Research Note

Control of *exuT* Activity for Galacturonate Transport by the Negative Regulator ExuR in *Erwinia chrysanthemi* EC16

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The negative regulatory protein ExuR in *Erwinia chrysanthemi* regulates expression of the galacturonate uptake (*exuT*) and utilization (*uxaA*, *uxaB*, *uxaC*) genes. We cloned and determined the nucleotide sequence of the *exuR* gene from *E. chrysanthemi* EC16. Analysis of the deduced amino acid sequence indicates that this protein possesses a helix-turn-helix motif and belongs to the GntR family of transcriptional repressors. Northern blot analysis and studies with transcriptional fusions of *exuT* in wild-type and *exuR* mutant backgrounds indicate that *exuT* transcription is deregulated in the *exuR* strain in vivo and in planta. [^{14}C]-galacturonic acid uptake was constitutively high under inducing and noninducing conditions in the *exuR* mutant. Maximal *exuT* transcription activity was observed within 8 h of bacterial inoculation into potato tubers, well before any visible symptoms of disease were detected. This suggests that ExuT transport activity in *E. chrysanthemi* is important in the early stages of disease development.

Additional keywords: digalacturonate, gene regulation, pectin utilization, plant disease.

The plant pathogen *Erwinia chrysanthemi* causes soft rot disease by secreting large amounts of several different plant cell wall-degrading enzymes. Among these different wall-degrading enzymes, pectate lyases have been identified as particularly important for *E. chrysanthemi* to macerate plant tissue (Collmer and Keen 1986; Keen and Tamaki 1986). Pectate lyases degrade pectate, a polymer of α-1,4-linked galacturonic acid (GalUA), to generate GalUA dimers and multimers with varying degrees of polymerization (Preston et al. 1992). The products of extracellular pectate degradation are transported into the bacterial cell, where they undergo intracellular catabolism to generate inducer molecules for the expression of higher levels of pectate lyases and other cell wall-degrading enzymes (Hugouvieux-Cotte-Pattat et al. 1996). Therefore, uptake of pectate degradation products is required for the in-

duction of virulence factors and represents an important event in the initial stages of the plant–bacterial interaction, leading to the initiation and progression of disease.

Uptake of GalUA in *E. chrysanthemi* EC16 is induced by pectate and its degradation products, saturated digalacturonic acid (GalUA)$_2$ and GalUA (San Francisco and Keenan 1993). The *exuT* gene encoding the transporter from this strain was cloned and sequenced, and the gene product was localized to the cytoplasmic membrane (Freeman and San Francisco, 1994; Haseloff et al. 1998). Mutants in *exuT* have a significantly lower capability to macerate potato tuber tissue than the wild-type parent (Haseloff et al. 1998). Information on the regulation of *exuT* expression has been restricted only to genetic analysis in *E. chrysanthemi* strains 3937 and B374 (Hugouvieux-Cotte-Pattat et al. 1983; Hugouvieux-Cotte-Pattat and Robert-Boudouy 1987). Wild-type *E. chrysanthemi* 3937 also can transport glucuronic acid (GulUA), an isomer of GalUA, with ExuT but cannot use GulUA as a sole carbon source for growth because this compound is unable to induce *exuT* expression (Hugouvieux-Cotte-Pattat et al. 1983). Spontaneous mutants capable of fermenting GulUA were isolated on the basis of their ability to grow on medium containing GulUA as a sole carbon source. This phenotype was attributed to the loss of a repressor function leading to the constitutive expression of the transport system, and the regulator was named ExuR, by analogy to the corresponding gene of *Escherichia coli* (Hugouvieux-Cotte-Pattat and Robert-Boudouy 1987; Nemoz et al. 1976; Portalier et al. 1980). Furthermore, Mu-*lac* insertions leading to constitutive uptake of [^{14}C]-GulUA were isolated, and the mutations were assigned to the regulator of *exuT* expression. These studies represent indirect evidence for the role of *exuR* in the regulation of *exuT*. In view of the importance of GalUA transport and metabolism in the pathogenicity of *E. chrysanthemi* (Beaulieu and Van Gijsegem 1990; Haseloff et al. 1998), we wished to more clearly elucidate the mechanism of *exuT* regulation.

