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Research Note

Synthesis and Secretion of *Erwinia chrysanthemi* Virulence Factors Are Coregulated

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Regulation of the transcription of four operons (*outS*, *outB*, *outT*, *outC-M*) coding for components of the protein secretion apparatus of *Erwinia chrysanthemi* has been studied. Regulation of the *outC* operon expression by *kdgR*, which is the repressor of all the genes involved in pectinolysis, requires the *outT* gene product, suggesting that OutT could be a transcriptional activator of *outC*. The gene *pecS*, which regulates *pel* and *celZ* gene expression, also negatively controls the *outC* operon in a manner independent of the regulation by OutT. All environmental conditions that affect pectate lyase production also modulate Out protein synthesis. Thus, pectate lyase synthesis and secretion are very tightly coregulated.

Additional keyword: cellulase.

Secretion of pectin-degrading enzymes is one determinant of the pathogenicity of the enterobacterium *Erwinia chrysanthemi*. The secreted proteins, a pectin-methylesterase (PME) and the five isoenzymes of pectate lyases (PLa, -b, -c, -d, -e) degrade pectin mainly to di- or trimers of galacturonate (Preston et al. 1992). These dimers and trimers enter the bacteria and are degraded through a five-step pathway into molecules used in the bacteria general metabolism (Condemine et al. 1986; Hugouvieux-Cotte-Pattat and Robert-Baudouy 1987). All genes coding for the enzymes involved in pectin degradation are negatively regulated by the KdgR repressor (Condemine and Robert-Baudouy 1987; Condemine and Robert-Baudouy 1991; Reverchon et al. 1991). Binding of KdgR to the regulatory regions of the genes it controls has been proven in vitro for a few genes (Nasser et al. 1992; Nasser et al. 1994). However, the regulatory regions of some genes regulated in vivo by *kdgR* are not able to bind purified KdgR protein in vitro (Nasser et al. 1994).

While the genes coding for the intracellular part of the pectin degradation pathway are only regulated by *kdgR*, the *pel* genes (coding for the pectate lyases) and the *pem* gene (coding for the PME) are subject to complex regulation (Hugouvieux-Cotte-Pattat et al. 1992). Physiological studies have shown that expression of these genes is sensitive to variations in environmental conditions and is dependent on temperature, osmolarity, growth phase, anaerobiosis, catabolic repression, and nitrogen or iron concentration (Hugouvieux-Cotte-Pattat et al. 1992; Sauvage et al. 1991). However, the five *pel* genes do not always respond in the same way and to the same extent to these signals. Regulatory genes controlling the expression of some or all the *pel* genes have been identified. *pecM* and *pecS* regulate negatively the expression of the five *pel* genes, *pem* and *celZ*, the gene encoding the endoglucanase EGZ (Reverchon et al. 1994). Other genes controlling *pel* gene expression are less well characterized.

Secretion from the periplasmic space to the the outer medium of PLs, PEM, and the endoglucanase EGZ occurs through the Out secretion system, a multiprotein complex encoded by the *out* gene cluster, a group of 15 genes organized in five operons (*outS*, *outB*, *outT*, *outCDEFGHIJKLM*, and *outO*) (Condemine et al. 1992; He et al. 1992; Lindeberg and Collmer 1992) (Fig. 1). Expression of two of the operons (*outT* and *outC*) is derepressed in a *kdgR* mutant (Condemine et al. 1992). Thus, they belong to the *kdg* regulon. Genes coding for proteins similar to those of the Out secretion system have been identified in several protein secreting bacteria. Among them, the *Klebsiella oxytoca* pullulanase secretion system is the one whose genetic organization is the most similar to the *out* secretion system (Pugsley 1993). All *K. oxytoca* secretion genes except one, *pulN*, have a homolog in *E. chrysanthemi* and only one *E. chrysanthemi* gene, *outT*, has no known homolog in *K. oxytoca* (Condemine et al. 1992; He et al. 1992; Lindeberg and Collmer 1992). *outT* has, in the *out* cluster, the position of the gene *pulA*, coding for the pullulanase, in the *pul* cluster (d'Enfert et al. 1987). However, it does not code for a PL or an endoglucanase. OutT has been characterized as a 12-kDa protein and it has no homology with any known protein. A mutation in *outT* results in a secretion minus phenotype (Condemine et al. 1992).

