RI; P, *Pst*I. The *Eco*RI site, indicated by an asterisk, originates from cosmid pAL2917. Interposon Ω was inserted at the *Eco*RI site of plasmid pTF20. Other details are given in the text.

the *ftc::lacZ* fusion in the presence of iron. However, three other clones displayed a true *Fur*⁻ phenotype and were checked further for a proper *fur* gene disruption by Southern blot hybridization. These three clones exhibited a correct insertion of the Ω interposon into the chromosomal *fur* gene (data not shown). To introduce this mutation into other genetic backgrounds, we decided to use the transduction technique. Transductants were selected in a top agar layer, where the mi-

croaerophilic conditions may reduce iron toxicity. With phage phiEC2, it was possible to transduce the *fur* null mutation into wild-type and different mutant backgrounds, although the transduction efficiency was very low in comparison to other markers (data not shown). These transductants were viable and could grow in aerobic conditions. It is likely that the environmental conditions used in transduction experiments allow the selection of a small population of *Fur*⁻ cells.

GATCGCCAGCCGGA	ACTTGACCAGCGAACGCGTCTCCA	ACTGGGTCAAGCAAATCAGCGAAGAGCTGAACCT	72																
GAAAGCACTGCAGGCCTGATT	CACCTGTGGCTCTGATACTAATGTACTCAGAGCCACATCTATGTAGATAGA		144																
TAAAACCTATCACAATCAGTGATA	CATTTTTTTAACTTTTCATTCCAACAGGGTACAATACCTCTTCGATCG		216																
GTCGGGTTGCATTTAATGACCTGTCTTGGCGTACAATATGTCTCTCGCAGGGAACA	ACTCTGAATACAGCGA		288																
GTTAGCAAGAATCATATTGTAAAAGTTCCGTGGT	<u>TTTCATTT</u> CGACGGCGCGGTTCTATAATTGAGACACAG		360																
TGACCTTTTTTTAAGCCGCGCCGATGATACTAGGCTAATAGCCTCTCAGATAAAAGCAAGTAAC	<u>AGGACT</u> AAATC		432																
M	T	D	N	N	T	A	L	K	K	A	G	L	K	V	T	L	17		
CGC	ATG	ACT	GAC	AAC	AAT	ACC	GCA	TTA	AAG	AAG	GCT	GGC	CTG	AAA	GTC	ACA	CTT	486	
P	R	L	K	I	L	E	V	L	Q	D	P	Q	C	H	H	V	S	35	
CCA	CGA	CTG	AAA	ATT	CTG	GAA	GTG	CTT	CAG	GAT	CCT	CAA	TGC	CAC	CAC	GTC	AGC	540	
A	E	D	L	Y	K	K	L	I	D	M	G	E	E	I	G	L	A	53	
GCG	GAA	GAC	TTA	TAC	AAA	AAG	CTG	ATT	GAT	ATG	GGT	GAA	GAG	ATT	GGT	CTG	GCC	594	
T	V	Y	R	V	L	N	Q	F	D	D	A	G	I	V	T	R	H	71	
ACC	GTT	TAC	CGC	GTG	CTC	AAC	CAG	TTT	GAC	GAC	GCC	GGC	ATC	GTT	ACC	CGT	CAT	648	
N	F	E	G	G	K	S	V	F	E	L	T	Q	Q	H	H	H	D	89	
AAC	TTC	GAA	GGC	GGC	AAA	TCG	GTT	TTC	GAA	CTC	ACG	CAG	CAG	CAC	CAT	CAT	GAC	702	
H	L	I	C	L	D	C	G	R	V	I	E	F	S	D	E	S	I	107	
CAT	CTG	ATC	TGT	CTG	GAT	TGC	GGT	CGG	GTG	ATT	GAA	TTC	AGC	GAC	GAG	TCT	ATC	756	
E	A	R	Q	R	E	I	S	E	K	H	G	I	K	L	T	N	H	125	
GAA	GCG	CGC	CAG	CGT	GAA	ATC	TCC	GAG	AAA	CAC	GGC	ATC	AAA	CTG	ACC	AAC	CAC	810	
S	L	Y	L	Y	G	H	C	S	S	G	D	C	R	E	D	D	N	143	
AGC	CTG	TAT	TTG	TAC	GGC	CAT	TGC	AGT	AGC	GGC	GAT	TGC	CGT	GAA	GAC	GAC	AAT	864	
A	H	N	E	R	*													149	
GCG	CAC	AAC	GAA	CGC	TAA	<u>TC</u> ACTCGATACAGAGAGAAAATAAAACCGCCGCGGCGGTTTATTTT												929	
TATCTTGT																		CCCCGGTAATCAGGCGGCAATCATCCCGCCAGCTGTCCCGCAAACCAACCGTCCGGTTGAACA	1001
CCAGATTATCCGCACTGGAATAAACGCGATCGGCGCAGAAATACCCTTCCCGCTCAA																		ACTGATAGGCTTTTT	1073
CAACTTCAGCCTGACCCAGACTGGCTTCCACAAAGCCCTGGGCGACAGTCAGCGAGTCCGGGTTGATAGTGG																		1145	
AGAGAAAATCCTCCGCCGCACCAGGGTTAGCCACGCTGAACAGACGGTCATACAGGCGGAACTGCGCCGGCA																		1217	
GCGCGTGAGCCGCCGATACCCAGTGGATCACGCCCTTACCTTGCGGCCGTCCGCCGGATCC																		1279	

Fig. 2. Nucleotide sequence of the *fur* gene. Position of a potential promoter P and a Shine-Dalgarno (SD) sequence are underlined. A putative terminator structure is indicated by arrows. The *fur* region nucleotide sequence has been deposited to the GenBank data base under accession no. AF016035.

