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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

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A novel *Erwinia carotovora* subsp. *carotovora* mutant designated RexZ, (regulator of exoenzymes) showed reduced production of the degradative exoenzymes. The *rexZ* gene product shows similarity to the KdgR regulatory protein from *Erwinia chrysanthemi*, described as the major repressor of the pectin catabolism pathway genes in the latter species. *In vitro* DNA–protein interaction experiments demonstrated that the synthesis of the RexZ protein is controlled by the cAMP–CRP (cAMP–receptor protein) complex. Western blot analysis also revealed the presence of a second KdgR homologue (distinct from RexZ) which, like RexZ, was present in all species of the genus *Erwinia* tested. The corresponding KdgR proteins from both *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* share a high level of sequence identity with the KdgR homologues from *E. chrysanthemi* and *Escherichia coli*. Although the *E. carotovora* subsp. *carotovora rexZ* regulatory region displayed specific interactions with both the purified *E. chrysanthemi* KdgR repressor and the partially purified *E. carotovora* subsp. *carotovora* KdgR, *in vivo* quantification revealed that the cellular level of RexZ protein was unaffected by the presence of pectic compounds. This study shows that the complex regulatory network governing virulence in the erwinias involves two totally distinct, but highly conserved, members of the IclR class of DNA binding proteins: RexZ and KdgR.

Keywords: phytopathogenicity, soft-rot, exoenzyme, transcriptional regulation

INTRODUCTION

The phytopathogenicity of several *Erwinia* species is correlated with the ability to produce and secrete plant cell wall degrading enzymes such as pectinases, cellulases (Cel) and proteases (Prt) (Collmer & Keen, 1986; Barras *et al*., 1994). The crucial role of these enzymes, particularly the pectate lyases (Pel), in the virulence of soft-rot *Erwinia* has been confirmed by the isolation of mutants exhibiting reduced virulence that are defective for production or secretion of these enzymes (Boccara *et al*., 1988; Hinton *et al*., 1989; Pirhonen *et al*., 1991). Exoenzyme production by soft-rot *Erwinia* species responds to several environmental conditions (Hugouvieux-Cotte-Pattat *et al*., 1996): presence of pectin-degradative products or plant extract, anaerobiosis, temperature, nitrogen starvation, osmolarity, catabolite repression, iron availability and growth phase.

In *Erwinia chrysanthemi* 3937, attention has been focused on the pectin degradation pathway, and the different steps of this metabolic pathway have been

Abbreviations: CRP, cyclic AMP receptor protein; KDG, 2-keto-3-deoxygluconate; OHHL, *N*-(3-oxohexanoyl)-L-homoserine lactone; PGA, polygalacturonate.

The GenBank accession numbers for the sequences reported in this paper are given in the text.

characterized. The degradation of the pectic compounds is initiated by extracellular pectinases, including two pectin methylesterases (encoded by *pemA* and *pemB*) (Laurent *et al*., 1993; Shevchik *et al*., 1996), five major isoenzymes of pectate lyases (encoded by *pelA*, *pelB*, *pelC*, *pelD* and *pelE*) and a set of secondary pectate lyases (Hugouvieux-Cotte-Pattat *et al*., 1996) which generate unsaturated digalacturonate. These latter compounds are transported into the bacterium, where they are catabolized by the products of the genes *ogl*, *kduI*, *kduD*, *kdgK* and *kdgA* (Condemine *et al*., 1986; Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1987; Reverchon & Robert-Baudouy, 1987). It has been demonstrated that the full expression of the pectin catabolism genes in *E. chrysanthemi* 3937 requires the presence of the cAMP–CRP (cAMP–receptor protein) complex (Reverchon *et al*., 1997; Nasser *et al*., 1997) and that the KdgR repressor essentially mediates the induction of this catabolic pathway in response to pectic compounds (Reverchon *et al*., 1991). *In vitro* experiments showed that the specific binding of the KdgR repressor to the operators of genes it regulates is inhibited in the presence of the pectin catabolic product 2-keto-3-deoxygluconate (KDG). Thus, it was proposed that KDG is the real intracellular inducer of the pectinolysis genes (Nasser *et al*., 1991, 1994). In addition to *kdgR*, two other loci that negatively regulate the expression of the pectinase genes, *pecS–pecM* and *pecT*, have also been characterized in *E. chrysanthemi*. However, the signal to which they respond remains unknown (Reverchon *et al*., 1994; Praillet *et al*., 1996; Surgey *et al*., 1996; Castillo & Reverchon, 1997).

