Comparative Study of Regulatory Mechanisms for Pectinase Production by *Erwinia carotovora* subsp. *carotovora* and *Erwinia chrysanthemi*

Hiroyuki Matsumoto,¹ Pongphen Jitareerat,¹ Yasuhiro Baba,² and Shinji Tsuyumu²

¹United Graduate School of Agricultural Science, Gifu University (Shizuoka University), 836 Ohya, Shizuoka 422-8529, Japan; ²Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan

Submitted 3 September 2002. Accepted 4 November 2002.

The production of pectinase, the major virulence determinant of soft-rot *Erwinia* species, is controlled by many regulatory factors. We focused on the major regulatory proteins, KdgR, CRP, Pir, and PecS, characterized mainly in *E. chrysanthemi*, and tested for their presence and function in the control of pectate lyase (Pel) and polygalacturonase (Peh) production in *E. carotovora* subsp. *carotovora*. Homologues of *kdgR* and *crp* but not of *pir* and *pecS* were detected by Southern blot analyses in *E. carotovora* subsp. *carotovora*. In fact, KdgR and CRP homologues of *E. carotovora* subsp. *carotovora* had high amino acid identities to those of *E. chrysanthemi*, including a complete match of the hypothetical helix-turn-helix DNA-binding motif. However, in Western blot analyses using anti-Pir (*E. chrysanthemi*) antibodies, a cross-reacting protein was present in both *Erwinia* species, although Pel production in *E. carotovora* subsp. *carotovora* was not further stimulated by adding plant extract into the medium containing PGA (polygalacturonic acid) in which hyperinduction by Pir has been reported in *E. chrysanthemi* EC16. When plasmids that contained each of these regulatory genes from *E. chrysanthemi* were introduced into *E. carotovora* subsp. *carotovora*, Pel production was controlled as predicted from their roles in *E. chrysanthemi*, except for PecS. PecS exerted a positive control in *E. carotovora* subsp. *carotovora*, in contrast to a negative control in *E. chrysanthemi*. DNA-binding assays demonstrated that KdgR, CRP, Pir, and PecS of *E. chrysanthemi* and KdgR and CRP homologues of *E. carotovora* subsp. *carotovora* could bind to the promoter regions of *pel-1*, *pel-3*, and *peh* of *E. carotovora* subsp. *carotovora*. Taken together, KdgR and CRP homologues of *E. carotovora* subsp. *carotovora* may regulate Pel and Peh production as in *E. chrysanthemi*. However, the presence of Pir and PecS homologues in *E. carotovora* subsp. *carotovora* was not identified in this study, though these proteins of *E. chrysanthemi* were functional on the promoter regions of the pectinase genes of *E. carotovora* subsp. *carotovora*.

Erwinia carotovora subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *E. chrysanthemi* are widely distributed in the world and cause serious damage on a wide variety of plants (Perombelon and Kelman 1980). Cell wall-degrading enzymes (Barras et al. 1994; Kotoujansky 1987), such as pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease

(Prt), are considered to be the main factors responsible for pathogenicity. These enzymes are produced and secreted into the external milieu in large quantities. Of these enzymes, Collmer and Keen (1986) noted that Pel and Peh play a crucial role in pathogenicity.

The production of these extracellular enzymes is affected by various environmental factors such as the presence of pectin and plant extracts, cell density, temperature, nitrogen starvation, oxygen or iron concentration, and osmolarity (Hugouvieux-Cotte-Pattat et al. 1992, 1996; Robert-Baudouy et al. 2001). Previous studies identified a number of regulatory genes involved in the production of these enzymes in *E. chrysanthemi* and *E. carotovora* subsp. *carotovora*, such as *pir* (*E. chrysanthemi*), *pecS-pecM* (*E. chrysanthemi*), *fur* (*E. chrysanthemi*), *hns* (*E. chrysanthemi*), *crp* (*E. chrysanthemi*), *aepA* (*E. carotovora* subsp. *carotovora*), *rexZ* (*E. carotovora* subsp. *carotovora*), *hor* (*E. carotovora* subsp. *carotovora*), *pehR-pehS* (*E. carotovora* subsp. *carotovora*), *expA-expS* (*E. carotovora* subsp. *carotovora*), *expM* (*E. carotovora* subsp. *carotovora*), *rpoS* (*E. carotovora* subsp. *carotovora*), *rsmA-rsmB-rsmC* (*E. carotovora* subsp. *carotovora*), *pecT* (*E. chrysanthemi*)/*hexA* (*E. carotovora* subsp. *carotovora*), *kdgR* (*E. chrysanthemi* and *E. carotovora* subsp. *carotovora*), and *expl-expR* (*E. chrysanthemi* and *E. carotovora* subsp. *carotovora*) (Andersson et al. 1999a, 1999b, 2000; Cui et al. 1995, 1999; Eriksson et al. 1998; Flego et al. 2000; Franza et al. 1999; Harris et al. 1998; Liu et al. 1999; Murata et al. 1991, 1994; Nasser et al. 1998, 2001; Nomura et al. 1998; Pirhonen et al. 1993; Reverchon et al. 1991, 1994, 1997; Surgey et al. 1996; Thomson et al. 1997, 1999).

In *E. chrysanthemi*, KdgR is the major negative regulatory protein responsible for the control of the expression of genes involved in the degradation and catabolism of pectic compounds (Nasser et al. 1994; Reverchon et al. 1991). In vitro experiments showed that specific binding of the KdgR repressor to the operators of these genes is inhibited by a pectic catabolic product, 2-keto-3-deoxygluconate (KDG) (Nasser et al. 1991). Pel production of *E. chrysanthemi* is further stimulated by the presence of one or more additional plant factors only in synergy with pectic compounds (Hugouvieux-Cotte-Pattat et al. 1992). Such hyperinduction of Pel is controlled by Pir activator in *E. chrysanthemi* EC16 (Nomura et al. 1998), although the chemical nature of the additional inducing factors in plant extracts is not yet known. From preliminary characterization of one inducing factor from carrot root, the factor could be an oligosaccharide (Bourson et al. 1993).

Besides these plant-responsive regulatory proteins in the bacteria, several global regulators have been reported. CRP

Corresponding author: S. Tsuyumu; Telephone and Fax: +81-54-238-4823; E-mail: tsuyumu@agr.shizuoka.ac.jp.

(cyclic AMP receptor protein) is required for the full expression of pectinolysis genes (Reverchon et al. 1997) mediated by the cAMP-CRP complex (Kolb et al. 1993). Other global regulators are PecS and PecT, which negatively regulate the expression of *pel* genes (Castillo and Reverchon 1997; Praillet et al. 1996; Reverchon et al. 1994; Surgety et al. 1996), although the signal or signals for those regulators are unknown.

Most of these factors are involved in other aspects of virulence, such as protein secretion, motility, EPS synthesis, iron capture, and the production of pigment, cellulase, and protease (Robert-Baudouy et al. 2001). In addition, recent results indicate several interactions between these regulatory systems (Andersson et al. 1999b; Cui et al. 1995, 1999, 2001; Hyytiäinen et al. 2001; Liu et al. 1999; Mukherjee et al. 1998, 2000; Reverchon et al. 1998). Robert-Baudouy and associates (2001) have indicated that regulation of pectinase synthesis in soft-rot *Erwinia* species seems to be a complex network involving multiple regulatory systems. However, only a few regulators have been found in both *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* to date; most others have been described in only one or the other. Even when common regulators have been reported, such as HexA (*E. carotovora* subsp. *carotovora*) or PecT (*E. chrysanthemi*) and ExpR (*E. carotovora* subsp. *carotovora* and *E. chrysanthemi*), their regulatory effects have been distinguishable in both species (Andersson et al. 2000; Harris et al. 1998). Thus, in this study, we tested for the presence of homologues of the representative regulatory genes *kdgR*, *crp*, *pir*, and *pecS* found in *E. chrysanthemi* and for their activities in the control of Pel and Peh production in *E. carotovora* subsp. *carotovora*.

RESULTS

Pel production of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains in various media.