Growth characteristics and pathogenicity of the *E. chrysanthemi fur* mutant.

The *fur* mutation was thus introduced into the wild-type strain 3937. The resulting mutant grew significantly less well than wild-type cells in minimal or rich medium (lower final cell density). Furthermore, in the presence of iron the generation time of the *fur* mutant was double that of the wild-type strain. In iron-restricted conditions, *Fur*⁻ and *Fur*⁺ cells showed nearly identical growth rates. The *fur* mutant was able to use glucose, glycerol, and PGA as carbon sources. Like the *E. coli* and *Vibrio cholerae fur* mutants, the *E. chrysanthemi fur* mutant was unable to use succinate. However, unlike these mutants, the *E. chrysanthemi fur* mutant remained capable of growing on fumarate plates. Unlike the wild-type strain, this mutant was found to grow less well in M63 medium than in Tris medium (data not shown). The latter was thus chosen for analyzing the expression of the reporter fusions. Finally, the *fur* mutant demonstrated a high level of mortality when kept more than 6 months at -80°C.

We examined the behavior of the *fur* mutant of strain 3937 after inoculation on African violets. *Fur*⁻ bacteria displayed an altered virulence on their host (Fig. 3). Indeed, this mutant gave a higher number of negative responses than the wild-type strain. Furthermore, the *fur* mutant proved to be less aggressive than the wild-type strain: a number of plants displaying localized macerations failed to give a systemic response in following days. This reduced pathogenicity of the *E. chrysanthemi fur* mutant on its host was explained by its altered growth capacity, as indicated by monitoring the bacterial growth in planta. Indeed, a 10-fold increase in viable counts (CFU) relative to the initial inoculum was detectable after 3 days in leaves inoculated with the wild-type strain. Within the same period of time, the population of the *fur* mutant decreased 10-fold.

Effect of the *fur* mutation on regulation of the achromobactin- and chrysobactin-iron transport systems.

We first examined the expression of a *fct::lacZ* fusion in *Fur*⁻ and *Fur*⁺ backgrounds during bacterial growth. In wild-type cells, the fusion was found to be strongly repressed under iron-rich conditions (Fig. 4). In a *fur* genotype, there was an increase in the *fct* transcriptional activity regardless of the iron concentration. However, partial iron regulation was still observed for the *fct* gene, in the absence of the Fur protein. These results were corroborated by assaying chrysobactin in culture supernatant fluids from the *fur* mutant of 3937, which appeared to be constitutively produced (data not shown). A fusion to the achromobactin biosynthetic gene (*acsA1::lacZ*) located immediately upstream from the ferriachromobactin permease operon (Mahé et al. 1995) was found to be constitutively overexpressed in a *fur* mutant (Fig. 4). These data show that in *E. chrysanthemi* the Fur protein negatively controls the genes involved in iron assimilation. However, this negative control may be indirect.

Effect of the *fur* mutation on *pel* gene transcription.

Sauvage and Expert (1994) have thoroughly analyzed *pelA::*, *pelB::*, *pelC::*, *pelD::*, and *pelE::uidA* activities in response to iron. However, these experiments were performed in deferrated M63 medium. Since *fur* mutants grew poorly in this

medium, which probably contains traces of iron because of a high phosphate concentration, we decided to use the low phosphate-containing Tris medium. Because of the difficulty involved in constructing *pel fur* double mutants, we restricted our analysis to *pelA*, *pelD*, and *pelE* genes. Indeed, transcription of *pelA* is independent of the iron concentration, whereas a moderate and a higher iron deficiency are necessary to induce transcription of *pelE* and *pelD*, respectively (Sauvage and Expert 1994; Masclaux et al. 1996). In a *fur* genotype, the *pelD* and *pelE* fusions were found to be derepressed regardless of the iron concentration (Fig. 5). Surprisingly, in the absence of the Fur protein, the β -glucuronidase activities conferred by these fusions were still stimulated by low iron levels. These data were corroborated by assaying the global Pel activity under similar conditions (Table 1). Nevertheless,

Fig. 3. Evolution of symptoms induced by *Erwinia chrysanthemi* 3937 and its *Fur*⁻ derivative on African violets (*Saintpaulia ionantha*). One leaf per plant was inoculated as detailed in Materials and Methods. The significant spreading phases of the disease were scored as follows: 1 = no maceration symptom; 2 = maceration localized at the inoculated area; 3 = maceration covering the inoculated leaf; and 4 = maceration invading the petiole and spreading toward the other aerial parts of the plant.

in a *Fur*⁻ background, the activity level of both fusions in the presence of iron was similar to that observed under low iron conditions for wild-type cells. Although PelD and PelE enzymes display the highest Pel-specific activity, this effect was not observed on the global Pel production (Table 1). In the absence of inducers, the detected Pel activity in culture supernatant fluids is very low, suggesting that secretion could be the limiting step. In contrast, *pelA* transcription was not activated by iron limitation and the *fur* null mutation did not result in a higher expression level of the *pelA::uidA* fusion (Fig. 5). Under conditions of pectinolysis, when PGA was used as a carbon source, the *fur* mutation did not cause an increase in the *pelD* and *pelE* transcriptional activity (data shown for *pelE* only; Fig. 5). However, under these conditions, iron deficiency still increased the expression of the *pelD* and *pelE* fusions. These results were confirmed by determining the global Pel activity in the same conditions of growth (Table 1). Col-

lectively, these data suggest that (i) the *E. chrysanthemi* Fur protein may act as a negative regulator of iron-regulated *pel* genes, and (ii) Fur may not be the only effector mediating the metal regulation of these genes. We attempted to introduce the *fur* mutation into several pectinase regulatory mutants, such as *kdgR*, *pecS*, and *crp* mutants. However, we failed to select such double mutants, which are probably lethal.