We have previously shown that the *outT* and *outC* operons are regulated in vivo by *kdgR* (Condemine et al. 1992). Although a putative KdgR binding sequence was found in the regulatory regions of these two operons, bandshift experiments have shown that only the *outT* regulatory region could bind the purified KdgR protein in vitro (Nasser et al. 1994). Therefore, regulation of *outC* by *kdgR* could not be explained by binding of KdgR preventing transcription of the gene. To test if a single transcript originating at the *outT* promoter and

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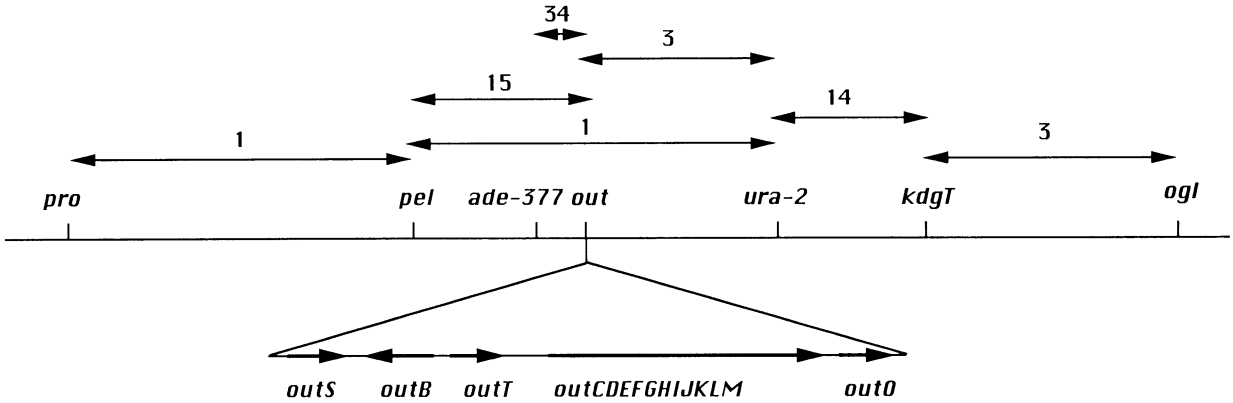

Fig. 1. Chromosomal mapping of the *out* cluster. The numbers above the arrow indicate the percentage of cotransfer of markers by chromosomal mobilization using the plasmid pULB110 (van Gijsegem et al. 1983). Organization of the genes within the *out* cluster is presented but the orientation of the cluster on the map has not been determined. The *pel* cluster is not detailed.

covering *outT* and the *outC* to *M* genes could exist, double mutants containing a *lacZ* reporter fusion into the *outC* operon (referred, for simplification, as *outC–lacZ* fusion) and a cassette insertion into *outT* or between *outT* and *outC* were constructed. Introduction of a kan^r cassette downstream of *outT* did not significantly change the level of expression of the *outC–lacZ* fusion in uninduced or induced conditions (Table 1). This absence of a polar effect suggested that the *outT* and the *outC* operons are not cotranscribed. Moreover, the distance between the two genes (800 bp) made this coupling unlikely. Insertion of the kan^r cassette inside *outT* reduced *outC* expression in all conditions tested (Table 1). Therefore, *outT* seems necessary for the expression of *outC*.