Pel production by *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains was confirmed to be induced in yeast extract-peptone (YP) medium containing polygalacturonic acid (PGA) but was repressed in YP medium containing glucose (Table 1). These results suggest that KdgR and CRP homologues of *E. carotovora* subsp. *carotovora* may function in a similar manner to *E. chrysanthemi*. The addition of potato extract or Chinese cabbage extract (data not shown) with PGA into YP or M63-glycerol minimal medium, however, did not hyperinduce Pel production in either *E. carotovora* subsp. *carotovora* strain or in *E. chrysanthemi* 3937, while *E. chrysanthemi* EC16 exhibited a typical hyperinduction in minimal medium. In previous work, *E. chrysanthemi* 3937 was shown

to hyperinduce Pel production in minimal medium containing PGA and plant extract, although the production was significantly affected by various environmental parameters, such as carbon source, temperature, and nitrogen or oxygen availability (Hugouvieux-Cotte-Pattat et al. 1992). Accordingly, minor differences in the incubation of bacterial cells, such as temperature, shaking speed, and the plant extracts used, might have concealed the hyperinduction of Pel in *E. chrysanthemi* 3937 in this study.

E. chrysanthemi *kdgR* and *crp* homologues in *E. carotovora* subsp. *carotovora*.

Southern blot analyses for *Eco*RI digests (Fig. 1) and for *Bam*HI digests (data not shown) of the total DNAs from *E. carotovora* subsp. *carotovora* EC1, *E. chrysanthemi* 3937, and *E. chrysanthemi* EC16 strains were performed using polymerase chain reaction (PCR) products of *kdgR*, *crp*, and *pecS* of *E. chrysanthemi* 3937 and *pir* of *E. chrysanthemi* EC16 as probes. Signal bands for *kdgR* and *crp* homologues were detected not only in *E. chrysanthemi* strains but also in *E. carotovora* subsp. *carotovora* EC1. Thus, *kdgR* and *crp* seem to be present both in *E. chrysanthemi* and in *E. carotovora* subsp. *carotovora*. In fact, *kdgR* has already been isolated and reported in other strains of *E. carotovora* subsp. *carotovora* (Liu et al. 1999; Thomson et al. 1999). However, no signal was detected for *pir* and *pecS* in *E. carotovora* subsp. *carotovora* EC1, even under the low-stringency hybridizing conditions of Southern blot analyses.

From a genomic library of *E. carotovora* subsp. *carotovora* EC1, clones from the regions homologous to *kdgR* and *crp* of *E. chrysanthemi* 3937 were obtained. Open reading frames (ORF) encoding 263 and 210 amino acid residues were found within the subclones of pKdgR(2.5E) and pCRP(3.5B), respectively. The KdgR homologue of *E. carotovora* subsp. *carotovora* EC1 had more than 98.1% and 92.8% amino acid identities to those of other strains of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* 3937, respectively. The CRP homologue of *E. carotovora* subsp. *carotovora* EC1 had 97.6% amino acid identity to that of *E. chrysanthemi* 3937. In either case, amino acid sequences corresponding to a potential helix-turn-helix (HTH) DNA-binding motif (based on the data in SWISS-PROT) completely matched those of *E. chrysanthemi* 3937 (Fig. 2).

Immunologically related Pir in *E. carotovora* subsp. *carotovora* strains.

In Western blot analyses of total cell lysates of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains using

Table 1. Pel activity^a of Ecc and Ech^b strains in various media

Medium	Strain							
	Ecc EC1		Ecc 71		Ech EC16		Ech 3937	
	Sup.	Total	Sup.	Total	Sup.	Total	Sup.	Total
YP	8.0 ± 1.4	11.1 ± 1.2	37.6 ± 1.6	39.3 ± 1.5	2.9 ± 1.2	7.6 ± 2.7	14.7 ± 7.	23.3 ± 5.9
YP+Glucose	0.6 ± 0.1	1.3 ± 0.1	1.9 ± 0.1	3.0 ± 0.4	3.2 ± 0.8	4.5 ± 1.6	5.8 ± 2.6	8.2 ± 2.7
YP+PGA	86.6 ± 6.8	93.6 ± 6.9	97.0 ± 4.1	102.7 ± 2.0	31.2 ± 2.6	36.3 ± 3.9	66.1 ± 10.2	80.7 ± 11.0
YP+PGA+Po ^c	92.3 ± 7.4	97.0 ± 13.0	126.7 ± 20.1	136.3 ± 18.1	35.5 ± 14.0	45.7 ± 12.6	67.2 ± 11.9	76.0 ± 13.2
M63 ^d	1.2 ± 0.8	2.6 ± 0.1	2.1 ± 0.2	14.9 ± 2.9	4.5 ± 0.5	NT ^e	15.9 ± 4.4	NT
M63+PGA	124.1 ± 32.7	138.5 ± 39.2	82.7 ± 5.3	93.4 ± 3.0	58.3 ± 44.7	NT	377.8 ± 64.5	NT
M63+PGA+Po	102.4 ± 4.4	106.7 ± 3.8	63.2 ± 7.7	75.6 ± 11.8	528.1 ± 196.5	NT	379.4 ± 79.0	NT

^a Pel activity was expressed as the U/OD₆₆₀ of the bacterial culture. Bacterial strains were grown in indicated medium at 27°C until early stationary phase and then fractionated. The data was expressed as the mean of at least three independent experiments.

^b Ecc = *Erwinia carotovora* subsp. *carotovora*; Ech = *E. chrysanthemi*.

^c Potato extract.

^d M63-glycerol minimal medium.

^e Not tested.

monoclonal antibodies raised against Pir of *E. chrysanthemi* EC16, a signal similar in molecular mass (about 30 kDa) to that estimated (30.1 kDa) for EC16 Pir (Nomura et al. 1998) was detected in all strains tested, although the signals were weaker than those of *E. chrysanthemi* EC16 (Fig. 3). Thus, Pir may be present not only in *E. chrysanthemi* strains but also in *E. carotovora* subsp. *carotovora* strains. Furthermore, proteins immunologically related to Pir were found in other *Erwinia* spp., including *E. carotovora* subsp. *atroseptica*, *E. herbicola* pv. *milletiae*, and *E. rhapontici*, but not in other bacteria, such as *Pseudomonas syringae* and *Agrobacterium tumefaciens* (data not shown). Though Pir has been shown to bind specifically to the promoter regions of *pel* genes (*pelA-E*) and pectin-catabolizing genes (*ogl*, *kdgK*) in *E. chrysanthemi* EC16 (Nomura et al. 1998), the protein may be required for a function other than that of a regulator, leading to the hyperinduction of Pel.

Dosage effects of *E. chrysanthemi* regulatory genes on Pel production in *E. carotovora* subsp. *carotovora*.

Introduction of the plasmid carrying a regulatory gene such as *kdgR*, *crp*, *pecT*, *hexA*, *aepA*, *gacA*, *gacS*, *expR*, *rsmA*, *aepH* (*rsmB*), or *rsmC* into soft-rot *Erwinia* species was shown to affect Pel production (Andersson et al. 2000; Castillo and Reverchon 1997; Cui et al. 1999, 2001; Harris et al. 1998; Liu et al. 1993; Ma et al. 2001; Mukherjee et al. 2000; Murata et al. 1994; Reverchon et al. 1997; Thomson et al. 1999), although the degree of effect to Pel production is somewhat dependent on the copy number of vector used. Thus, we tested such gene dosage effects in *E. carotovora* subsp. *carotovora* EC1-N (Table 2) after introduction of the plasmids carrying the individual gene *kdgR*, *crp*, or *pecS* of *E. chrysanthemi* 3937 or *pir* of *E. chrysanthemi* EC16. In YP medium, the additional *kdgR* copies decreased Pel production, while the presence of *crp* or *pir* increased Pel production, compared with that of the vector control (Fig. 4). Accordingly, Pel production in *E. carotovora* subsp. *carotovora* may be regulated by the homologues of these molecules in a manner similar to that in *E. chrysanthemi* (Nomura et al. 1998; Reverchon et al. 1991, 1997). Unexpectedly, *pecS* increased Pel production in *E. carotovora* subsp. *carotovora* EC1-N, although it has been reported as a negative regulator in *E. chrysanthemi* 3937 (Reverchon et al. 1994). When bacteria were grown in the medium containing PGA, no clear positive effects on Pel production by CRP, Pir, or PecS were observed. Surprisingly, the presence of *kdgR* increased Pel production in *E. carotovora* subsp. *carotovora* EC1-N compared with that in the vector control. High production of

KdgR might contribute to the activation of Pel production in the medium containing PGA by an unknown mechanism.