DISCUSSION

In vitro and in planta experiments have shown that, in *E. chrysanthemi* 3937, iron is a signal for differential expression of genes encoding Pel. (Sauvage and Expert 1994; Lojkowska et al. 1995; Masclaux et al. 1996). In many gram-negative bacteria, the Fur repressor protein mediates metalloregulation. This work was aimed at elucidating whether the *E. chrysanthemi fur* homologue is responsible for iron regulation of the *pel* genes. We isolated and cloned the *E. chrysanthemi fur* gene by complementing an *E. coli Fur*⁻ mutant. The *E. chrysanthemi fur* gene encodes a protein showing 89% identity (96% similarity) with its *E. coli* counterpart. The failure to clone this gene in a multicopy plasmid, together with the difficulty in constructing a *fur* null mutant and in transducing the corresponding mutation, strengthens the idea that this protein not only monitors iron regulation but is also an important regulator of cell physiology (Litwin and Calderwood 1994; Hall and Foster 1996). Moreover, the concentration of this regulatory protein seems to be tightly regulated (Watnick et al. 1997). The role of Fur in the control of the chrysobactin- and achromobactin-dependent iron transport systems was examined by analyzing the expression of *lacZ* transcriptional fusions to the *fct* and *acs* genes, respectively. In the absence of the Fur product, both genes are highly transcribed even in the presence of the iron. It is worth noting that in *fur* mutants the transcription levels were higher than the induced levels observed in the wild type. This effect may result from the fact that, in *fur*-deficient cells, iron can no longer give metallo-repression. However, the *acs::lacZ* fusion showed a constitutive expression, whereas partial iron regulation was still observed for the *fct* gene. Fur thus seems to be the major regulator for iron transport functions in *E. chrysanthemi*, although some other effectors may control the genes of the chrysobactin system. Indeed, in many bacteria additional regulatory mechanisms occur under iron-limiting conditions (for a review see Crosa 1997). Positive regulation exerted by siderophores has been described in *E. coli*, *Yersinia pestis*, and pseudomonads (Crosa 1997; Fetherston et al. 1996). However, it seems unlikely that such a similar type of regulation occurs in *E. chrysanthemi*, since the strain used in this study harbors a fusion in the *fctcb*sCEBA operon and does not synthesize chrysobactin and the cognate outer membrane receptor.

The contribution of Fur to the control of *pel* genes by iron was investigated by analyzing the transcription of *pelA*, *pelD*, and *pelE* genes, which are differentially regulated by this metal. The transcriptional activity of *pelA* was not affected by the *fur* mutation. Disruption of the *fur* gene results in an increase in *pelD::uidA* and *pelE::uidA* expression, which, however, remains in some way iron regulated. These data indicate that the Fur protein may be a repressor of the iron-regulated *pel* genes or may control some other effectors involved in this regulation. Several other regulatory genes have been shown to

Fig. 4. Expression of the chromosomal *fct34::lacZ* and *acsA1::lacZ* gene fusions in *Fur*⁺ (squares) and *Fur*⁻ (circles) cells of *Erwinia chrysanthemi* L37. Strains were grown in iron rich (FeCl_3 : 30 μM) (solid symbols) or iron poor (EDDHA: 30 $\mu\text{g}/\text{ml}$) (open symbols) conditions. 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.

control pectinolysis, such as those encoding the KdgR, PecS, PecT, and CRP proteins. The inability to transduce the *fur* mutation in the corresponding mutants prevented us from analyzing the possible interactions between these regulatory pathways. Nevertheless, it seems unlikely that Fur is an activator of the *kdgR* or/and *pecS* genes, since the transcriptional level of the *pelA* gene was not affected in a *Fur*⁻ mutant. A

thorough inspection of the promoter region of the *E. chrysanthemi* 3937 *pelD* and *pelE* genes revealed two well-conserved Fur boxes (13 out of 19 nucleotides) located 30 and 50 bp upstream from the -35 promoter site of *pelD* and *pelE*, respectively (Fig. 6). The presence of Fur boxes strengthens the hypothesis of a direct control by Fur for the iron-regulated *pel* genes. Usually the Fur box overlaps the -35 or -10 promoter

Fig. 5. Expression of chromosomal *pel::uidA* gene fusions in *Fur*⁺ (squares) and *Fur*⁻ (circles) cells of *Erwinia chrysanthemi* L37. Strains were grown in iron rich (FeCl_3 : 30 μM) (solid symbols) or iron poor (EDDHA: 30 $\mu\text{g}/\text{ml}$) (open symbols) conditions. Bacterial growth was assessed by measuring optical density (OD) at 600 nm. As an example, the graph *pelE::uidA* + PGA shows the expression of the fusion for cells grown in the presence of pectic inducers. Experiments were performed three times and standard deviations are shown. β -Glucuronidase activity is expressed in nanomoles of paranitrophenol liberated per minute, per unit of optical density at 600 nm.