The gene encoding the presumptive ExuR was cloned (Freeman and San Francisco 1994), and the nucleotide sequence deposited under GenBank accession no. U35884. The deduced amino acid sequence of this gene indicates the presence of a polypeptide with a hydrophilic profile containing an N-terminal, helix-turn-helix motif (ProfileScan in the PROSITE database), which is characteristic of the GntR fam-

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ily of transcriptional regulators (Haydon and Guest 1991) (Fig. 1). This open reading frame is 777 bp and predicted to encode a protein of 259 amino acids, with a calculated molecular mass of 31,081 kDa. A homology search of the DNA and protein databases with the deduced amino acid sequence revealed a strong homology with the *E. coli* ExuR (76% identity), UxuR (48% identity), and FarR (37% identity) regulatory proteins, suggesting a regulatory function for the gene (Altschul et al. 1997). Examination of the 5′ upstream sequences of *exuR* indicate the presence of potential −10 and −35 consensus sequences (TATCAT and TTTCTG, respectively) corresponding to a prokaryotic promoter region. A putative ribosome binding sequence (GAGCAGG) (Shine and Dalgarno 1974) is located four bases upstream of the ATG start codon.

In order to study the role of *exuR* in the regulation of *exuT* expression, an *E. chrysanthemi exuR* strain was constructed by marker-exchange mutagenesis (Roeder and Collmer 1985). Previously, Northern blot analysis revealed that *exuT* transcripts are inducible in the presence of GalUA in wild-type *E. chrysanthemi* EC16 (Haseloff et al. 1998). In the *E. chrysanthemi exuR* strain, high levels of *exuT* transcripts were ob-

served in uninduced (glycerol grown) and induced (GalUA grown) cultures (Fig 2). These levels of *exuT* transcripts were comparable to the levels in the induced wild-type cells and indicate the loss of regulatory control over *exuT* transcription. These results confirm that *exuT* expression is regulated by ExuR at the transcriptional level and that *exuT* transcription is constitutive in the *exuR* mutant.