The effect of a *kdgR* mutation was tested on the expression of the *outC–lacZ* fusion in the mutants described above. As expected, the *kdgR* mutation led to an elevated level of expression of the *outC–lacZ* fusion (Table 1). Introduction of a *kdgR* mutation into the *outT* mutant did not change the level of expression of the *outC–lacZ* fusion, which remained lower than in the wild-type strain. Thus, a functional OutT protein seems necessary to the regulation of *outC* by *kdgR*. In this mutant, addition of inducer led to a 1.5-fold increase of the level of expression of the fusion, indicating that a second regulatory gene could control *outC* expression. When the *kdgR* mutation was introduced into the strain containing the kan^r cassette between *outT* and *outC*, expression of *outC* was still constitutive but at a lower level than in the strain without the kan^r cassette. The cassette was introduced four nucleotides downstream of the *outT* stop codon. This could destabilize the *outT* mRNAs and reduce their half-life and the concentration of the OutT protein in the bacteria, leading to a lower level of expression of *outC*. These experiments suggest that *outT* could be an activator of *outC* expression. Inactivation of KdgR by the inducer, KDG, would induce the synthesis of OutT which would in turn initiate *outC* expression (Fig. 2). In this model a functional KdgR-box is not needed in front of *outC* for a regulation of this gene by *kdgR*. The same type of regulation could exist for other genes of the *kdg* regulon in front of which no functional KdgR binding site has been found (*kdgA*, *kduD*, *kdgC*) (Nasser et al. 1994). Existence of a molecule interacting with OutT to induce a conformational change allowing its activation might not be necessary: The level of activation could depend directly on the concentration

Table 1. Effect of *outT*, *kdgR*, and *pecS* mutations on the expression of an *outC-lacZ* operon fusion[a]

Additional mutation	Inducer	β-Galactosidase levels of strains with the following genotypes	
		outC-lacZ	*outC-lacZ kdgR*
None	–	65 ± 9	222 ± 42
	+	180 ± 10	289 ± 50
outT	–	43 ± 10	7 ± 6
	+	65 ± 16	76 ± 13
kan^r between *outT* and *outC*	–	74 ± 18	118 ± 27
	+	146 ± 30	161 ± 20
pecS	–	160 ± 22	593 ± 52
	+	313 ± 32	617 ± 43
outT pecS	–	138 ± 12	117 ± 19
	+	232 ± 26	238 ± 32

[a] The bacteria were grown to early stationary phase in M63 + glycerol medium with or without addition of galacturonate and polygalacturonate. β-Galactosidase assays were performed on toluenized cell cultures. β-Galactosidase activity was assayed according to Miller (1972) and is expressed in nanomoles of *o*-nitrophenol formed per minute per milligram (dry weight) of bacteria.

of OutT. We could not prove, by bandshift assay, an interaction of OutT with the regulatory region of *outC*. These experiments were performed with crude bacterial extracts that probably contain a very low OutT concentration. This could result from the low expression of the protein: *outT* codon usage is that of a weakly expressed protein. A low level of expression is a characteristic of activator proteins (Raibaud and Schwarz 1984).

In an *outT* mutant, the residual *outC* expression remains inducible by PGA. To test if this effect could be mediated by one of the regulatory genes controlling *pel* gene expression, mutations in some of these genes were transduced into a strain containing the *outC–lacZ* fusion. Introduction of a *pecS* mutation led to an elevated expression of the *outC–lacZ* fusion in uninduced and induced conditions (Table 1) but did not change the expression of *outT*, *outB*, and *outS* (data not shown). The effect of *pecS* on *outC* does not require OutT since an elevated expression of *outC* could also be observed in the absence of *outT* (Table 1) (Fig. 2). In the double *outT*