sites of iron-regulated genes. In addition to their unusual position, these iron boxes overlap the binding sites for the cAMP-CRP complex that were recently characterized by DNase I footprinting experiments (Nasser et al. 1997). In the Fur paradigm, the Fe(II) Fur dimer inhibits transcription initiation by blocking the entry of the RNA polymerase (Escobar et al. 1997, 1998). Both proteins could compete for occupation of the same site and could be replaced by one another according to the iron status of the cell. Furthermore, when present at high concentration, the Fur protein can polymerize around its binding site (Le Cam et al. 1994). Here, we speculate that Fur could behave as an antiactivator of *pel* gene transcription by blocking the action of CRP. Indeed, Nasser et al. (1997) showed that CRP activates the transcription of pectinase-encoding genes. In the absence of pectic inducers, when KdgR is active, CRP is still capable of significantly increasing the transcription of *pelB*, *pelC*, *pelD*, and *pelE* genes (Reverchon et al. 1997). From these data, a model can be drawn in which CRP already bound to its target site can immediately activate *pel* gene transcription, when KdgR dissociates from its operator sequence. Such an event may cause a small increase in Pel activity that is sufficient to turn on the whole machinery involved in pectin degradation. Our hypothesis is that Fur would hinder CRP binding and subsequent gene activation. This hypothesis could explain the higher transcriptional activity of *pelD* and *pelE* observed in the *fur* mutant. It is noteworthy that the operator regions from both genes display the highest affinity for the cAMP-CRP complex (Nasser et al. 1997). Furthermore, the lower affinity for KdgR of the *pelE* promoter in comparison to that of the *pelD* gene could explain the difference in iron limitation required for activation of those genes.

This model raises some puzzling questions: for example, what does happen in the presence of iron and pectic inducers? In this case the repressor KdgR is inactivated, but the active Fur product would prevent activation by CRP. We now have some indication that the negative metalloregulation mediated by Fur is partially relieved upon conditions of pectinolysis (D. Expert, *personal communication*). Thus, in these conditions pectinase production could still be activated. Secondly, our hypothesis does not explain the iron regulation of *pel* gene transcription that still occurs in a *fur* null mutant. One can imagine the existence of another, not yet identified effector responsible for the upregulation observed under iron starvation. Another possibility would be that the regulatory system of the pectinase-encoding genes is modified according to the

iron status of the cell. In this hypothesis, iron deficiency would directly or indirectly modulate the level of repression caused by KdgR and/or other regulators. Further understanding of the precise mechanisms involved in this complex regulatory circuit requires a molecular study in which the genuine contribution of the *E. chrysanthemi* Fur protein will be investigated.

MATERIALS AND METHODS

Strains and media.

The bacterial strains and bacteriophage and plasmids used in this work are described in Tables 2 and 3, respectively. Nutrient-rich media were LB and MacConkey (Miller 1972). Tris medium was used as minimal medium, containing 2 g per liter of glucose, glycerol, or polygalacturonate as a carbon source (Franza et al. 1991). It was supplemented with EDDHA (ethylene-diamine-*N,N'*-bis[2-hydroxyphenyl]acetic acid), purchased from Sigma (St. Louis, MO) at a final concentration of 30 µg/ml in order to provide iron-limited conditions. For iron-rich conditions, FeCl₃ was added at a final concentration of 30 µM. When necessary, the following antibiotics (Sigma) were used, at these final concentrations (all per ml): ampicillin (Amp), 50 µg; chloramphenicol (Cm), 30 µg; kanamycin (Km), 25 µg; spectinomycin (Spec), 30 µg; streptomycin (Sm), 100 µg; or tetracycline (Tc), 25 µg. TA7 was used for

Fur box consensus	G A T A A T G A T A A T C A T T A T C
<i>pelD</i>	<u>G A T A A A A T</u> <u>T A A T C A</u> G C C T C
<i>pelE</i>	A T T A A T A A A A A C C A T T G T C
<i>fct</i>	<u>G C A A A T G T A</u> <u>A A T G G T T C T C</u>

Fig. 6. Schematic view of the promoter regions of the *pelD* and *pelE* genes from *Erwinia chrysanthemi* 3937. Promoter elements, identified by primer extension experiments, KdgR boxes, and cyclic AMP (cAMP) receptor protein (CRP) binding sites confirmed by footprinting experiments (Nasser et al. 1997) are shown. Potential Fur boxes (see below) are underlined. Sequence alignment of the putative Fur boxes found in the promoter region of the *pelD*, *pelE*, and *fct* genes from *E. chrysanthemi* 3937 is presented. Conserved nucleotides are boxed. The Fur box from the *fct* gene is functional in *Escherichia coli* (Sauvage et al. 1996).

Table 1. Pectate lyase production in *E. chrysanthemi* 3937 Fur⁺ and Fur⁻ strains^a

Strain	PGA		Glycerol	
	-Fe	+Fe	-Fe	+Fe
Fur ⁺	0.70 ± 0.08	0.20 ± 0.02	0.030 ± 0.005	0.010 ± 0.002
Fur ⁻	0.70 ± 0.09	0.20 ± 0.03	0.045 ± 0.006	0.010 ± 0.003

^a Cells were grown in Tris medium supplemented with glycerol or polygalacturonic acid (PGA). Tris Medium was made iron rich or iron deprived by the addition of 30 µM FeCl₃ or EDDHA (30 µg/ml), respectively. Samples were collected on late logarithmic-phase cultures for determination of pectate lyase-specific activity. Specific activity is expressed as micromoles of products liberated per minute per milligram (dry weight) of bacteria. Results reported are the averages of three independent experiments; standard deviations are indicated.

soft agar overlays (Franza et al. 1991). For recombinant plasmid selection, IPTG (isopropyl- β -D-thiogalactopyranoside) and X-Gal (Boehringer, Mannheim, Germany) were added at final concentrations of 250 and 40 μ g/ml, respectively. Carbon source utilization was monitored on M9 agar plates, after 5 days of aerobic growth at 30°C (Miller 1972). For gene marker exchange by homologous recombination, the minimal Low Phosphate medium was used (Roeder and Collmer 1985). This medium contained (g per liter): Tris (12.1), $\text{MgSO}_4 \cdot (\text{H}_2\text{O})_7$ (0.1), $(\text{NH}_4)_2\text{SO}_4$ (1), Citrate $(\text{Na})_3$ (0.5). It was adjusted to pH 7 with HCl 10 N. Potassium phosphate was added at a final concentration of 250 μ M, after autoclaving.