Binding of *E. chrysanthemi* KdgR, CRP, Pir, and PecS to the promoter regions

of *E. carotovora* subsp. *carotovora* *pel-1*, *pel-3*, and *peh*.

Using purified KdgR, CRP, and PecS of *E. chrysanthemi* 3937 and Pir of *E. chrysanthemi* EC16, gel-mobility shift assays were conducted to test the abilities of the proteins to bind to the promoter regions of *pel-1*, *pel-3*, and *peh* of *E. carotovora* subsp. *carotovora*. The promoter region of *E. chrysanthemi* *pelE*, which has been reported to bind to all of these proteins, was used as the positive control (Nomura et al. 1998; Rouanet et al. 1999). Bands of all the promoter fragments tested were shifted by these *E. chrysanthemi* regulatory proteins (Fig. 5), indicating that the promoters of *E. carotovora* subsp. *carotovora* have recognition sites for these *E. chrysanthemi* regulatory proteins. The binding efficiencies of KdgR, CRP, and Pir of *E. chrysanthemi* to the promoter regions of *E. carotovora* subsp. *carotovora* seemed to be lower than those to *E. chrysanthemi* *pelE*, except in the case of PecS. In binding assays using purified KdgR and CRP of *E. carotovora* subsp. *carotovora* EC1, however, the binding efficiency did not significantly differ compared with those of *E. chrysanthemi* (data not shown). Thus, the low binding efficiencies of KdgR and CRP (*E. chrysanthemi* and *E. carotovora* subsp. *carotovora* equally) to the promoter regions of *E. carotovora* subsp. *carotovora* seem to result from a difference in the promoter sequences in which these regulatory molecules bind. In the case of Pir, the binding efficiencies to the promoter regions of *E. carotovora* subsp. *carotovora* were indistinguishable from that of *E. chrysanthemi* *pelE* in binding assays under low-ionic strength conditions (Fig. 5). However, no band of the *E. chrysanthemi*-Pir complex with the *E. carotovora* subsp. *carotovora* promoter regions was observed at high-ionic strengths, while the band with *E. chrysanthemi* *pelE* was clearly observed under similar conditions (data not shown). Thus, the binding efficiencies of *E. chrysanthemi* Pir to the *E. carotovora* subsp. *carotovora* promoter regions seemed to be lower than that to *E. chrysanthemi* *pelE*. On the other hand, PecS formed an intense band with both the *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* promoter regions, even in high-ionic strength conditions, and a broad region was protected in DNaseI footprinting assays, depending on the concentration of protein (data not shown). These results may be due to the fact that PecS has been reported to bind preferably at the AT-rich region in *E. chrysanthemi* 3937 (Praillet et al. 1996).

Fig. 1. Southern blot analyses of *EcoRI*-digested DNAs from *Erwinia carotovora* subsp. *carotovora* EC1, *E. chrysanthemi* EC16, and *E. chrysanthemi* 3937 using *E. chrysanthemi* regulatory genes as the probes. The hybridization was done at 60°C. Lane M, DIG-labeled λ -HindIII marker; lanes 1 to 3, total *EcoRI*-digested DNAs of *E. carotovora* subsp. *carotovora* EC1, *E. chrysanthemi* EC16, and *E. chrysanthemi* 3937.

Induction of *E. carotovora* subsp. *carotovora* *pel-1*, *pel-3*, and *peh* and *E. chrysanthemi* *pelE*

in *E. carotovora* subsp. *carotovora* and in *E. chrysanthemi*.

Tn7 integrants that have a single insertion of each of the promoter-*lux* fusions into their chromosomes (Shen et al. 1992) were constructed for the expression studies of *E. carotovora* subsp. *carotovora* *pel-1*, *pel-3*, and *peh* and *E. chrysanthemi* *pelE*, both in *E. carotovora* subsp. *carotovora* EC1-N and in *E. chrysanthemi* EC16. In YP medium containing PGA, expression of each of these genes was induced in both species (Fig. 6). These data suggest that KdgR of both species may be functional on the promoter regions of these genes of both species. It is noteworthy that, in both species, *E. chrysanthemi* *pelE* expression seemed to be tightly repressed in YP medium and highly induced in the medium containing PGA compared with expression of *E. carotovora* subsp. *carotovora* *pel-1*, *pel-3*, and *peh*. On the other hand, *E. carotovora* subsp. *carotovora* *peh* expression was highly inducible in *E. carotovora* subsp. *carotovora* EC1-N grown in YP medium containing PGA but not in *E. chrysanthemi* EC16. Therefore, some other positive factor or factors for the full induction of *E. carotovora* subsp. *carotovora* *peh* expression may be present in *E. carotovora* subsp. *carotovora* but not in *E. chrysanthemi*.

Expression of *E. carotovora* subsp. *carotovora* *pel-1*, *pel-3*, and *peh* in a *pir*-deficient mutant of *E. chrysanthemi* EC16.

The expression of *E. carotovora* subsp. *carotovora* *pel-1* and *peh* in a *pir*-deficient mutant (K2367) of *E. chrysanthemi*

EC16 was lower than in the wild-type strain grown in M63-glycerol minimal medium containing PGA and potato extract in which *Pel* activity is hyperinduced (Fig. 7). Thus, *Pir* of *E. chrysanthemi* EC16 may act as the positive factor for the expression of *E. carotovora* subsp. *carotovora* *pel-1* and *peh* under these conditions. The expression level of *E. carotovora* subsp. *carotovora* *pel-3* in *E. chrysanthemi* EC16 was not high, even under the hyperinductive conditions relative to the other cases. We can not conclude, then, that *Pir* of *E. chrysanthemi* EC16 is functional on the promoter region of *E. carotovora* subsp. *carotovora* *pel-3* in this experiment.

DISCUSSION

KdgR and CRP homologues of *E. carotovora* subsp. *carotovora* EC1 had high amino acid identities to those of *E. chrysanthemi* 3937 (92.8 and 97.6%, respectively), including a complete match of the hypothetical HTH motif. As expected from this information, KdgR and CRP homologues of *E. carotovora* subsp. *carotovora* EC1 were indistinguishable from those of *E. chrysanthemi* 3937 in DNA-binding assays (data not shown). Therefore, the lower binding efficiencies of KdgR and CRP (*E. chrysanthemi* and *E. carotovora* subsp. *carotovora* equally) to the promoter regions of *E. carotovora* subsp. *carotovora* *pel* genes and *peh*, compared with those of *E. chrysanthemi* *pelE*, seemed to result from the differences in their binding regions on these genes. Among the *pel* genes in *E.*

Ecc EC1	1'	MASADLDKQPDSSVSVLKVFGLIQLALGEEREIGITELSQRVMSKSTVYRFLQTMKSLGY
		** *****
Ech 3937	1"	MAIADLDKQPDSSVSVLKVFGLIQLALGEEREIGITELSQRVMSKSTVYRFLQTMKSLGY
	61'	VAQEGESEKYSLLTKLFELGAKALQNVDLIRSADIQMRELSALTRETIHLGALDESDGIVY

	61"	VAQEGESEKYSLLTKLFELGAKALQNVDLIRSADIQMRELSALTRETIHLGALDESDIVY
	121'	IHKIDSMYNLRMYSRIGRRNPLHSTAIGKVLLAWDRSEVEEVLSTVEFTRSTPHTLCSA
		***** **.* **.* **.*
	121"	IHKIDSMYNLRMYSRIGRRNPLHSTAIGKVLLAWDRSEVEEVLSTVEFTRSTPHTIGST
	181'	EALLNQLDVRQGYGEDNEEQEGLRCIAVPVDFRFGVVIAGLSISFPTIRFSEENKHE
		. **.* *****
	181"	EELLPLQLDLVRQGYGEDNEEQEGLRCIAVPVDFRFGVVIAGLSISFPTIRFSEDNKHE
	241'	YVATLHTAARNISEQMGYHDYPF
		*** *****
	241"	YVAMLHTAARNISDQMGYHDYPF
Ecc EC1	1'	MVLGKPQTDPTLEWFLSHCHIHKYPKSTLIHQGEKAETLYYIVKGSVAVLKDEEGKEM

