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-methyl esterase (PME) and the five isoenzymes of pectate lyases (PLa, -b, -c, -d, -e) degrade pectin mainly to di- or trimers of galacturonic acid (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 of 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 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, outCDEFGHIJKLMNOP, 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 a 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
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' 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) makes this coupling unlikely. Insertion of the kan' 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' cassette between outT and outC, expression of outC was still constitutive but at a lower level than in the strain without the kan' 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 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. 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 pUL8110 (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.](image-url)
**Table 2.** Effect of various environmental conditions on the transcription of outT-lacZ, outC-lacZ, outB-uidA, and outS-uidA fusions

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>outT-lacZ</th>
<th>outC-lacZ</th>
<th>outB-uidA</th>
<th>outS-uidA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol 30°C</td>
<td>73</td>
<td>70</td>
<td>2200</td>
<td>3980</td>
</tr>
<tr>
<td>Glycerol +galacturonate+PGA 30°C</td>
<td>132</td>
<td>159</td>
<td>1750</td>
<td>4250</td>
</tr>
<tr>
<td>Glycerol 37°C</td>
<td>7</td>
<td>8</td>
<td>1050</td>
<td>3440</td>
</tr>
<tr>
<td>Glycerol +galacturonate+PGA 37°C</td>
<td>29</td>
<td>33</td>
<td>1060</td>
<td>3290</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>55</td>
<td>43</td>
<td>395</td>
<td>811</td>
</tr>
<tr>
<td>High osmolarity</td>
<td>29</td>
<td>37</td>
<td>851</td>
<td>2075</td>
</tr>
<tr>
<td>Glucose</td>
<td>44</td>
<td>101</td>
<td>612</td>
<td>2730</td>
</tr>
<tr>
<td>Low nitrogen</td>
<td>27</td>
<td>26</td>
<td>280</td>
<td>1160</td>
</tr>
</tbody>
</table>

*Each fusion was assayed in the absence or presence of inducing compounds at different temperatures or in different physiological conditions. Standard conditions were obtained by cultivation in M63 medium containing glycerol as carbon source and shaking at 200 rpm. Carbon sources were added at 2 g/liter except galacturonate 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 (NH₄)₂SO₄ supplemented with arginine (200 µg/ml) as nitrogen source. High osmolarity was obtained by adding 0.3 M NaCl to M63 medium. β-Galactosidase 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.

The 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 uninuced 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 galacturonate (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...
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 tran-
scriptional activator MalT regulates pullulanase synthesis and
secretion (d’Enfert et al. 1987), and in *P. aeruginosa* where sev-
eral exoproteins and the Xcp secretion system are ex-
pressed 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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