Mating, transformation, and transduction methods.

The *E. chrysanthemi* genomic library constructed in cosmid pLA2917 was propagated in the ED8767 strain of *E. coli*. Tri-parental matings with the helper plasmid pRK2013 were performed as described previously (Franza et al. 1991). *E. coli* cells were transformed by the CaCl_2 procedure and *E. chrysanthemi* bacteria were electroporated as described by Sambrook et al. (1989). Phage phiEC2 transductions were per-

formed as described previously, except that 3.5 ml of soft agar overlay (TA7) was used for plating on 90-mm petri dishes (Franza et al. 1991).

DNA methods.

DNA manipulations (plasmid isolation, cloning, and electrophoresis) were carried out as described by Sambrook et al. (1989). The cosmid library of *E. chrysanthemi* was constructed in vector pLA2917 linearized with *Bgl*II, using *Sau*3A partially digested genomic DNA. The ligated DNA was packaged in vitro with the Gold Kit packaging extracts as recommended by the supplier (Stratagene, La Jolla, CA). The packaged extracts were transduced into *E. coli* ED8767. Tc^r clones were selected and screened for kanamycin sensitivity. All cloning experiments were performed in the DH5 α strain of *E. coli*. DNA/DNA hybridization analysis was performed by Denhardt's method, described by Sambrook et al. (1989). DNA sequencing was performed on double-strand plasmid by the dideoxynucleotide chain termination method with Sequenase version 2.0 and [α - ^{35}S]dATP (Amersham, Little Chalfont, England). When necessary, DMSO (dimethyl sulfoxide;

Table 2. Bacterial strains and phage used in this study

Strain	Characteristics	Reference or source
<i>Erwinia chrysanthemi</i>		
3937	Wild-type strain	Our collection
3937 <i>fur</i>	<i>fur</i> with an interposon Ω inserted at the <i>Eco</i> RI site, Spec^r	This study
L37	Lac^- derivative from 3937	Our collection
L37 <i>acsA1::lacZ</i>	Acs^- derivative from L37, Km^r	Mahé et al. 1995
L37 <i>fc1::lacZ34</i>	Fct^- and Cbs^- , Km^r	Masclaux and Expert 1995
L37 <i>acsA1::lacZ fur</i>	Acs^- and Fur^- , Km^r and Spec^r	This study
L37 <i>fc1::lacZ 34fur</i>	Fct^- , Cbs^- , and Fur^- , Km^r and Spec^r	This study
L37 <i>pelA::uidA</i>	PelA^- , Km^r	Masclaux and Expert 1995
L37 <i>pelD::uidA</i>	PelD^- , Km^r	Masclaux and Expert 1995
L37 <i>pelE::uidA</i>	PelE^- , Km^r	Masclaux and Expert 1995
L37 <i>pelA::uidA fur</i>	PelA^- and Fur^- , Km^r and Spec^r	This study
L37 <i>pelD::uidA fur</i>	PelD^- and Fur^- , Km^r and Spec^r	This study
L37 <i>pelE::uidA fur</i>	PelE^- and Fur^- , Km^r and Spec^r	This study
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> ($\phi 80\text{lacZ}\Delta\text{M15}$) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi</i> <i>relA1</i>	Sambrook et al. 1989
ED8767	<i>supE44</i> <i>supF58</i> <i>hsdS3</i> ($\text{r}_B^- \text{m}_B^-$) <i>recA56</i> <i>galK2</i> <i>galT22</i> <i>metB1</i>	Sambrook et al. 1989
H1618	<i>thr</i> <i>lac</i> <i>ser</i> <i>fhuA</i> <i>rpsL</i> <i>thi</i> <i>hsdR</i> <i>fhu::</i> MudI(Aplac) <i>fur-31</i>	Hantke 1984
Phage		
PhiEC2	General transducing phage of <i>Erwinia chrysanthemi</i>	Résibois et al. 1984

Table 3. Plasmids used in this study

Plasmids or cosmids	Characteristics	Reference or source
Cosmids		
pLA2917	21-kb mobilizable cosmid, Tc^r and Km^r	Allen and Hanson 1985
pCSF	30-kb <i>Sau</i> 3A fragment of genomic DNA from <i>Erwinia chrysanthemi</i> cloned in the <i>Bgl</i> II site of the Km^r gene from pLA2917, Fur^+ and Tc^r	This study
Plasmids		
pRK2013	Mobilization helper plasmid, Km^r	Figurski and Helinski 1979
pUC19	2.7-kb vector, Amp^r	Sambrook et al. 1989
pBC	3.4-kb vector derived from pUC19, Cm^r	Stratagene, La Jolla, CA
pRK767	10.7-kb mobilizable vector, Tc^r , RK2 derivative	Gill and Warren 1988
pWSK29	5.4-kb low copy number vector, Amp^r , pSC101 derivative	Wang and Kushner 1991
pTF20	1.2-kb <i>Pst</i> I- <i>Bam</i> HI fragment from pCSF cloned in pWSK29, Tc^r	This study
pTF21	1.2-kb <i>Pst</i> I- <i>Bam</i> HI fragment from pCSF cloned in pRK767, Tc^r	This study
pTF22	0.7-kb <i>Pst</i> I- <i>Eco</i> RI fragment from pCSF cloned in pUC19, Amp^r	This study
pTF23	0.5-kb <i>Eco</i> RI- <i>Bam</i> HI fragment from pCSF cloned in pUC19, Amp^r	This study
pTF24	2.7-kb <i>Eco</i> RI fragment from pCSF cloned in pUC19, Amp^r	This study
pTF25	Interposon Ω cloned into the <i>Eco</i> RI site of the <i>fur</i> gene from pTF20, Amp^r and Spec^r	This study
pTF26	3.2-kb <i>Pst</i> I- <i>Bam</i> HI fragment from pTF25, containing <i>fur::\Omega</i> , cloned in pBC Cm^r and Spec^r	This study