Ech 3937	1"	MVLGKPQTDPTLEWFLSHCHIHKYPKSTLIHQGEKAETLYYIVKGSVAVLKDEEGKEM
	61'	ILSYLNQGDFIGELGLFEQGERSAWVRAKTACEVAEISYKKFRQLIQVNPDIIMRLSAQ
		***** *
	61"	ILSYLNQGDFIGELGLFEQGERSAWVRAKTACEVAEISYKKFRQLIQVNPDIIMRLSSQ
	121'	MASRLQVTSEKVGNLAFLDVTGRIAQTLLNLAKQPDAMTHPDGMQIKITRQEIQIVGCS
		** *****
	121"	MARRLQVTSQKVGNLGFLDVTGRIAQTLLNLAKQPDAMTHPDGMQIKITRQEIQIVGCS
	181'	RETVGRILKMLEQNLISAHGKTIVVYGTR

	181"	RETVGRILKMLEQNLISAHGKTIVVYGTR

KdgR

CRP

Fig. 2. Alignments of deduced amino acid sequences of KdgR and CRP homologues of *Erwinia carotovora* subsp. *carotovora* EC1 with those of *E. chrysanthemi* 3937. Potential helix-turn-helix motifs are underlined. Identical amino acids and similar amino acids are shown as asterisks and dots, respectively.

chrysanthemi 3937, expression of *pelD*, a homologue of *pelE* in *E. chrysanthemi* EC16, is most severely affected by changes in culture conditions, including induction by pectic compounds (Hugouvieux-Cotte-Pattat et al. 1992). In this study, the expression of *E. chrysanthemi pelE* (EC16) was also highly induced relative to that of *E. carotovora* subsp. *carotovora pel* genes and *peh*, both in *E. chrysanthemi* and in *E. carotovora* subsp. *carotovora*. Although the binding studies were carried out in vitro while the expression studies were done in vivo, the different binding efficiencies of KdgR and CRP (and Pir) to the promoter regions of *E. carotovora* subsp. *carotovora* (*pel-1*, *pel-3*, and *peh*) and *E. chrysanthemi* (*pelE*) may reflect different controls for the level of expression of each gene, such as the induction efficiency by pectic compounds, rather than a difference in the regulatory mechanisms of pectinase genes in both species.

Surprisingly, in the medium containing PGA, the increase in the dosage of gene *kdgR* of *E. chrysanthemi* 3937 elevated Pel production in *E. carotovora* subsp. *carotovora* EC1-N compared with that in the vector control (Fig. 4). Because KdgR of *E. chrysanthemi* 3937 was shown to be released from the operator by the interaction with KDG in vitro (Nasser et al. 1991), the activation effect by its gene dosage may result from one or more indirect mechanisms, such as the interaction of released KdgR with one or more other negative regulatory molecules.

On the other hand, the gene dosage effect of *crp* was not clearly observed in the medium containing PGA (Fig. 4). From the analyses of simultaneous binding of KdgR and CRP to the promoter regions of several pectinolysis genes in *E. chrysanthemi*, Nasser and associates (1997) showed that binding of both proteins could be either competitive or independent. Even in the case of competition, however, KdgR is preponderant because the preferential and exclusive binding of KdgR leads to the prevention of RNA polymerase binding. Thus, release of KdgR repressors from the operators may be critical for the activation of Pel production. In a noninducing medium, the increase in the dosage of gene *crp* seems to alleviate the prevention of RNA polymerase binding mediated by KdgR (Fig. 4). Peh production in *E. carotovora* subsp. *carotovora* strains seemed not to be subjected to catabolite repression in YP or M63-glycerol minimal medium containing glucose, although it was induced in medium containing PGA (data not shown). The weak binding in vitro of CRP to the promoter region of *E. carotovora* subsp. *carotovora* *peh* may not reflect the situation in vivo.

Although a protein immunologically related to Pir in the genus *Erwinia* was detected by Western blot analyses using

monoclonal antibodies raised against Pir of *E. chrysanthemi* EC16, amino acid identity of Pir between *E. chrysanthemi* EC16 and *E. chrysanthemi* 3937 is 91.5% (85.5% at DNA level), even in same species. In the case of PecS, its homologue in *Erwinia carotovora* species was not detected even by Western blot analysis using antibodies raised against PecS of *E. chrysanthemi* 3937 (Praillet et al. 1996). Accordingly, overall structures of the Pir and PecS homologues in *E. carotovora* subsp. *carotovora* may not be similar to those of *E. chrysanthemi*, even if the homologues exist between the species. However, both proteins of *E. chrysanthemi*, in fact, elevated Pel production in *E. carotovora* subsp. *carotovora* and bound to the promoter regions of *E. carotovora* subsp. *carotovora pel* genes and *peh*. Thus, the binding sites for these regulators should be present.

In *E. carotovora* subsp. *carotovora* strains, the use of plant extracts did not hyperinduce Pel activity in YP or M63-glycerol minimal medium containing PGA in this study. In addition, extra copies of *pir* from *E. chrysanthemi* EC16 could not clearly elevate Pel production in *E. carotovora* subsp. *carotovora* EC1-N in the presence of PGA relative to that of the vector control, nor was induction seen after the addition of plant extract to the medium (data not shown). For some reason, the use of plant extract in the presence of PGA seemed to be ineffective for hyperinducing Pel in *E. carotovora* subsp. *carotovora*. One or more plant extract molecules might be unable to enter *E. carotovora* subsp. *carotovora* cells in view of the fact that the expression of *E. carotovora* subsp. *carotovora pel-1* and *peh* in *E. chrysanthemi* EC16 was under the control of *pir* and was hyperinduced by the addition of plant extract into the medium containing PGA. As to Pir function, Pir was reported to bind to a DNA sequence that overlaps the KdgR-binding site, competing with KdgR in *E. chrysanthemi* EC16 (Nomura et al. 1998). Accordingly, Pir may behave as an antirepressor against KdgR to control *pelE* transcription in *E. chrysanthemi* EC16. Also, PecS, which was initially isolated as a repressor from *E. chrysanthemi* 3937, has recently been shown to act as an activator of the transcription of polygalacturonase genes *pehV*, *pehW*, and *pehX* (Nasser et al. 1999). In a review by Robert-Baudouy and associates (2001), the mechanism of antirepression exerted by Pir or PecS was suggested as an efficient way to increase the transcription of a limited set of genes controlled by the main repressor KdgR. These observations are correlated with the finding that gene dosage effects of *pir* or *pecS* of *E. chrysanthemi* were not clearly observed in *E. carotovora* subsp. *carotovora* in the medium containing PGA, as they were for *crp* (Fig. 4). Thus, *E. chrysanthemi* Pir and PecS

Fig. 3. Western blot analyses of total cell lysates from *Erwinia chrysanthemi* and from *E. carotovora* subsp. *carotovora* strains using monoclonal antibodies raised against Pir of *E. chrysanthemi* EC16. Bacterial cells were grown in M63-glycerol minimal medium containing 0.2% polygalacturonic acid and 1% potato extract at 27°C until early stationary phase. Lane M, prestained molecular weight marker; lane P, purified Pir of *E. chrysanthemi* EC16; lanes 1 to 4, total cell lysates of *E. chrysanthemi* strains of EC16, 3937, EC183, and D7; lanes 5 to 8, total cell lysates of *E. carotovora* subsp. *carotovora* strains of EC1, 71, T29, and DB15.

homologues in *E. carotovora* subsp. *carotovora* seem to function merely as antirepressors against KdgR for the control of Pel and, possibly, of Peh production.

In this study, we found that KdgR and CRP homologues of *E. carotovora* subsp. *carotovora* may regulate Pel and Peh pro-

duction as they do in *E. chrysanthemi*. However, the presence of Pir and PecS homologues in *E. carotovora* subsp. *carotovora* was not concluded here, although these proteins of *E. chrysanthemi* were functional on the promoter regions of pectinase genes of *E. carotovora* subsp. *carotovora*.