Operon (transcription) fusions of *exuT–lux* were constructed in wild-type and *exuR* strains with the Tn*7–lux* system (Shen et al. 1992). Use of this system allows fusions to be inserted into the Tn*7 att* site on the chromosome, preventing insertion into functional genes. The strain used in these studies possesses a functional wild-type *exuT* allele. Southern blot analysis with the *lux* gene as a probe was carried out to confirm that only a single copy of the gene fusion was present on the chromosome. These single-copy operon fusions were used to obtain a detailed profile of *exuT* expression in relation to different phases of bacterial growth in culture and during plant tissue maceration. In vivo studies were carried out by monitoring bioluminescence of cells grown in glycerol or GalUA at regular time points for a period of 15 h (Fig. 3). Starter cultures were grown in M9 glycerol overnight before inoculation
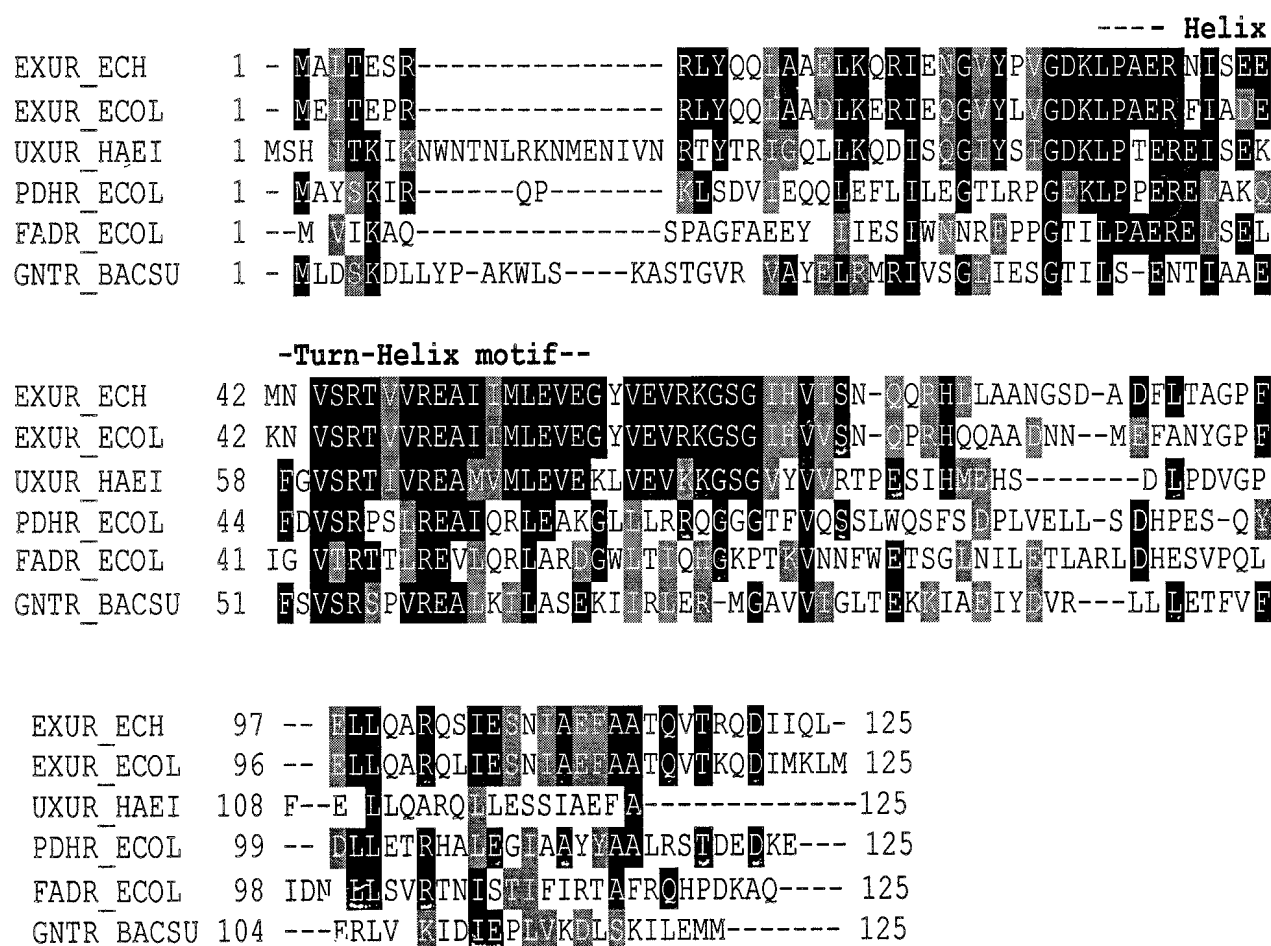
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                                                              ---- Helix
EXUR_ECH     1 - MALTESR--------------- RLYQQLAALKQRIENGVYPVGDKLPAERNISEE
EXUR_ECOL    1 - METTEPR--------------- RLYQQLAADLKERIEQGVYLVGDKLPAERFIADE
UXUR_HAEI    1 MSHLTKIKNWNTNLRKNMENIVN RTYTRIGQLLKQDISQGIYSLGDKLPTEREISEK
PDHR_ECOL    1 - MAYSKIR------QP------- KLSDVIEQQLEFLILEGTLRPGEKLPPERELAKQ
FADR_ECOL    1 --M VIKAQ--------------SPAGFAEEY IIESIWNRFPPGTILPAERELSEL
GNTR_BACSU   1 - MLDSKDLLYP-AKWLS----KASTGVR VAYELRMRIVSGLIESGTILS-ENTIAAE

               -Turn-Helix motif--
EXUR_ECH    42 MN VSRTVVREAIIMLEVEGYVEVRKGSG IHVISN-QQRHLLAANGSD-A DFLTAGPF
EXUR_ECOL   42 KN VSRTVVREAIIMLEVEGYVEVRKGSG LHVVSN-QPRHQQAALNN--MLFANYGPF
UXUR_HAEI   58 FGVSRTIVREAMVMLEVEKLVEVKKGSG VYVVRTPESIHMEHS-------D LPDVGP
PDHR_ECOL   44 FDVSRPSLREAIQRLEAKGLLLRRQGGGTFVQSSLWQSFS DPLVELL-S DHPES-QY
FADR_ECOL   41 IG VIRTTLREVIQRLARLGWLT QHGKPTKVNNFWETSGINILHTLARL DHESVPQL
GNTR_BACSU  51 FSVSRSPVREALKILASEKIIRLER-MGAVVIGLTEKKIAEIYLVR---LL LETFVF