Sigma) was added in the annealing mixture (5% final concentration). Data were analyzed with the software package GCG provided by Bisance (Dessen et al. 1990). The sequence of the *E. chrysanthemi fur* gene has been submitted to GenBank under the accession number AF016035.

Construction of a *fur* null mutant.

The interposon Ω coding for spectinomycin resistance was inserted into the *EcoRI* site of the *E. chrysanthemi fur* gene cloned in pTF20, generating plasmid pTF25. The disrupted *fur* gene was then cloned in plasmid pBC, which is unstable in *E. chrysanthemi*. The resulting plasmid, pTF26, was introduced into *E. chrysanthemi* L37 harboring the *ftc34::lacZ* fusion by electroporation. Transformants were purified once on LB agar plates containing Cm and Spec. Overnight cultures in LB with Cm and Spec were 20-fold diluted in Low Phosphate medium supplemented with glucose and Spec in order to promote plasmid destabilization with exchange recombination of the disrupted DNA insert into the *E. chrysanthemi* chromosome. After 24 h of growth, the culture was further diluted 20-fold in the same minimal medium. After four passages, the cells were plated on LB agar plates containing Spec. Individual colonies were then streaked on plates containing Spec + Cm and Spec only. Forty Spec^r Cm^s clones were isolated and streaked on MacConkey plates containing lactose and spectinomycin, with or without 20 μ M FeCl₃. Clones displaying a constitutive expression of the reporter fusion (i.e., red colonies in the presence of iron) were further analyzed.

Determination of β -galactosidase, β -glucuronidase, and Pel activities in bacterial culture.

An inoculum of an overnight LB culture was 60-fold diluted in Tris medium containing glycerol and appropriate antibiotics. The inoculated culture was divided in two subcultures, in which EDDHA (30 μ g/ml) or FeCl₃ (30 μ M) was added. Cultures were grown aerobically at 30°C. Samples were collected and immediately frozen. Enzymatic activities were assayed as reported previously (Sauvage and Expert 1994; Masclaux et al. 1996). β -Glucuronidase activity is expressed in nanomoles of paranitrophenol liberated per minute, per unit of optical density at 600 nm. Specific Pel activity is expressed as micromoles of products liberated per minute per milligram (dry weight) of bacteria. Glycerol was replaced by polygalacturonic acid (PGA) as a carbon source for pectinolysis-inducing conditions. Each experiment was performed three times.

Pathogenicity assay.

Pathogenicity was tested on potted African violets (*Saintpaulia ionantha*), cv. Blue Rhapsody, as reported by Expert and Toussaint (1985). Bacterial cells were plated on LB agar medium and incubated 24 h at 30°C. Cells were suspended in a NaCl solution (9 g per liter) to give an optical density at 600 nm of 0.3. One leaf per plant was inoculated with 100 μ l of the resulting suspension. Plant growth conditions are the same as those described by Sauvage and Expert (1994). Progression of the symptoms was scored daily for 16 days.

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LITERATURE CITED

- Achenbach, L. A., and Yang, W. 1997. The *fur* gene from *Klebsiella pneumoniae*: Characterization, genomic organization and phylogenetic analysis. *Gene* 185:201-207.
- Allen, L. N., and Hanson, R. S. 1985. Construction of broad-host-range cosmid cloning vectors: Identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. *J. Bacteriol.* 161:955-962.
- Bagg, A., and Neilands, J. B. 1987. Ferric uptake regulation protein act as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *E. coli*. *Biochemistry* 26:5471-5477.
- Barras, F., Van Gijsegem, F., and Chatterjee, A. K. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. *Annu. Rev. Phytopathol.* 32:201-234.
- Biegel Carson, S. D., Thomas, C. E., and Elkins, C. 1996. Cloning and sequencing of a *Haemophilus ducreyi fur* homolog. *Gene* 176:125-129.
- Calderwood, S. B., and Mekalanos, J. J. 1988. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operator fusion plasmid. *J. Bacteriol.* 170:1015-1017.
- Chen, L., James, L. P., and Helmann, J. D. 1993. Metalloregulation in *Bacillus subtilis*: Isolation and characterization of two genes differentially regulated by metal ions. *J. Bacteriol.* 175:5428-5437.
- Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. *Annu. Rev. Phytopathol.* 24:383-409.
- Coy, M., and Neilands, J. B. 1991. Structural dynamics and functional domains of the Fur protein. *Biochemistry* 30:8201-8210.
- Crosa, J. H. 1997. Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. *Microbiol. Mol. Biol. Rev.* 61:319-336.
- de Lorenzo, V., Giovannini, F., Herrero, M., and Neilands, J. B. 1988a. Metal ion regulation of gene expression: Fur repressor-operator interaction at the promoter region of the aerobactin system of pColV-K30. *J. Mol. Biol.* 203:875-884.
- de Lorenzo, V., Herrero, M., Giovannini, F., and Neilands, J. B. 1988b. Fur (ferric uptake regulation) protein and CAP (catabolite activator protein) modulate transcription of *fur* gene in *Escherichia coli*. *Eur. J. Biochem.* 173:537-546.
- Dessen, P., Fondrat, C., Valencien, C., and Mugnier, C. 1990. Bisance: A French service for access to biomolecular sequence databases. *CABIOS* 6:355-356.
- Enard, C., Dirole, A., and Expert, D. 1988. Systemic virulence of *Erwinia chrysanthemi* 3937 requires a functional iron assimilation system. *J. Bacteriol.* 170:2419-2426.
- Escobar, L., de Lorenzo, V., and Perez-Martin, J. 1997. Metalloregulation in vitro of the aerobactin promoter of *Escherichia coli* by the Fur (ferric uptake regulation) protein. *Mol. Microbiol.* 26:799-808.
- Escobar, L., Perez-Martin, J., and de Lorenzo, V. 1998. Coordinated repression in vitro of the divergent *fepA-fes* promoters of *Escherichia coli* by the iron uptake regulation Fur protein. *J. Bacteriol.* 180:2579-2582.
- Expert, D., Enard, C., and Masclaux, C. 1996. The role of iron in plant host-pathogen interactions. *Trends Microbiol.* 4:232-237.
- Expert, D., Sauvage, C., and Neilands, J. B. 1992. Negative transcriptional control of iron transport in *Erwinia chrysanthemi* involves an iron-responsive two factor system. *Mol. Microbiol.* 6:2009-2017.
- Expert, D., and Toussaint, A. 1985. Bacteriocin-resistant mutants of *Erwinia chrysanthemi*: Possible involvement of iron acquisition in phytopathogenicity. *J. Bacteriol.* 163:221-227.
- Fetherston, J. D., Bearden, S. W., and Perry, R. D. 1996. YbtA, an AraC-type regulator of the *Yersinia pestis* pesticin/yersiniabactin receptor. *Mol. Microbiol.* 22:315-325.

- Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* 76:1648-1652.
- Franza, T., Enard, C., Van Gijsegem, F., and Expert, D. 1991. Genetic analysis of the *Erwinia chrysanthemi* 3937 chrysobactin iron-transport system: Characterisation of a gene cluster involved in uptake and biosynthetic pathway. *Mol. Microbiol.* 5:1319-1329.
- Franza, T., and Expert, D. 1991. The virulence-associated chrysobactin iron uptake system of *Erwinia chrysanthemi* 3937 involves an operon encoding transport and biosynthetic functions. *J. Bacteriol.* 173:6874-6881.
- Ghassemian, M., and Straus, N. A. 1996. Fur regulates the expression of iron stress genes in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Microbiology* 142:1469-1476.
- Gill, P. R., and Warren, G. J. 1988. An iron-antagonized fungistatic that is not required for iron assimilation from a fluorescent rhizosphere pseudomonad. *J. Bacteriol.* 170:163-170.
- Hall, H. K., and Foster, J. W. 1996. The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. *J. Bacteriol.* 178:5683-5691.
- Halliwell, B., and Gutteridge, J. M. C. 1985. Oxygen is poisonous: An introduction to oxygen toxicity and free radicals. Pages 1-19 in: *Free Radicals in Biology and Medicine*. B. Halliwell and J. M. C. Gutteridge, eds. Clarendon, Oxford University Press, Oxford.
- Hantke, K. 1984. Cloning of the repressor protein gene of iron regulated systems in *Escherichia coli*. *Mol. Gen. Genet.* 197:337-341.
- Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., and Reverchon, S. 1996. Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annu. Rev. Microbiol.* 50:213-257.
- Hugouvieux-Cotte-Pattat, N., Dominguez, H., and Robert-Baudouy, J. 1992. Environmental conditions affect transcription of the pectinase genes of *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 174:7807-7818.
- Laurent, F., Kotoujansky, A., Labesse, G., and Bertheau, Y. 1993. Characterization and overexpression of the *pem* gene encoding pectin methyl-esterase of *Erwinia chrysanthemi* 3937. *Gene* 131:17-25.
- Le Cam, E., Frechon, D., Barry, M., Fourcade, A., and Delain, D. 1994. Observation of binding and polymerization of Fur repressor onto operator-containing DNA with electron and atomic force microscopes. *Proc. Natl. Acad. Sci. USA* 91:11816-11820.
- Litwin, C. M., and Calderwood, S. B. 1994. Analysis of the complexity of gene regulation by Fur in *Vibrio cholerae*. *J. Bacteriol.* 176:240-248.
- Lojkowska, E., Masclaux, C., Boccarda, M., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. 1995. Characterization of the *pelL* gene encoding a novel pectate lyase of *Erwinia chrysanthemi* 3937. *Mol. Microbiol.* 16:1183-1195.
- Mahé, B., Masclaux, C., Rauscher, L., Enard, C., and Expert, D. 1995. Differential expression of two siderophore-dependent-iron acquisition pathways in *Erwinia chrysanthemi* 3937: Characterization of a novel ferrisiderophore permease of the ABC transporter family. *Mol. Microbiol.* 18:33-43.
- Masclaux, C., and Expert, D. 1995. Signalling potential of iron in plant-microbe interactions: The pathogenic switch of iron transport in *Erwinia chrysanthemi*. *Plant J.* 7:121-128.
- Masclaux, C., Hugouvieux-Cotte-Pattat, N., and Expert, D. 1996. Iron is a triggering factor for differential expression of *Erwinia chrysanthemi* 3937 pectate lyases during pathogenesis of African violets. *Mol. Plant-Microbe Interact.* 9:198-205.
- Messiaen, J., and Van Cutsem, P. 1993. Defense gene transcription in carrot cells treated with oligolacturonides. *Plant Cell Physiol.* 34:1117-1123.
- Messiaen, J., and Van Cutsem, P. 1994. Pectic signal transduction in carrot cells: Membranes, cytosolic and nuclear responses induced by oligolacturonides. *Plant Cell Physiol.* 35:677-689.
- Miller, J. F. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Nasser, W., Reverchon, S., Condemine, G., and Robert-Baudouy, J. 1994. Specific interaction of *Erwinia chrysanthemi* KdgR repressor with different operators of genes involved in pectinolysis. *J. Mol. Biol.* 236:427-440.
- Nasser, W., Reverchon, S., and Robert-Baudouy, J. 1992. Purification and functional characterisation of the KdgR protein, a major repressor of pectinolytic genes of *Erwinia chrysanthemi*. *Mol. Microbiol.* 6:257-265.