In the related soft-rot species, *Erwinia carotovora* subsp. *carotovora*, analysis of regulatory mutants has allowed the identification of several loci involved in the regulation of exoenzyme genes: *expI/carI/hslI*, *hor*, *aepA*, *aepH* (*rsmB*), *rsmA* and *hexA*. The gene *expI/carI/hslI*, a homologue of the *Vibrio fischeri luxI* gene, directs the production of an autoinducer molecule identical to the one synthesized by LuxI and called *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) (Jones *et al*., 1993; Pirhonen *et al*., 1993; Salmond *et al*., 1995; Chatterjee *et al*., 1995). OHHL regulates the synthesis of Pel, polygalacturonase (Peh), Cel and Prt and the production of the antibiotic carbapenem in *E. carotovora* subsp. *carotovora* (Jones *et al*., 1993; Pirhonen *et al*., 1993). Similarly, the Hor protein has also been found to regulate exoenzyme and antibiotic production in *E. carotovora* (Thomson *et al*., 1997). Mutations in the genes *aepA* and *aepH* (*rsmB*) have been shown to affect extracellular enzyme synthesis (Murata *et al*., 1991; Liu *et al*., 1993). AepA activates the transcription of *pel-1*, which encodes the major Pel in *E. carotovora* subsp. *carotovora* strain 71 (Liu *et al*., 1993; Murata *et al*., 1994), and the RNA transcript of *aepH* (*rsmB*) activates the synthesis of Pel, Peh, Cel and Prt by way of its interaction with RsmA (Liu *et al*., 1998). The negative regulatory gene, *rsmA*, when carried on a multicopy plasmid has been shown to suppress the production of the depolymerizing enzymes, the synthesis of OHHL and the extent of plant pathogenicity in several soft-rotting *Erwinia* spp. (Cui *et al*., 1995). Although the number of regulatory inputs governing exoenzyme production is extensive, few regulators common to both *E. chrysanthemi* and *E. carotovora* have been described to date. Even where a common regulator has been reported, such as HexA (*E. carotovora*)/PecT (*E. chrysanthemi*), its regulatory effects are not the same in both species (Harris *et al*., 1998).

In this paper, we describe the identification, cloning and characterization of an *E. carotovora* regulatory gene, *rexZ*, the product of which displays homology with the *E. chrysanthemi* KdgR repressor protein. However, unlike KdgR, RexZ acts as an activator of exoenzyme production and therefore virulence. We describe the construction of an *E. carotovora rexZ* mutant by reverse genetics and the analysis of the *E. carotovora rexZ* mutant phenotype with respect to synthesis of depolymerizing enzymes. In addition, the modulation of RexZ protein synthesis, in *E. carotovora*, was also investigated. This study also shows that *E. carotovora* contains a homologue of the *E. chrysanthemi* KdgR in addition to the novel RexZ protein; both are members of the IclR class of DNA-binding proteins. Furthermore, sequence analysis of KdgR homologues, isolated from *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*, showed that the N-terminus of these proteins is different from that previously reported for the *E. chrysanthemi* KdgR protein, but agreed well with the *Escherichia coli* KdgR protein sequence. Finally, the functional interchangeability of the *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* KdgR virulence repressors was investigated.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids are described in Table 1. *Erwinia* spp. and *Escherichia coli* were grown at 30 °C and 37 °C respectively, in LB medium or M63 minimal medium (Miller, 1972) supplemented with a carbon source (0·2%, except PGA and pectin, 0·4%) and, when required, with amino acids (40 μg ml^{-1}). Antibiotics were used at the following final concentrations: streptomycin (Sm) and ampicillin (Ap), 100 μg ml^{-1}; kanamycin (Km) and chloramphenicol (Cm), 50 μg ml^{-1}; tetracycline (Tc), 20 μg ml^{-1}.