EXUR_ECH    97 -- ELLQARQSIESNIAFEAATQVTRQDIIQL- 125
EXUR_ECOL   96 -- ELLQARQLIESNIAEEAATQVTKQDIMKLM 125
UXUR_HAEI  108 F--E ILQARQLLESSIAEFA-------------125
PDHR_ECOL   99 -- DLLETRHALEGIAAYVAALRSTDEDKE--- 125
FADR_ECOL   98 IDN LLSVRTNISTLFIRTAFRQHPDKAQ---- 125
GNTR_BACSU 104 ---FRLV KIDIEPLVKDLSKILEMM------- 125
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Fig. 1. Alignment of the N-terminal region of ExuR with homologs of the GntR family of regulatory proteins. EXUR_ECH: Repressor for galacturonate uptake and utilization genes from *Erwinia chrysanthemi*. EXUR_ECOL: Repressor for galacturonate uptake and utilization genes from *Escherichia coli*. UXUR_HAE1: Repressor glucuronate transport and utilization genes from *Haemophilus influenzae*. PDHR_ECOL: Repressor for pyruvate dehydrogenase from *E. coli*. FADR_ECOL: Fatty acid-responsive gene from *E. coli*. GNTR_BACSU: Repressor for gluconate transport and utilization genes from *Bacillus subtilis*.

into media to monitor *exuT* expression. Wild-type cells grown on glycerol (uninduced) had basal levels of *exuT–lux* activity, reaching a maximal value of 5.3×10^7 cpm after 10 h (luciferase activity on a cell-density basis) (Fig. 3). When these cells were grown in GalUA as an inducer, luciferase activity increased rapidly and attained levels approximately 2.5 times the basal values observed with uninduced cultures (solid circles). Highest levels of expression were observed during the mid-log phase and declined rapidly to basal levels before the onset of stationary phase. These results indicate that *exuT–lux* expression is induced by GalUA in wild-type cells. The expression profile of the *exuT–lux* fusion was different in the *exuR* strain compared with the wild type. Luciferase activity of the uninduced *exuR* mutant (Fig. 3) reached levels comparable to the induced wild-type cultures. The peak value was slightly higher than the wild-type cells grown in GalUA, and *exuT–lux* expression remained relatively high, even in stationary phase cultures. This suggests that *exuT–lux* expression is deregulated in the *exuR* mutant. The expression profile of *exuT* transcription in the *exuR* mutant in the presence of GalUA (Fig. 3) was unexpected, comprising a distinct "lag phase" before reaching higher levels in early stationary phase cultures. The reason for this effect is unclear and may represent additional controls such as KdgR on this important transport system. Sequence analysis of the 5′ *exuT* region indicated the presence of a conserved KdgR binding box (Haseloff et al. 1998). How this protein or any others interact in *exuT* expression can only be speculated at this time.

The relevance of *exuR* during potato tuber infections was studied by monitoring *exuT–lux* expression in wild-type and *exuR* strains. Cored potato cubes were inoculated with approximately 10^7 bacterial cells and incubated in scintillation vials in a humid environment at 30°C. Luciferase activity was monitored at regular intervals in a scintillation counter. The strain bearing the *exuR* mutation resulted in an earlier onset of maceration symptoms compared to the wild type, and luciferase activity in the *exuR* strain increased rapidly and reached higher levels (approximately 1.5 fold) than the wild-type strain (data not shown). Thus, the *exuR* mutation appears to influence the rate and extent of potato tissue maceration. Observations also were made to assess the temporal expression pattern of *exuT* during plant tissue maceration with potato tuber tissue. Transcription of *exuT* increased rapidly over the first 5 h, peaked at 7 h, and declined to basal levels at 34 h postinoculation (Fig. 4). Interestingly, initial visible symptoms of potato tuber maceration were observed at approximately 10 to 12 h postinoculation, and host tissue was macerated thoroughly at 22 to 34 h postinoculation. Previous studies with a *pelE–lux* fusion in *E. chrysanthemi* inoculated into potato tubers showed that *pelE* transcription increased continually and peaked at approximately 20 h postinoculation (Gold et al. 1992; Shen et al. 1992). Our data strongly suggest that *exuT* transcription is important in the very early stages of the plant–microbe interaction. One possible explanation for the lower levels of *exuT* transcription following visible signs of tissue maceration could be the availability of alternate carbon sources, some of which may catabolite repress transcription of the gene.