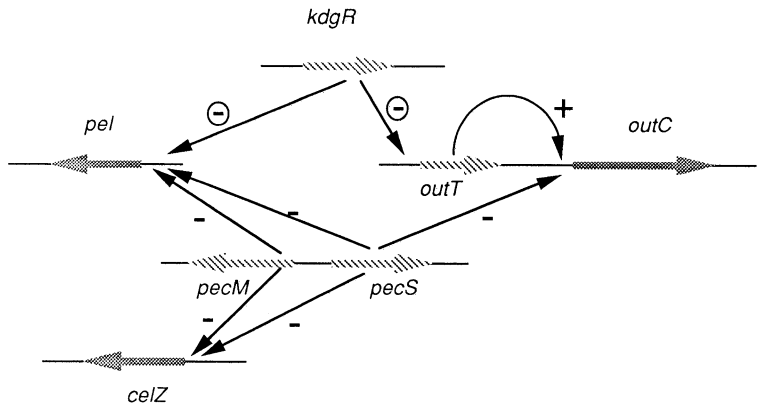

Fig. 2. Regulation of the *outC* operon, *celZ* and *pel* genes by *kdgR*, *outT*, *pecS*, and *pecM*. Only one *pel* gene is represented. Dashed arrows represent regulatory genes, gray arrows structural genes. + and – near the black arrows indicate positive and negative regulation. These signs are circled when a regulatory protein-operator interaction has been proven.

Table 2. Effect of various environmental conditions on the transcription of *outT-lacZ*, *outC-lacZ*, *outB-uidA*, and *outS-uidA* fusions[a]

Growth condition	β-Galactosidase or β-glucuronidase activity of bacteria with the following genotype:			
	outT-lacZ	*outC-lacZ*	*outB-uidA*	*outS-uidA*
Glycerol 30°C	73	70	2200	3980
Glycerol +galacturonate+ PGA 30°C	132	159	1750	4250
Glycerol 37°C	7	8	1050	3440
Glycerol +galacturonate+ PGA 37°C	29	33	1060	3290
Anaerobiosis	55	43	395	811
High osmolarity	29	37	851	2075
Glucose	44	101	612	2730
Low nitrogen	27	26	280	1160

[a] Each fusion was assayed in the absence or presence of inducing compounds at different temperatures or in different physiological conditions by cultivation in specific media. Standard conditions were obtained by cultivation in M63 medium containing glycerol as carbon source and with shaking at 200 rpm. Carbon sources were added at 2 g/liter except polygalacturonate that was added at 4 g/liter. Anaerobic culture were realized by overlaying the culture medium with paraffin oil. Fumarate (2.5%) was added as electron acceptor. Nitrogen starvation was performed in the following medium: M63 deprived of $(NH_4)_2SO_4$ supplemented with arginine (200 μg/ml) as nitrogen source. High osmolarity was obtained by adding 0.3 M NaCl to M63 medium. β-Glucuronidase was assayed according to Novel *et al.*(1974). β-Galactosidase and β-glucuronidase activities are expressed in nanomoles of *o*-nitrophenol or *p*-nitrophenol formed per minute per milligram (dry weight) of bacteria.

pecS mutant, the level of *outC* expression was lower than in a *pecS* mutant, confirming the activator role of OutT. In such a mutant, the level of expression of the *outC* operon was high enough to restore protein secretion to a wild-type level (data not shown). The effect of *pecS* and *kdgR* mutations was cumulative since expression of the *outC–lacZ* fusion was higher in the double *kdgR pecS* mutant than in any of the single mutants (Table 1). Surprisingly, *pecM*, which controls *celZ* expression (Reverchon et al. 1994), did not regulate *outC* (data not shown). However, the low induction observed in an *outT*, an *outT pecS* or an *outT kdgR pecS* mutant (Table 1) in which *kdgR*-mediated induction no longer occurs, suggests the existence of another protein responsible for this PGA-mediated induction.

The effect of environmental conditions known to affect *pel* gene expression was tested on *out* gene expression. Growth at 37°C strongly reduced *outT* and *outC* expression but moderately reduced that of *outB* and *outS* (Table 2). Growth in a medium with low nitrogen concentration, high osmolarity or in anaerobiosis reduced the expression of the four operons (Table 2). These results could explain the inhibition of secretion of PL observed at high osmolarity by Prior et al. (1994). Growth with glucose as sole carbon source slightly repressed *outT*, *outB*, and *outS*. While only high temperature and low nitrogen concentration significantly reduced PL synthesis (Hugouvieux-Cotte-Pattat et al. 1992), all conditions led to a reduction of the expression of part or all of the *out* operons.