Exoenzyme liquid and plate assays. Pectinase and cellulase activities were detected using plate assays as described by Andro *et al*. (1984). Protease activity was determined using the plate assay of Hankin & Anagnostakis (1975). Samples of overnight culture to be tested were spotted onto the appropriate enzyme assay plate and incubated at 30 °C for 24 h. Liquid enzyme assays of cellulase and protease activity were performed as described previously (Hinton & Salmond, 1987). Total specific activity, expressed as ΔA_{550} mg^{-1} min^{-1} ml^{-1} and ΔA_{436} mg^{-1} min^{-1} ml^{-1}, for cellulase and protease respectively, was determined. Liquid assays of pectate lyase activity were performed on toluenized cell extracts as described previously (Moran *et al*., 1968).

Recombinant DNA techniques. All molecular biological techniques have been described previously (Ausubel *et al*., 1987), unless otherwise stated. Nucleotide sequence analysis of *rexZ*

was performed by the chain-termination method (Sanger *et al*., 1977) on random sonicated templates cloned into the M13 vector mp18 or mp19. PCR (using *Taq* DNA polymerase, NEB; annealing temperature 43 °C) was used to amplify the *E. carotovora* subsp. *carotovora* SCRI193 *rexZ* gene, using primers EcK1 (CGGGATCCGTTGCGTTATTATTG) and Eck2 (GGTCTAGAAGCAGATGGAGTATTGG), from chromosomal DNA. PCR (using *Taq* DNA polymerase, NEB; annealing temperature 42 °C) was also used to amplify an *E. chrysanthemi kdgR*-specific gene probe using primers EHR1 (ATGGATGATATTAATCGG) and EHR2 (ACGGATAA-TCGTGGTATC) complementary to the terminal 5′ and 3′ sequences of the *kdgR* ORF, respectively. For Southern blots the *kdgR*-specific gene probe was labelled using the 'non-radioactive' DIG-11-dUTP kit (Boehringer Mannheim).

The *E. carotovora* subsp. *carotovora* SCRI193 mini-library was constructed in pACYC184 (Chang & Cohen, 1978). Chromosomal DNA was prepared and digested with *Eco*RV, before being size-fractionated by agarose gel electrophoresis. Size-fractionated DNA of between 4 kb and 7·5 kb was recovered from the agarose gel using a Geneclean Kit (BIO 101) and ligated into pACYC184, also digested with *Eco*RV. The ligated products were used to transform *Escherichia coli* DH1 by electroporation. Transformants were selected on nutrient media supplemented with Cm. The *kdgR* gene homologue of *E. carotovora* subsp. *carotovora* SCRI193 was sequenced using universal forward and reverse M13 primers (Pharmacia). Additional primers were designed to complete the sequencing: KD1 (GCTCAGGCATTGATAGGG), KD3 (CACATTTTATGGCGCGG) and KD5 (CGGGATCCTG-TGGACTATCTTACC). The *kdgR* gene homologues from *E. carotovora* subsp. *carotovora* strains F14 and F148, and *E. carotovora* subsp. *atroseptica* strain SCRI27, were sequenced using the pBluescript primers T3 and T7 (Stratagene). The nucleotide sequence of the *kdgR* homologues reported in this study was generated on an Applied Biosystems 373 automated sequencing machine using *Taq* FS (Perkin-Elmer). Nucleotide sequence data were analysed using the MAC MOLLY TETRA program (SoftGene, Berlin) or on BLAST, searching the GenBank/EMBL and SWISS-PROT databases.

Marker-exchange mutagenesis of the *rexZ* gene. A 2·8 kb *Bam*HI fragment of pMO11, carrying *rexZ*, was cloned in pKNG101 (Kaniga *et al*., 1991) also digested with *Bam*HI, to yield pSMG38. The *rexZ* gene was insertionally inactivated by the cloning of a 1266 bp *Nla*IV fragment of pACYC177 (Chang & Cohen, 1978), encoding resistance to kanamycin, into pSMG38 at the unique *Sca*I site. The resulting plasmid, pSMG39, was used to marker-exchange *E. carotovora* subsp. *carotovora* ATTn*10* as previously described (Kaniga *et al*., 1991).