To observe the effect of the *exuR* mutation on ExuT transport activity, [^{14}C]-GalUA uptake was studied in the wild-type and *exuR* mutant. [^{14}C]-GalUA uptake activity remained at basal levels in wild-type cells grown under noninducing conditions (Fig. 5) but increased approximately twofold in cells grown in the presence of the inducer. These results are consistent with previous reports indicating inducible GalUA uptake
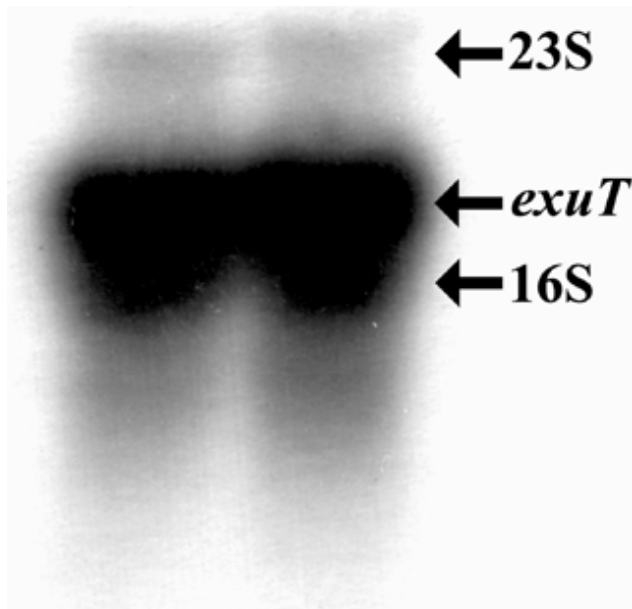

Fig 2. Analysis of *exuT* transcription by Northern blot hybridization. Total RNA isolated from *Erwinia chrysanthemi exuR* mutant grown on M9 salts medium with 0.2% glycerol (uninduced, lane 1) or galacturonic acid (induced, lane 2) was separated on a 1.2% formaldehyde gel. A 1.1-kb *Kpn*I fragment of the *exuT* gene was used as the probe.
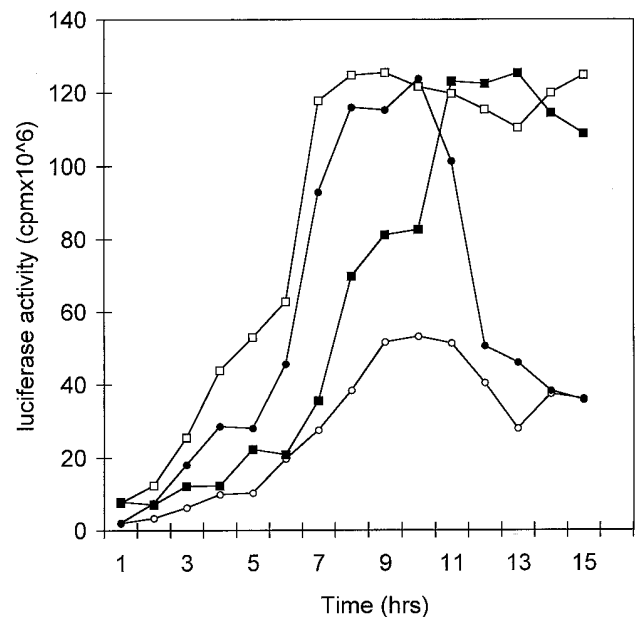