PL synthesis is growth-phase dependent (Hugouvieux-Cotte-Pattat et al. 1992). Lindeberg and Collmer (1992) observed that the expression of the *outC* operon was also growth-phase dependent while Ji et al. (1987) had shown that expression of an uncharacterized *out-lacZ* fusion (now localized in the *outC* operon) was constant during growth. To solve this contradiction, expression of *outS*, *outB outT*, and *outC* was followed at different growth stages, in uninduced conditions or galacturonate-induced conditions. A growth-phase dependent expression of *outT* and *outC* was observed (two- to threefold increase of the specific activity at the end of the exponential phase) only in induced conditions (Fig. 3). This could explain the contradiction between previous experiments since Lindeberg and Collmer (1992) performed their experiments in induced conditions and Ji et al. (1987) in uninduced conditions. A weak increase of *outB* and *outS* expression (20 to 30%) was also observed at the end of exponential growth when bacteria were grown in the presence of galaturonate (data not shown). To test if the increased expression of *outC* could result from an increased OutT concentration at the end of the exponential growth phase, the same experiment was repeated in an *outT outC-lacZ* mutant (Fig. 3). *outC* expression was still growth-phase dependent in this double mutant, indicating that this effect was not dependent on OutT concentration. A growth-phase-dependent expression of *outC* was also observed in a *pecS* mutant (Fig. 3). Thus, *outC* growth-phase regulation does not seem to occur