Purification of the *E. carotovora* subsp. *carotovora* KdgR protein for N-terminal sequencing. Using the pUC19 −48 reverse oligonucleotide primer (NEB) and primer KD6 (CCCAAGCTTCAGTGATGGTGATGGTGATGGAAAGG-GTAATCGTGGTAGC) the *E. carotovora* subsp. *carotovora kdgR* gene was amplified by PCR from plasmid pREP4. Primer KD6 was designed such that six codons encoding histidine residues were incorporated into the 3′ end of the *kdgR* ORF. The resultant amplified PCR product was digested with *Hin*dIII and ligated into pUC19, digested with the same enzyme, and used to transform *Escherichia coli* DH1. Transformants were selected on nutrient media supplemented with Ap. The KdgR-His tagged protein was purified from cultures grown to early stationary phase using Ni-NTA resin (Qiagen) as described in the manufacturer's instructions. Purified protein, after SDS-PAGE, was blotted on to PVDF membrane (Boehringer Mannheim) and visualized using Ponceau-S stain (Sigma). N-terminal sequencing was performed on an Applied Biosystems automated peptide sequencer.

Preparation of *E. carotovora* subsp. *carotovora* cell extract for gel retardation assays. Cells from a 1 litre culture of *E. carotovora* subsp. *carotovora* SCRI193 were pelleted, washed and resuspended in 50 ml extraction buffer (10 mM HEPES/NaOH pH 7·9, 4 mM Tris/HCl pH 7·9, 1 mM EDTA, 5 % glycerol, 1 mM PMSF, 1 mM DTT). Crude protein extract was obtained by disrupting bacteria at 13·8 MPa in a French press (AMINCO). Crude extract was then centrifuged to remove cell debris and the supernatant was submitted to fractionated precipitation with ammonium sulphate using: 0–20, 20–40, 40–70 and 70–100 % saturation. The precipitated protein fractions were resuspended in a small volume of extraction buffer and dialysed against the same buffer to remove any residual ammonium sulphate. The 20–40 % ammonium sulphate saturation protein fraction was identified, by Western blot analysis, as containing KdgR. This fraction constituted the partially purified KdgR fraction used for band-shift assays.

Preparation of operator fragments for binding studies. The regulatory regions of *rexZ* and the *E. chrysanthemi pelE* genes were cloned into pBluescript (Table 1) and end-labelled as previously described (Nasser *et al*., 1997). These labelled fragments were further purified with the Qiagen quick extraction kit.

Gel retardation assays and footprinting with DNase I. These assays were performed as previously described (Nasser *et al*., 1997). Gel retardation assays were conducted using various amounts of either crude protein extract (2–20 μg) or purified CRP or KdgR protein.

Production of RexZ in *Escherichia coli* and generation of RexZ-specific antibodies. The *rexZ* ORF was amplified by PCR using primers which introduced a unique *Nde*I restriction site at the ATG initiation codon and an *Eco*RI site after the stop codon. By cloning the 798 bp amplified *Nde*I–*Eco*RI fragment into the pT7-7 vector, to yield pWS2, the *rexZ* ATG start codon was fused to the T7 promoter. Expression of *rexZ* was performed using the methods described by Tabor & Richardson (1985). Crude protein extract was obtained by disrupting bacteria at 13·8 MPa in a French pressure cell (AMINCO). Cell debris and insoluble material was recovered by centrifugation at 20000 ***g*** for 20 min. The expressed RexZ protein contained within the resultant pellet was further purified by preparative SDS-PAGE. Gel fragments containing RexZ were excised, crushed, suspended in phosphate-buffered saline (10 mM Na_2HPO_4, 1·76 mM KH_2PO_4, 138 mM NaCl, 2·7 mM KCl) and injected subcutaneously into a New Zealand White rabbit. KdgR-specific antibodies were also generated, in a similar manner, using purified KdgR protein (Nasser *et al*., 1992). The anti-RexZ and anti-KdgR antibodies were purified from rabbit antisera, as previously described (Praillet *et al*., 1996; Sakakibara *et al*., 1991). The recovered antisera reacted specifically with either RexZ or KdgR.