Fig. 3. In vivo luciferase activity of the wild-type and *exuR* strains containing the *exuT–lux* operon fusions. Circles denote the wild-type strain and squares represent the *exuR* mutant. Open and solid symbols represent data obtained from cultures grown in M9 salts with 0.2% glycerol (uninduced) and 0.2% galacturonic acid (induced), respectively. Values represent the average of three replicate treatments. The experiment was repeated three times.
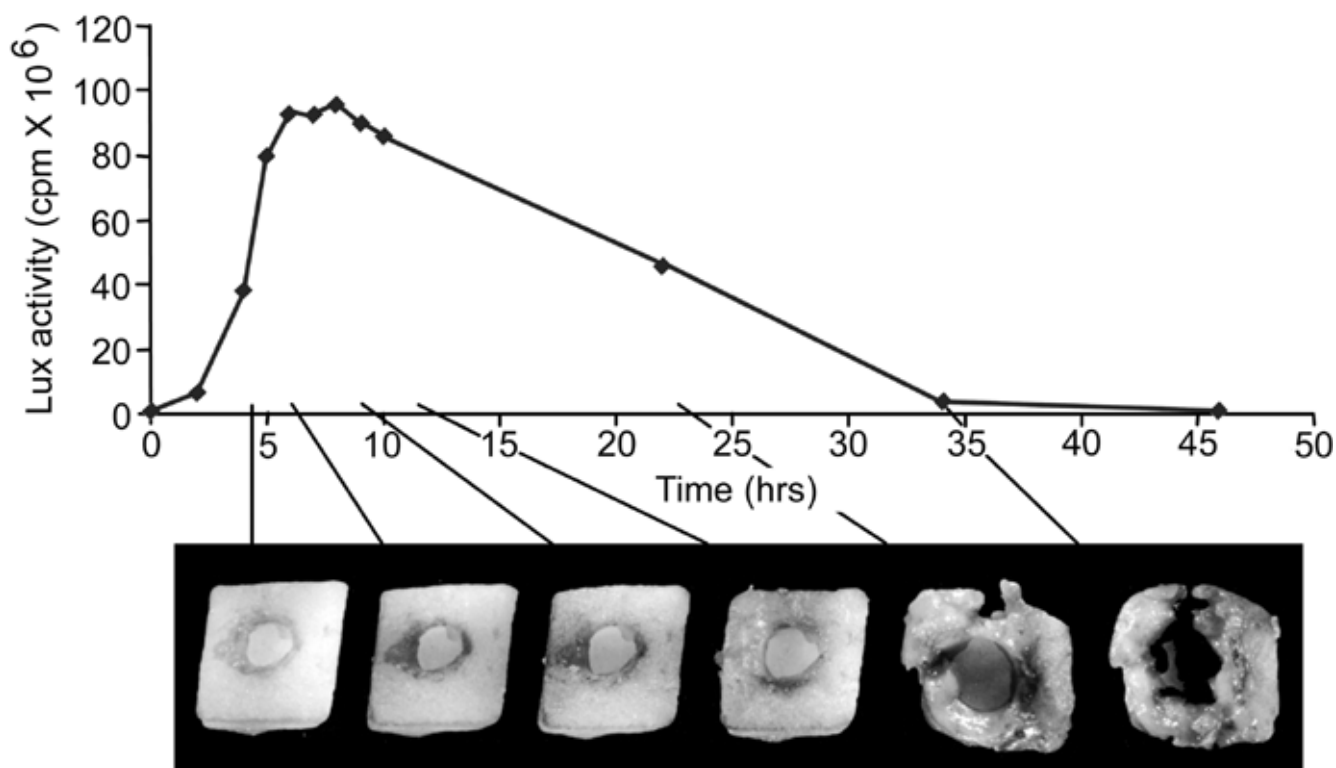
Fig 4. Temporal expression of *exuT* during potato tuber maceration. Bacteria carrying the *exuT–luxAB* fusion were inoculated into cored potato cubes and incubated in a humid environment at 30°C. These data represent an average of 19 replications over the time course. Pictures represent a visualization of the progression of tissue maceration over the time course of the experiment.
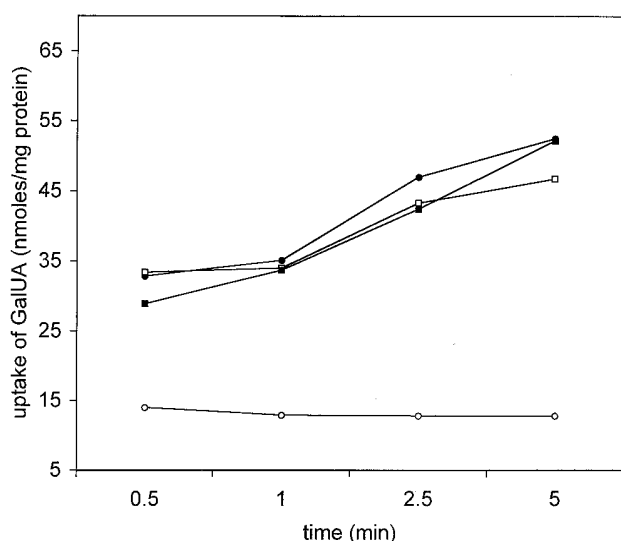