Table 2. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
EC1	Wild type	Laboratory collection
EC1-N	Spontaneous Nal ^r mutant of EC1, Nal ^r	This study
71	Wild type	A. K. Chatterjee, University of Missouri
T29	Wild type	A. K. Chatterjee
DB15	Wild type	A. K. Chatterjee
<i>Erwinia chrysanthemi</i>		
EC16	Wild type	A. K. Chatterjee
K2367	<i>pir</i> ⁻ derivative of EC16, Km ^r	Nomura et al. 1998
3937	Wild type	Kotoujansky et al. 1982
EC183	Wild type	A. K. Chatterjee
D7	Wild type	A. K. Chatterjee
<i>Escherichia coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74 deoR recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL nupG</i>	Invitrogen, Carlsbad, CA, U.S.A.
HB101	F ⁻ <i>hsdS</i> (<i>r</i> _B ⁻ <i>m</i> _B ⁻) <i>thi pro leu lacY ara xyl supE recA13</i>	Sambrook et al. 1989
BL21(DE3)	F ⁻ <i>dcm ompT hsdS gal</i> λ (DE3)	Novagen, Madison, WI, U.S.A.
Plasmid		
pLAFR3	Broad host range vector, Tet ^r	Staskawicz et al. 1987
pUC118	Cloning vector, Amp ^r	Takara, Tokyo, Japan
pHSG298	Cloning vector, Km ^r	Takara
pCR-Script SK(+)	Cloning vector, Amp ^r	Stratagene, La Jolla, CA, U.S.A.
pBluescript SK(-)	Cloning vector, Amp ^r	Stratagene
pET-21a(+)	T7 overexpression vector, Amp ^r	Novagen
pHSK728	Tn7- <i>lux</i> transcriptional fusion vector, Amp ^r , Sp ^r /Stm ^r	Shen et al. 1992
pMON7181	Helper plasmid carrying genes for Tn7 transposition, Gm ^r	Barry 1988
pMON7184	Helper plasmid carrying genes for Tn7 transposition, Tet ^r	Barry 1988
pRK2013	Helper plasmid carrying genes for conjugation, Km ^r	Figurski and Helinski 1979
pKdgR(2.5E)	2.5-kb <i>EcoRI</i> fragment containing <i>kdgR</i> of EC1 cloned into the same site of pUC118	This study
pCRP(3.5B)	3.5-kb <i>BamHI</i> fragment containing <i>crp</i> of EC1 cloned into the same site of pUC118	This study
pKdgR-3937	1.3-kb PCR product containing <i>kdgR</i> of 3937, processed with <i>BamHI</i> and <i>HindIII</i> , cloned into the same sites of pLAFR3	This study
pCRP-3937	1.2-kb PCR product containing <i>crp</i> of 3937, processed with <i>EcoRI</i> and <i>HindIII</i> , cloned into the same sites of pLAFR3	This study
pPir-EC16	1.2-kb PCR product containing <i>pir</i> of EC16, processed with <i>EcoRI</i> and <i>HindIII</i> , cloned into the same sites of pLAFR3	This study
pPecS-3937	0.8-kb PCR product containing <i>pecS</i> of 3937, processed with <i>EcoRI</i> and <i>HindIII</i> , cloned into the same sites of pLAFR3	This study
pET-K3	801-bp PCR product containing <i>kdgR</i> ORF of 3937, processed with <i>NdeI</i> and <i>BamHI</i> , cloned into the same sites of pET21-a(+)	This study
pET-C3	655-bp PCR product containing <i>crp</i> ORF of 3937, processed with <i>NdeI</i> and <i>BamHI</i> , cloned into the same sites of pET21-a(+)	This study
pET-PIR	pET21-a(+) derivative containing <i>pir</i> ORF of EC16 under the T7 promoter	Nomura et al. 1999
pTP1	pT7-5 (T7 overexpression vector) derivative containing <i>pecS</i> ORF of 3937 under the T7 promoter, Amp ^r	Prailliet et al. 1996
pET-KE	801-bp PCR product containing <i>kdgR</i> ORF of EC1, processed with <i>NdeI</i> and <i>BamHI</i> , cloned into the same sites of pET21-a(+)	This study
pET-CE	655-bp PCR product containing <i>crp</i> ORF of EC1, processed with <i>NdeI</i> and <i>BamHI</i> , cloned into the same sites of pET21-a(+)	This study
pPEL1-R	323-bp PCR product containing <i>pel-1</i> promoter region (-174 to +131) of 71, processed with <i>XmaI</i> and <i>PstI</i> , cloned into the same sites of pCR-Script SK(+)	This study
pPEL3-R	298-bp PCR product containing <i>pel-3</i> promoter region (-199 to +77) of 71, processed with <i>XmaI</i> and <i>PstI</i> , cloned into the same sites of pCR-ScriptSK(+)	This study
pPEH-R	286-bp PCR product containing <i>peh</i> promoter region (-68 to +218) of EC1, treated with Klenow fragment, cloned into the <i>HincII</i> site of pHSG298	This study
pPEH-RL	<i>BamHI-PstI</i> fragment containing <i>peh</i> promoter region from pPEH-R cloned into the same sites of pBluescript SK(-)	This study
pHS001	2.3-kb <i>HindIII-SmaI</i> fragment containing <i>pelE</i> of EC16 cloned into the same sites of pUC118	K. Nomura, lab collection
pPEL1-LUX	<i>XmaI-EcoRI</i> fragment containing <i>pel-1</i> promoter region from pPEL-1R cloned into the same sites of pHSK728	This study
pPEL3-LUX	<i>XmaI-EcoRI</i> fragment containing <i>pel-3</i> promoter region from pPEL-3R cloned into the same sites of pHSK728	This study
pPEH-LUX	<i>BamHI-EcoRI</i> fragment containing <i>peh</i> promoter region from pPEH-RL cloned into the same sites of pHSK728	This study
pPELE-LUX	<i>NdeI-MluI</i> fragment (-150 to +110) containing <i>pelE</i> promoter region from pHS001, blunted with Klenow fragment, cloned into <i>SmaI</i> site of pHSK728	Nomura et al. 1998

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media.

Bacterial strains and plasmids used in this study are described in Table 2. The strains of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* were grown in YP medium (1% peptone, 0.5% yeast extract, pH 6.8) at 27°C. *Escherichia coli* strains were grown in LB (Luria-Bertani) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) at 37°C. M63 medium (Miller 1972) containing 0.2% glycerol was used as minimal medium. When required, carbon sources and antibiotics were added as follows: PGA (0.2%), glucose (0.2%), nalidixic acid (Nal, 30 µg per ml), ampicillin (50 µg per ml), kanamycin (50 µg per ml), streptomycin (Stm, 25 µg per ml), spectinomycin (Sp, 100 µg per ml), and tetracycline (Tet, 12.5 µg per ml). Plate media were solidified by the addition of 1.5% agar. Plant extracts were prepared by autoclaving vegetables (100 g/l) that had been cut into small pieces. Resultant supernatant ($1/10$ vol) was added to the medium.

Pel activity assay.

Bacterial cells were grown at 27°C until early stationary phase by measuring optical cell density at 660 nm (OD_{660}) (BACTOMONITOR BACT-500, Intertech Inc., Tokyo). The culture supernatant was prepared by centrifuging 1 ml of the culture at 15,000 rpm for 2 min. To determine total activity, the supernatant of the sonicated bacterial culture was used after sedimenting the cell debris. One ml of the cell suspension was sonicated two times for 20 s (Ultrasonic Disrupter UD-200, Tomy, Tokyo) on ice. Pel activity was assayed, essentially as described by Zink and associates (1985). Sample solution (10 µl) was added to 990 µl of the reaction buffer (0.05% PGA, 0.1 M Tris-HCl, pH 8.5, 0.1 mM $CaCl_2$), prewarmed at 30°C. Following a thorough mixing, the increase in the OD_{230} was measured every minute (Ultrospec 3000, Pharmacia Biotech, Cambridge, U.K.). One unit of Pel activity was defined as the enzyme that produced a change in absorbance of 0.001 at 230 nm in one minute.

Recombinant DNA techniques.

Preparation of total and plasmid DNA, PCR, restriction digestion, ligation, DNA electrophoresis, electroporation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were done essentially as described by Ausubel and associates (1987) or Sambrook and associates (1989). EMBL3 *Bam*HI Arms Cloning System (Promega, Madison, WI, U.S.A.) was used for the construction of genomic library of *E. carotovora* subsp. *carotovora* EC1 according to instructions. Nucleotide sequence analysis was performed with a DNA-Autosequencer (Li-cor, Lincoln, NE, U.S.A.). Restriction and modifying enzymes were purchased from Nippon Gene (Toyama, Japan) and New England Biolabs (Beverly, MA, U.S.A.). Primer sequences used in this study are available upon request.