Immunoblotting. After separation on a 12 % SDS-PAGE gel, proteins were electrotransferred onto a Hybond-C membrane using a 1017 Macrophor system (Pharmacia LKB). The membranes were washed and challenged with anti-RexZ or anti-KdgR antibodies diluted to 1/200 and 1/500, respectively,

Table 1. Bacterial strains and plasmids

Strain	Genotype or description*	Source or reference
Escherichia coli		
NM522	Δ(*lac–proAB*) *thi hsd5 supE* [F′ *proAB*$^+$ *lacI*^{q}*lacZ*ΔM15]	Stratagene
K38	HfrC (*λ*)*pho*A*4 pit-10 tonA22 ompF627 relA1*	Russel & Model (1984)
DH1	F$^-$ *recA1 endA1 thi-1 hsdR17 gyrA*96 ($r_k^- m_k^-$) *supE44 relA1*	Hanahan (1983)
CC118	Δ(*ara–leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(am)*recA1*	Herrero *et al.* (1990)
CC118(*λpir*)	As CC118, lysogenised with *λpir* phage	Herrero *et al.* (1990)
***Erwinia* spp.**		
Erwinia chrysanthemi		
3937	Wild-type strain isolated from *Saintpaulia ionanthia*	Kotoujansky *et al.* (1982)
A903	*lmrT*^{c} *lacZ2 kdgR*::Tn*5*	Condemine & Robert-Baudouy (1987)
EC16	Wild-type	A. Chatterjee, Univ. of Missouri
E. carotovora subsp. *carotovora*		
SCRI193	Wild-type	Hinton & Salmond (1987)
ATTn*10*	Wild-type, restrictionless	McGowan *et al.* (1996)
SM2	ATTn*10*, *rexZ*::*kan*	This study
PNP22	ATTn*10*, CarI$^-$	McGowan *et al.* (1995)
F14	Wild-type	General lab stock
F148	Wild-type	General lab stock
E. carotovora subsp. *atroseptica*		
SCRI31	Wild-type	Hinton *et al.* (1985)
SCRI27	Wild-type	M. Perombelon, Scottish Crop Research Institute
E. amylovora		
SCRI449	Wild-type	M. Perombelon, Scottish Crop Research Institute
E. herbicola		
SCRI424	Wild-type	M. Perombelon, Scottish Crop Research Institute
Plasmids		
pUC19	Cloning vector, Ap^r *lacZ′*	Yanisch-Perron *et al.* (1985)
cWU142	pSF6 harbouring the carbapenem biosynthetic cluster, *kduI*, *kdgT* and *rexZ*	McGowan *et al.* (1995)
pMO11	pUC19 derivative harbouring *rexZ* of *E. carotovora* subsp. *carotovora* ATTn*10*	McGowan *et al.* (1997)
pBluescript	Ap^r *lacZ′*	Stratagene
pSR1175	pBluescript harbouring the *pelE* regulatory region of *E. chrysanthemi* 3937	Nasser *et al.* (1994)
pWS1	pBluescript harbouring the *rexZ* regulatory region of *E. carotovora* subsp. *carotovora* ATTn*10*	This work
pACYC177	Cloning vector, Ap^r Kn^r	Chang & Cohen (1978)
pKNG101	*sacB* marker-exchange vector, Str^r	Kaniga *et al.* (1991)
pSMG38	pKNG101 derivative harbouring *rexZ* of *E. carotovora* subsp. *carotovora* ATTn*10*	This work
pSMG39	pKNG101 derivative harbouring *rexZ*::*kan* insertion	This work
pT7-7	Ap^r	Tabor & Richardson (1985)
pWS2	pT7-7 harbouring the coding region of *rexZ*	This work
pULB113	RP4::Mu3A	Van Gijsegem & Toussaint (1982)
pROU2	pULB113 derivative harbouring *kduD*, *kdgR* and *ogl* genes of *E. chrysanthemi* 3937	Reverchon & Robert-Baudouy (1987)