Fig. 5. Uptake of [^{14}C]- galacturonic acid (GalUA) of the wild-type and *exuR* mutant. Circles and squares denote the wild-type and *exuR* mutant, respectively. The cultures were grown in M9 salts with 0.2% glycerol (uninduced) or 0.2% GalUA (induced) and are denoted in the graph by open and closed symbols, respectively. The data points are average (+/− standard error) of three independent experiments.

activity in wild-type *E. chrysanthemi* EC16. In the *exuR* mutant, [^{14}C]-GalUA uptake activity was constitutively high under inducing and noninducing conditions. These results suggest that GalUA uptake is deregulated in the *exuR* mutant,

which indicates a loss-of-repressor function. Results presented in this paper provide convincing evidence that *exuT* expression correlates with the early events of plant–microbe interactions and that ExuR is the major regulator of *exuT* transcription. Studies are under way to identify *exuT* DNA sequences that interact with the repressor, ExuR. Identification of these sequences through DNA mobility shift and footprint analyses will aid in the identification of other genes that may be regulated by ExuR and other members of the GntR family of repressors.

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LITERATURE CITED

Altschul, S. F., Stephen, F., Thomas, L. M., Alejandro, A. S., Zang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Beaulieu, C., and Van Gijsegem, F. 1990. Identification of plant-inducible genes in *Erwinia chrysanthemi* 3937. J. Bacteriol. 172:1569-1575.

Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. 24:383-409.

Freeman, T. L., and San Francisco, M. J. D. 1994. Cloning of a galactu-

ronic acid uptake gene from *Erwinia chrysanthemi* EC16. FEMS Microbiol. Lett. 118:101-106.

Gold, S., Nishio, S., Tsuyumu, S., and Keen, N. T. 1992. Analysis of the *pelE* promoter in *Erwinia chrysanthemi* EC16. Mol. Plant-Microbe Interact. 5:170-178.

Haseloff, B. J., Freeman, T. L., Valmeekam, V., Melkus, M. W., Oner, F., Valachovic, M. S., and San Francisco, M. J. D. 1998. The *exuT* gene of *Erwinia chrysanthemi* EC16: Nucleotide sequence, expression, localization, and relevance of the gene product. Mol. Plant-Microbe Interact. 11:270-276.

Haydon, D. J., and Guest, J. R. 1991. A new family of bacterial regulatory proteins. FEMS Microbiol. Lett. 79:291-296.

Hugouvieux-Cotte-Pattat, N., and Robert-Baudouy, J. 1987. Hexuronate catabolism in *Erwinia chrysanthemi*. J. Bacteriol. 169:1223-1231.

Hugouvieux-Cotte-Pattat, N., Quesneau, Y., and Robert-Baudouy, J. 1983. Aldohexuronate transport system in *Erwinia carotovora*. J. Bacteriol. 154:663-668.

Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., and Reverchon, S. 1996. Regulation of pectinolysis in *Erwinia chrysanthemi*. Annu. Rev. Microbiol. 50:213-257.

Keen, N. T., and Tamaki, S. 1986. Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high level expression in *Escherichia coli*. J. Bacteriol. 168:595-606.

Nemoz, G., Robert-Baudouy, J., and Stoeber, F. 1976. Physiological and genetic regulation of the aldohexuronate transport system. J. Bacteriol. 127:706-718.

Portalier, R., Robert-Boudouy, J., and Stoeber, F. 1980. Regulation of *Escherichia coli* K-12 hexuronate system genes: *exu* regulon. J. Bacteriol. 143:1095-1107.

Preston, J. F., III, Rice, J. D., Ingram, L. O., and Keen, N. T. 1992. Differential depolymerization mechanisms of pectate lyases secreted by *Erwinia chrysanthemi* EC16. J. Bacteriol. 174:2039-2942.

Roeder, D. L., and Collmer, A. 1985. Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. J. Bacteriol. 164:51-56.

San Francisco, M. J. D., and Keenan, R. W. 1993. Uptake of galacturonic acid in *Erwinia chrysanthemi* EC16. J. Bacteriol. 175:4263-4265.

Shen, H., Gold, S. E., Tamaki, S. J., and Keen, N. T. 1992. Construction of a Tn*7*–*lux* system for gene expression studies in Gram-negative bacteria. Gene 122:27-34.

Shine, J., and Dalgarno, L. 1974. 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA 71:1342-1346.