Southern blot hybridization.

Southern blot hybridization was performed essentially as described by Sambrook and associates (1989). For preparation and detection of the probe, PCR DIG Labeling Mix (Roche, Mannheim, Germany) and DIG Chem-Link Labeling and Detection Set (Roche) were used, respectively. Procedures were done according to the manufacturer's instructions.

Preparation of monoclonal antibodies.

Monoclonal antibodies were generated essentially as described by Ausubel and associates (1987). Mouse (BALB/c line, 7 weeks old) was immunized using 100 µl of 500 µg of *E. chrysanthemi* EC16 per ml purified Pir after mixing with an equal volume of complete (first time) or incomplete Bacto Freund's adjuvant (2 weeks after the first injection, at intervals of 1 week, three times) (Difco, Detroit, MI, U.S.A.). One week after the last booster injection, spleen cells were isolated and fused with mouse myeloma NS-1 cells. Of 233 hybridoma cell lines, a single clone that produced useful antibodies was selected by repeated cloning and screening of the single hybridoma cells. The culture supernatant resulting from sedimentation of the cells by centrifugation at 2,500 rpm for 10 min was used as the monoclonal antibody.

Fig. 4. Pel activity of *Erwinia carotovora* subsp. *carotovora* EC1 containing clone plasmids for *E. chrysanthemi* regulatory molecules. Plasmid DNA was introduced into *E. carotovora* subsp. *carotovora* EC1-N by triparental mating using HB101 harboring pRK2013 as the helper strain. The bacterial cells were grown at 27°C until early stationary growth phase in indicated medium. Data are expressed as the mean of Pel-specific activity (U/OD₆₆₀) of sonicated bacterial cultures from at least three independent experiments.

Western blot hybridization.

After proteins were separated on a 10% SDS-PAGE gel, the gel was washed in transfer buffer C (25 mM Tris-base, 40 mM boric acid, 15% methanol, 0.04% SDS) for 20 min. Using a PolyBlot Transfer System (American Bionetics, Hayward, CA, U.S.A.), proteins in the gel were blotted onto a Hybond-P PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Assembly was as follows: the anode to the

cathode, two No. 1 filter papers (Whatman, Maidstone, U.K.) presoaked in transfer buffer A (0.3 M Tris-base, 15% methanol, 0.04% SDS), two filter papers presoaked in transfer buffer B (25 mM Tris-base, 15% methanol, 0.04% SDS), a PVDF membrane pretreated with 100% methanol for 5 s and then soaked in transfer buffer C, a gel presoaked in transfer buffer C, and two filter papers presoaked in transfer buffer C. Proteins were electrotransferred at 0.8 mA/cm² for 1 h, followed by electro-

Fig. 5. Gel-mobility shift assays of the promoter regions of *Erwinia carotovora* subsp. *carotovora* *peh*, *pel-1*, and *pel-3* and *E. chrysanthemi* *pelE* using purified *E. chrysanthemi* KdgR, CRP, Pir, and PecS under low-ionic strength conditions. **A**, Lanes 0 to 5 correspond to incubation with 0, 10, 25, 40, 55, and 110 nM purified KdgR, respectively. **B**, Lanes 0 to 5 correspond to incubation with 0, 20, 40, 80, 160, and 320 nM purified CRP (cyclic AMP receptor protein), respectively. **C**, Lanes 0 to 4 correspond to incubation with 0, 1.5, 6, 25, and 100 nM purified Pir, respectively. **D**, Lanes 0 to 4 correspond to incubation with 0, 20, 40, 80, and 160 nM purified PecS, respectively.

transfer at 2.5 mA/cm² for 30 min. After the transfer, the membrane was rinsed in PBS buffer (137 mM NaCl, 2.5 mM KCl, 8 mM Na₂HPO₄·12H₂O, 1.5 mM KH₂PO₄) and then blocked in the same buffer containing 5% skim milk and 0.3% TritonX-100 for 2 h at room temperature. Then, the membrane was washed three times in PBS buffer containing 0.3% TritonX-100 (washing buffer) for 15 min. After sedimenting anti-Pir monoclonal-antibody-producing cells by centrifugation at 2,500 rpm for 10 min, one-third volume of the culture supernatant in washing buffer was used as the primary antibody. The membrane was incubated for 2 h at room temperature. After three 15-min buffer washes, the membrane was incubated with a 1/1000 volume of a secondary antibody (AP conjugated anti-mouse antibody, Biolabs, Beverly, MA, U.S.A.) in washing buffer for 2 h at room temperature. Following three 15-min buffer washes, the membrane was developed in AP luminescent buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂·6H₂O) containing 0.1% 4-nitro blue tetrazolium (NBT) and 0.1% X-phosphate (BCIP).

Preparation of operator fragments for binding study.

DNA fragments cut from the plasmids, pPEL-1R (*XmaI*-*PstI*), pPEL-3R (*XmaI*-*PstI*), pPEH-R (*XmaI*-*PstI*), and pHS001 (*NdeI*-*MluI*) (Table 2) cut with restriction enzymes were end-labeled with 100 µCi of [α -³²P]dCTP (3,000 Ci/mmol; 1 Ci = 37 GBq) and the Klenow fragment of DNA polymerase I. These labeled fragments were electrophoresed, excised, and extracted using QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany).

Overexpression and purification of regulatory proteins.

T7 overexpression vector constructs containing *kdgR*, *crp*, *pir*, or *pecS* were introduced into *Escherichia coli* BL21(DE3) by electroporation using Cell-Porator (set at 18.4 kV/cm, 300 µF, and 4 ohms; Gibco BRL Life Technologies, Gaithersburg, MD). Among the transformants, single cells that were sufficiently overexpressing the protein were selected by incubation in 3 ml of LB medium, as described later. Then, the cells were streaked onto plates of LB agar and were incubated at 37°C overnight. The freshly prepared cells were collected and grown in 400 ml of LB liquid medium at 37°C until cell density reached an OD₆₆₀ approximately equal to 0.4. At this point, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture for an additional 2-h incubation. Cells were harvested by centrifugation at 8,000 rpm for 5 min, washed, and resuspended in 50 ml of buffer A (12 mM HEPES-NaOH pH 8.0, 4 mM Tris-HCl, pH 8.0, 1 mM EDTA-NaOH pH 8.0, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT], 10% glycerol). Crude cell extracts were obtained by sonication of washed bacterial suspension five times (2 min each) in ice-cold conditions. The sonicated crude extract was then centrifuged at 11,000 rpm for 30 min. The supernatant was fractionated by ammonium sulfate precipitation. The fraction precipitated with 20 to 40% ammonium sulfate (KdgR), 40 to 60% CRP, 25 to 40% Pir, or 55 to 70% PecS was resuspended in 5 ml of buffer A and dialyzed against three liters of the same buffer overnight. After removing insolubilized proteins of the dialysate by centrifugation at 11,000 rpm for 10 min, the dialysate was degassed for 10 min and applied to a

Fig. 6. Activity of *Erwinia carotovora* subsp. *carotovora* *pel-1*, *pel-3*, and *peh* and *E. chrysanthemi* *pelE* promoter-*lux* fusions in *E. carotovora* subsp. *carotovora* EC1 and in *E. chrysanthemi* EC16. One-tenth volume of overnight cultures in YP medium of *E. carotovora* subsp. *carotovora* EC1-N and *E. chrysanthemi* EC16 integrants was added to the same medium and was incubated at 27°C until mid-exponential growth phase (OD₆₆₀ = approximately 0.4). Following the preincubation, polygalacturonic acid (PGA) was added to final concentration of 0.2% for the induction. After 2 h incubation, photon production by the strains was measured every hour. The data was expressed as the mean of peak Lux-specific activities from at least three independent experiments.

Heparin affinity column (POROS HE/P, 10 mm × 100 mm; Boehringer Mannheim, Mannheim, Germany). The column was washed with buffer A containing 0.1 M KCl and was eluted with a linear gradient of KCl from 0.1 to 1 M at a flow rate of 5 ml/min. Several fractions (2 ml/tube) that formed a maximum peak in a UV curve were pooled and dialyzed against three liters of buffer A containing 50 mM KCl. Proteins prepared by this two-step purification procedure were estimated to be single by staining with Coomassie Brilliant Blue R250 after SDS-PAGE (data not shown). Purified protein solutions were stored at -20°C after adding 100% glycerol (50%, final). The protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, U.S.A.) solution with bovine serum albumin (BSA) as the standard.