- Nasser, W., Robert-Baudouy, J., and Reverchon, S. 1997. Antagonistic effect of CRP and KdgR in the transcription control of the *Erwinia chrysanthemi* pectinolysis genes. *Mol. Microbiol.* 26:1071-1082.
- Neema, C., Lauthère, J.-P., and Expert, D. 1993. Iron deficiency induced by chrysobactin in *Saintpaulia ionantha* leaves inoculated with *Erwinia chrysanthemi*. *Plant Physiol.* 102:967-973.
- Persmark, M., Expert, D., and Neilands, J. B. 1989. Isolation, characterization and synthesis of chrysobactin, a compound with a siderophore activity from *Erwinia chrysanthemi*. *J. Biol. Chem.* 264:3187-3193.
- Praillet, T., Nasser, W., Robert-Baudouy, J. and Reverchon, S. 1996. Purification and functional characterization of PecS: A regulator of virulence factor synthesis in *Erwinia chrysanthemi*. *Mol. Microbiol.* 20:391-402.
- Praillet, T., Reverchon, S., and Nasser, W. 1997. Mutual control of the PecS/PecM couple, two proteins regulating virulence-factor synthesis in *Erwinia chrysanthemi*. *Mol. Microbiol.* 24:803-814.
- Prince, R. W., Cox, D. C., and Vasil, M. L. 1993. Coordinate regulation of siderophore and exotoxin A production: Molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. *J. Bacteriol.* 175:2589-2598.
- Résibois, A., Colet, M., Faellen, M., Schoonejans, E., and Toussaint, A. 1984. Phi-EC2 a new generalized transducing phage of *Erwinia chrysanthemi*. *Virology* 137:102-112.
- Reverchon, S., Expert, D., Robert-Baudouy, J., and Nasser, W. 1997. The cyclic AMP receptor protein is the main activator of pectinolysis genes in *Erwinia chrysanthemi*. *J. Bacteriol.* 179:3500-3508.
- Reverchon, S., Nasser, W., and Robert-Baudouy, J. 1991. Characterization of *kdgR*, a gene of *Erwinia chrysanthemi* that regulates pectin degradation. *Mol. Microbiol.* 5:2203-2216.
- Roeder, D. L., and Collmer, A. 1985. Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. *J. Bacteriol.* 164:51-56.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sauvage, C., and Expert, D. 1994. Differential regulation by iron of *Erwinia chrysanthemi* pectate lyases: Pathogenicity of iron transport regulatory (*cbr*) mutants. *Mol. Plant-Microbe Interact.* 7:71-77.
- Sauvage, C., Franza, T., and Expert, D. 1996. Analysis of the *Erwinia chrysanthemi* ferrichrysobactin receptor gene: Resemblance to the *Escherichia coli fepA-fes* bidirectional promoter region and homology with hydroxamate receptors. *J. Bacteriol.* 178:1227-1231.
- Schäffer, S., Hantke, K., and Braun, V. 1985. Nucleotide sequence of the iron regulatory gene *fur*. *Mol. Gen. Genet.* 200:110-113.
- Shevchik, V. E., Condemine, G., Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. 1996. Characterization of pectin methyl-esterase B, an outer membrane lipoprotein of *Erwinia chrysanthemi* 3937. *Mol. Microbiol.* 16:745-763.
- Staggs, T. M., and Perry, R. D. 1992. Fur regulation in *Yersinia* species. *Mol. Microbiol.* 6:2507-2516.
- Stojiljkovic, I., Bäuml, A. J., and Hantke, K. 1994. Fur regulon in Gram-negative bacteria: Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. *J. Mol. Biol.* 236:531-545.
- Stojiljkovic, I., and Hantke, K. 1995. Functional domains of the *Escherichia coli* ferric uptake regulator protein Fur. *Mol. Gen. Genet.* 247:199-205.
- Surgey, N., Robert-Baudouy, J., and Condemine, G. 1996. The *Erwinia chrysanthemi pecT* gene regulates pectinase gene expression. *J. Bacteriol.* 178:1593-1599.
- Thomas, C. E., and Sparling, P. F. 1996. Isolation of a *fur* mutant of *Neisseria gonorrhoeae*. *J. Bacteriol.* 178:4224-4232.
- Tomalsky, M. E., Salinas, P., Actis, L. A., and Crosa, J. H. 1994. Characterization of the *Vibrio anguillarum fur* gene: Role in regulation of expression of the FatA outer membrane protein and catechols. *J. Bacteriol.* 176:213-220.
- Wang, R. F., and Kushner, S. R. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* 100:195-199.
- Watnick, P. I., Eto, T., Takahashi, H., and Calderwood, S. B. 1997. Purification of *Vibrio cholerae* Fur and estimation of its intracellular abundance by antibody sandwich enzyme-linked immunoabsorbent assay. *J. Bacteriol.* 179:243-247.