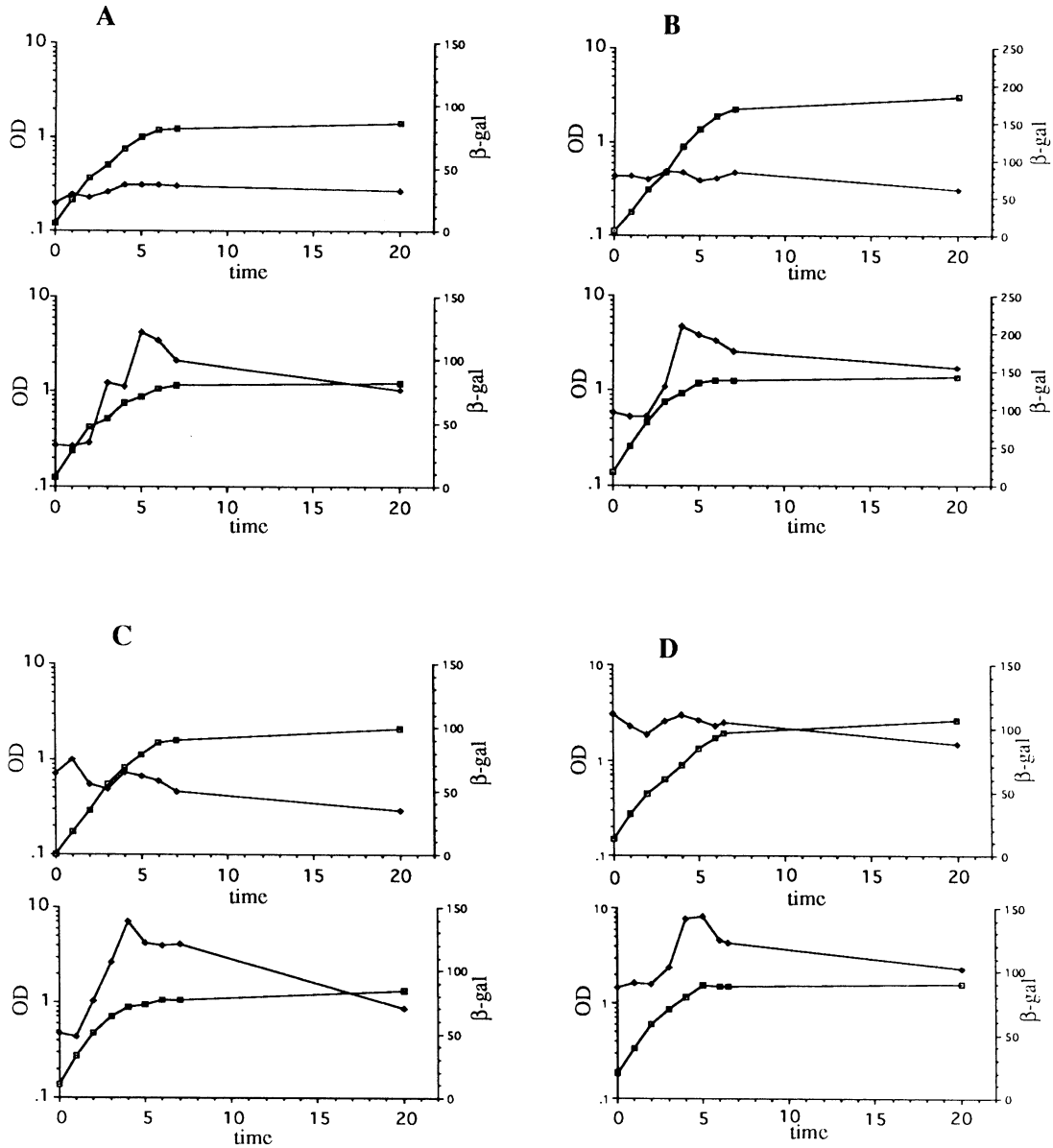

Fig. 3. Growth and expression of an *outT-lacZ* and *outC-lacZ* fusion strains in various backgrounds. Growth (□) of the different fusion strains in M63 minimal medium (upper part of each panel) or supplemented with galacturonate (bottom part) was followed by measuring OD_{600}. β-Gal activity (◇) was determined on each sample and was expressed in nanomoles of *o*-nitrophenol formed per minute per milligram (dry weight) of bacteria. **A,** *outT-lacZ* mutant strain; **B,** *outC-lacZ* mutant strain; **C,** *outT outC-lacZ* double mutant strain. **D,** *pecS outC-lacZ* double mutant strain.

through one of the identified regulators, *kdgR*, *outT,* or *pecS*. The growth-phase-dependent regulation of the *pel* genes is more visible in induced than in uninduced condition (40- versus 3-fold factors). Growth-phase regulation of *outC* in uninduced conditions may occur but remain unnoticed because of the low inducibility of the gene.

The chromosomal localization of the *out* cluster has been confirmed by cotransfer experiments. The order previously determined of the *ade*-377 and *out* markers on the *E. chrysanthemi* map (Ji et al. 1989) has been inverted. Two previously unmapped genes (*ura-2* and *kdgT*, coding for the KDG permease) have been localized in this region. The order of genes in this region was determined as *pro pel* cluster (*hmpX pelA pelE pelD pecY pem) ade-377 out* cluster (*outS outB outT outCDEFGHIJKLM outO) ura-2 kdgT ogl* (Fig. 1).

CONCLUSION

E. chrysanthemi has developed regulation mechanisms that link the synthesis of pectinases and cellulase with that of their secretion machinery (Fig. 2). The *kdgR*-mediated regulation allows the synthesis and secretion of pectinases when pectin is present in the culture medium. The *pecS*-mediated regulation seems to be more global since it controls the synthesis and secretion of pectinases and cellulases. The signal to which this regulation responds is unknown but could be specific to the plant-bacteria interaction. Coregulation by environmental conditions (temperature, growth phase, osmolarity, and nitrogen concentration) adds another level of complexity to this system. Other examples of coregulation between the synthesis of a secreted protein and that of its secretion ma-

chinery have been described in *K. oxytoca*, where the transcriptional activator MalT regulates pullulanase synthesis and secretion (d'Enfert et al. 1987), and in *P. aeruginosa* where several exoproteins and the Xcp secretion system are expressed in the late-log phase (Akrim et al. 1993). This might be a general situation but remains to be confirmed in other bacteria.

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