Gel-mobility shift assay.

Standard procedures were performed as described by Ausubel and associates (1987) with minor modifications. Gel-mobility shift assays were carried out in 10 µl of reaction mixtures containing 12 mM HEPES-NaOH, pH 8.0, 4 mM Tris-HCl, pH 8.0, 0.1 mM EDTA-NaOH, pH 8.0, 50 mM KCl, 10 mM MgCl₂, 10mM CaCl₂, 0.1 mM PMSF, 1 mM DTT, 10% glycerol, 1 µg poly(dI-dC)-(dI-dC) (Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.) as the bulk carrier DNA, 2 µg BSA, 10 fmol of labeled DNA probe (5 × 10⁴ cpm), and various amounts of purified protein. After incubation for 30 min at 27°C, the reaction mixtures were loaded onto a 4% polyacrylamide gel (15 × 15 cm) in low-ionic strength buffer (6.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, pH 6.0, and 1 mM EDTA-NaOH, pH 8.0) or in high-ionic strength buffer (50 mM Tris-HCl, pH 8.5, 380 mM glycine, and 2 mM EDTA-NaOH, pH 8.0) and electrophoresed in the same buffer

at 20 mA for 2 h. The gel was then vacuum-dried and exposed to Hyperfilm (Amersham Pharmacia Biotech). For CRP, reaction mixtures, gel, and electrophoresis buffer contained 100 µM cAMP.

Insertion of promoter-lux fusions into *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* chromosomes.

To construct promoter-lux fusions, promoter regions of *pel-1* from pPEL1-R, *pel-3* from pPEL3-R, and *peh* from pPEH-RL were cloned into the multicloning sites of pHSK728 (Shen et al. 1992), yielding pPEL1-LUX, pPEL3-LUX, and pPEH-LUX, respectively (Table 2). For *E. carotovora* subsp. *carotovora* EC1, these constructs were electroporated into an *Escherichia coli* HB101 harboring a helper plasmid pRK2013 for conjugation and a helper plasmid pMON7184 (Barry 1988) for Tn7 transposition. Then, the transformants were conjugated with EC1-N, which is a spontaneous Nal^r mutant of EC1. The Nal^r, Sp^r, and Tet^r transconjugants were selected. They were subcultured at least five times in YP medium without antibiotics. Colonies resistant to Nal and Sp but sensitive to Tet were selected tentatively as candidates. Single insertion into the chromosome was confirmed by Southern blot analysis using Sp/Stm gene (3.8-kb *Pst*I-*Eco*RV fragment of pHSK728) as the probe. For *E. chrysanthemi* EC16, constructs were directly introduced by electroporation (9.4 kV/cm, 160 µF, and 4 ohms) into EC16 containing a helper plasmid pMON7181 for Tn7 transposition. Then, the transformants resistant to Sp and Gm were selected. The rest of the procedures were performed as for *E. carotovora* subsp. *carotovora* EC1.

Bioluminescence assay.

Photon production by the promoter-lux fusion integrants was measured 10 times for 1 s by a chemiluminescence detector (Hamamatsu Photonics, Hamamatsu, Japan) every hour. The mean of 10 readings (counts per s) in 1 ml divided by OD₆₆₀ was used as the Lux-specific activity (cps/OD₆₆₀). Peak Lux-specific activity during the incubation of each experiment was recorded. Data were expressed as the mean of peak Lux-specific activities from at least three independent experiments.

ACKNOWLEDGMENT

We are very grateful to K. Sayama and H. Dora for technical assistance with preparation of monoclonal antibodies. We also thank A. K. Chatterjee, the late N. T. Keen, and W. Nasser for generously providing strains of *Erwinia* spp., pMON7184, and pTP1, respectively. Thanks are extended to B. E. Hazen for revising the manuscript. Supported in part by grant-in-aid (no. 12052210) and by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (no. 13073).

LITERATURE CITED

- Andersson, R. A., Eriksson, A. R. B., Heikinheimo, R., Mae, A., Pirhonen, M., Koiv, V., Hyttiainen, H., Tuikkala, A., and Palva, E. T. 2000. Quorum sensing in the plant pathogen *Erwinia carotovora* subsp. *carotovora*: The role of *expR_{Ecc}*. Mol. Plant-Microbe Interact. 13:384-393.
- Andersson, R. A., Koiv, V., Norman-Setterblad, C., and Pirhonen, M. 1999a. Role of RpoS in virulence and stress tolerance of the plant pathogen *Erwinia carotovora* subsp. *carotovora*. Microbiology 145:3547-3556.
- Andersson, R. A., Palva, E. T., and Pirhonen, M. 1999b. The response regulator ExpM is essential for the virulence of *Erwinia carotovora* subsp. *carotovora* and acts negatively on the sigma factor RpoS (σ^s). Mol. Plant-Microbe Interact. 12:575-584.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1987. Current Protocols in Molecular Biology. John Wiley and Sons, New York.
- Barras, F., van Gijsegem, F., and Chatterjee, A. K. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu. Rev. Phytopathol. 32:201-234.
- Barry, G. F. 1988. A broad-host-range shuttle system for gene insertion

Fig. 7. Activity of *Erwinia carotovora* subsp. *carotovora* *pel-1*, *pel-3*, and *peh* promoter-lux fusions in *pir*-deficient mutant of *E. chrysanthemi* EC16 (K2367). Promoter-lux fusion integrants in *E. chrysanthemi* EC16 and in K2367 were grown at 27°C in M63-glycerol minimal medium containing 0.2% polygalacturonic acid and 1% potato extract. When cell density reached OD₆₆₀ = approximately 1.0, photon production by the strains was measured every hour. The data was expressed as the mean of peak Lux-specific activities from at least three independent experiments.

- into the chromosomes of gram-negative bacteria. *Gene* 71:75-84.
- Bourson, C., Favey, S., Reverchon, S., and Robert-Baudouy, J. 1993. Regulation of the expression of a *pelA::uidA* fusion in *Erwinia chrysanthemi* and demonstration of the synergistic action of plant extract with polygalacturonate on pectate lyase synthesis. *J. Gen. Microbiol.* 139:1-9.
- Castillo, A., and Reverchon, S. 1997. Characterization of the *pecT* control region from *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 179:4909-4918.
- Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. *Annu. Rev. Phytopathol.* 24:383-409.
- Cui, Y., Chatterjee, A., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Identification of a global repressor gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, *N*-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp. *J. Bacteriol.* 177:5108-5115.
- Cui, Y., Mukherjee, A., Dumenyo, C. K., Liu, Y., and Chatterjee, A. K. 1999. *rsmC* of the soft-rotting bacterium *Erwinia carotovora* subsp. *carotovora* negatively controls extracellular enzyme and harpin_{Ecc} production and virulence by modulating levels of regulatory RNA (*rsmB*) and RNA-binding protein (RsmA). *J. Bacteriol.* 181:6042-6052.
- Cui, Y., Chatterjee, A., and Chatterjee, A. K. 2001. Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory *rsmB* RNA, extracellular enzymes, and harpin_{Ecc}. *Mol. Plant-Microbe Interact.* 14:516-526.
- Eriksson, A. R. B., Andersson, R. A., Pirhonen, M., and Palva, E. T. 1998. Two-component regulators involved in the global control of virulence in *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 11:743-752.
- 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. U.S.A.* 76:1648-1652.
- Flego, D., Marits, R., Eriksson, A. R. B., Koiv, V., Karlsson, M.-B., Heikinheimo, R., and Palva, E. T. 2000. A two-component regulatory system, *pehR-pehS*, controls endopolygalacturonase production and virulence in the plant pathogen *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 13:447-455.
- Franza, T., Sauvage, C., and Expert, D. 1999. Iron regulation and pathogenicity in *Erwinia chrysanthemi* 3937: Role of the Fur repressor protein. *Mol. Plant-Microbe Interact.* 12:119-128.
- Harris, S. J., Shih, Y.-L., Bentley, S. D., and Salmond, G. P. C. 1998. The *hexA* gene of *Erwinia carotovora* encodes a LysR homologue and regulates motility and the expression of multiple virulence determinants. *Mol. Microbiol.* 28:705-717.
- Hugouvieux-Cotte-Pattat, N., Dominguez, H., and Robert-Baudouy, J. 1992. Environmental conditions affect the transcription of the pectinase genes of *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 174:7807-7818.
- Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., and Reverchon, S. 1996. Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annu. Rev. Microbiol.* 50:213-257.
- Hyttinen, H., Montesano, M., and Palva, E. T. 2001. Global regulators ExpA (GacA) and KdgR modulate extracellular enzyme gene expression through the RsmA-*rsmB* system in *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 14:931-938.
- Kolb, A., Busby, S., Buc, H., Garges, S., and Adhya, S. 1993. Transcriptional regulation by cAMP and its receptor protein. *Annu. Rev. Biochem.* 62:749-795.
- Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot *Erwinias*. *Annu. Rev. Phytopathol.* 25:405-430.
- Kotoujansky, A., Lemattre, M., and Boistard, P. 1982. Utilization of a thermosensitive episome bearing transposon Tn10 to isolate Hfr donor strains of *Erwinia carotovora* subsp. *chrysanthemi*. *J. Bacteriol.* 150:122-131.
- Liu, Y., Murata, H., Chatterjee, A., and Chatterjee, A. K. 1993. Characterization of a novel regulatory gene *aepA* that controls extracellular enzyme production in the phytopathogenic bacterium *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 6:299-308.
- Liu, Y., Jiang, G., Cui, Y., Mukherjee, A., Ma, W.-L., and Chatterjee, A. K. 1999. *kdgR_{Ecc}* negatively regulates genes for pectinases, cellulase, protease, Harpin_{Ecc}, and a global RNA regulator in *Erwinia carotovora* subsp. *carotovora*. *J. Bacteriol.* 181:2411-2422.
- Ma, W.-L., Cui, Y., Liu, Y., Dumenyo, C. K., Chatterjee, A., and Chatterjee, A. K. 2001. Molecular characterization of global regulatory RNA species that control pathogenicity factors in *Erwinia amylovora* and *Erwinia herbicola* pv. *gypsophylae*. *J. Bacteriol.* 183:1870-1880.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Mukherjee, A., Cui, Y., Ma, W.-L., Liu, Y., Ishihama, A., Eisenstark, A., and Chatterjee, A. K. 1998. RpoS (sigma-S) controls expression of *rsmA*, a global regulator of secondary metabolites, harpin, and extracellular proteins in *Erwinia carotovora*. *J. Bacteriol.* 180:3629-3634.
- Mukherjee, A., Cui, Y., Ma, W.-L., Liu, Y., and Chatterjee, A. K. 2000. *hexA* of *Erwinia carotovora* ssp. *carotovora* strain Ecc71 negatively regulates production of RpoS and *rsmB* RNA, a global regulator of extracellular proteins, plant virulence and the quorum-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. *Environ. Microbiol.* 2:203-215.
- Murata, H., McEvoy, J. L., Chatterjee, A., Collmer, A., and Chatterjee, A. K. 1991. Molecular cloning of an *aepA* gene that activates production of extracellular pectolytic, cellulolytic, and proteolytic enzymes in *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 4:239-246.
- Murata, H., Chatterjee, A., Liu, Y., and Chatterjee, A. K. 1994. Regulation of the production of extracellular pectinase, cellulase, and protease in the soft rot bacterium *Erwinia carotovora* subsp. *carotovora*: Evidence that *aepH* of *E. carotovora* subsp. *carotovora* 71 activates gene expression in *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *Escherichia coli*. *Appl. Environ. Microbiol.* 60:3150-3159.
- Nasser, W., Condemine, G., Plantier, R., Anker, D., and Robert-Baudouy, J. 1991. Inducing properties of analogs of 2-keto-3-deoxygluconate on the expression of pectinase genes of *Erwinia chrysanthemi*. *FEMS (Fed. Eur. Microbiol. Soc.) Lett.* 81:73-78.
- Nasser, W., Reverchon, S., Condemine, G., and Robert-Baudouy, J. 1994. Specific interactions of *Erwinia chrysanthemi* KdgR repressor with different operators of genes involved in pectinolysis. *J. Mol. Biol.* 236:427-440.
- 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.
- Nasser, W., Bouillant, M. L., Salmond, G. P. C., and Reverchon, S. 1998. Characterization of the *Erwinia chrysanthemi* *expl-expR* locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules. *Mol. Microbiol.* 29:1391-1405.
- Nasser, W., Shevchik, V. E., and Hugouvieux-Cotte-Pattat, N. 1999. Analysis of three clustered polygalacturonase genes in *Erwinia chrysanthemi* 3937 revealed an anti-repressor function for the PecS regulator. *Mol. Microbiol.* 34:641-650.
- Nasser, W., Faelen, M., Hugouvieux-Cotte-Pattat, N., and Reverchon, S. 2001. Role of the nucleoid-associated protein H-NS in the synthesis of virulence factors in the phytopathogenic bacterium *Erwinia chrysanthemi*. *Mol. Plant-Microbe Interact.* 14:10-20.
- Nomura, K., Nasser, W., Kawagishi, H., and Tsuyumu, S. 1998. The *pir* gene of *Erwinia chrysanthemi* EC16 regulates hyperinduction of pectate lyase virulence genes in response to plant signals. *Proc. Natl. Acad. Sci. U.S.A.* 95:14034-14039.
- Nomura, K., Nasser, W., and Tsuyumu, S. 1999. Self-regulation of Pir, a regulatory protein responsible for hyperinduction of pectate lyase in *Erwinia chrysanthemi*. *Mol. Plant-Microbe Interact.* 12:385-390.
- Perombelon, M. C. M., and Kelman, A. 1980. Ecology of the soft rot *Erwinias*. *Annu. Rev. Phytopathol.* 18:361-387.
- Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2467-2476.
- 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.
- 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.
- Reverchon, S., Nasser, W., and Robert-Baudouy, J. 1994. *pecS*: A locus controlling pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi*. *Mol. Microbiol.* 11:1127-1139.
- 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., Bouillant, M. L., Salmond, G. P. C., and Nasser, W. 1998. Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in *Erwinia chrysanthemi*. *Mol. Microbiol.* 29:1407-1418.
- Robert-Baudouy, J., Nasser, W., Condemine, G., Reverchon, S., Shevchik, V. E., and Hugouvieux-Cotte-Pattat, N. 2001. Pectic enzymes of *Erwinia chrysanthemi*, regulation and role in pathogenesis: Pages 221-268 in: *Advances in Molecular Genetics of Plant-Microbe Interactions*. Vol. 5. G. Stacey, and N. T. Keen, eds. American Phytopathological Society Press, St. Paul, MN, U.S.A.
- Rouanet, C., Nomura, K., Tsuyumu, S., and Nasser, W. 1999. Regulation

- of *pelD* and *pelE*, encoding major alkaline pectate lyases in *Erwinia chrysanthemi*: Involvement of the main transcriptional factors. J. Bacteriol. 181:5948-5957.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Shen, H., Gold, S. E., Tamaki, S. J., and Keen, N. T. 1992. Construction of a Tn7-*lux* system for gene expression studies in gram-negative bacteria. Gene 122:27-34.
- Staskawicz, B. J., Dahlbeck, D., Keen, N. T., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.
- Surgey, N., Robert-Baudouy, J., and Condemine, G. 1996. The *Erwinia chrysanthemi* *pecT* gene regulates pectinase gene expression. J. Bacteriol. 178:1593-1599.
- Thomson, N. R., Cox, A., Bycroft, B. W., Stewart, G. S. A. B., Williams, P., and Salmond, G. P. C. 1997. The Rap and Hor proteins of *Erwinia*, *Serratia* and *Yersinia*: A novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. Mol. Microbiol. 26:531-544.
- Thomson, N. R., Nasser, W., McGowan, S., Sebaihia, M., and Salmond, G. P. C. 1999. *Erwinia carotovora* has two KdgR-like proteins belonging to the IclR family of transcriptional regulators: Identification and characterization of the RexZ activator and the KdgR repressor of pathogenesis. Microbiology 145:1531-1545.
- Zink, R. T., Engwall, J. K., McEvoy, J. L., and Chatterjee, A. K. 1985. *recA* is required in the induction of pectin lyase and carotovoricin in *Erwinia carotovora* subsp. *carotovora*. J. Bacteriol. 164:390-396.