Table 1 *(cont.)*

Strain	Genotype or description*	Source or reference
pREP1	pACYC184 harbouring the *E. carotovora* subsp. *carotovora* SCRI193 *kdgR*	This work
pREP4	pUC19 harbouring a 2·1 kb *Eco*RV–*Sph*I subclone of pREP1	This work
pREP27	pBluescript harbouring the *E. carotovora* subsp. *atroseptica* SCRI27 *kdgR*	This work
pREP14	pBluescript harbouring the *E. carotovora* subsp. *carotovora* F14 *kdgR*	This work
pREP148	pBluescript harbouring the *E. carotovora* subsp. *carotovora* F148 *kdgR*	This work

* Genotype symbols are according to Bachmann (Basham & Bateman, 1975). *lmrT*^{c} indicates that the transport system encoded by *lmrT*, which mediates entry of lactose, melibiose and raffinose into the cells, is constitutively expressed. *lacZ′* indicates that 3′ end of this gene is truncated.

in T-TBS containing 1% gelatin as previously described (Praillet *et al*., 1996). Membranes were developed using the ECL kit (Amersham), according to the manufacturer's instructions.

RESULTS

Sequence analysis

During analysis of the *E. carotovora* subsp. *carotovora* strain ATTn*10* genes responsible for production of the *β*-lactam antibiotic carbapenem, a partial ORF was identified downstream from these *car* genes and transcribed on the opposite strand (McGowan *et al*., 1996). This ORF potentially encoded the C-terminus of a protein with homology to the KduI protein of *E. chrysanthemi*. It was decided to extend the sequence in the region, in order to identify the 5′ end of this gene, on the cosmid cWU142.

Following restriction mapping of cWU142, to identify *Eco*RI fragments contiguous with the 3′ end of the carbapenem biosynthetic cluster, two fragments were cloned in pUC19 (Yanisch-Perron *et al*., 1985). These fragments were either cloned directly in M13mp18 or were sonicated and the resulting fragments ligated into M13mp19. Analysis of the resulting subclones by the dideoxynucleotide chain-termination method (Sanger *et al*., 1977) generated 3080 bp of DNA sequence. Part of this sequence has already been reported (McGowan *et al*., 1996) and therefore the novel sequence reported here will be lodged with GenBank under the already existing accession number, U17224.

Upon translation of the DNA sequence generated, several ORFs were identified. The previously recognized partial ORF was completed. It was predicted to encode a protein of 276 amino acids, with a molecular mass of 31071 Da, which presents high homology with the 5-keto-4-deoxyuronate isomerase (KduI) of *E. chrysanthemi* (65% aa identity) and with two proteins of *Escherichia coli* and *Bacillus subtilis* (66% and 47% aa identity, respectively). Accordingly we have designated this gene *kduI*. Two other ORFs were identified. Based on homologies with previously described genes, one of these ORFs was designated *kdgT*. *kdgT* is predicted to encode a protein of 318 amino acids with a molecular mass of 32581 Da which shares 54% and 74% aa identity with the 2-keto-3-deoxygluconate transporter of *B. subtilis* and *Escherichia coli*, respectively. The predicted *E. carotovora* subsp. *carotovora* KdgT protein also displayed high homology with 318 out of 398 residues of the *E. chrysanthemi* KdgT protein. The N-terminus of the *E. chrysanthemi* KdgT protein is significantly longer than all those of the known KdgT homologues (data not shown). In the absence of experimental data therefore, it is possible that the previously assigned translational start site of the *E. chrysanthemi kdgT* ORF is incorrect and that its true start site lies at a position analogous to that in the other previously reported *kdgT* homologues.

The third ORF whose predicted product has homology with the KdgR protein of *E. chrysanthemi* was originally referred to as unpublished data in a previous paper (Salmond *et al*., 1994). However, on completion of the sequence and the analysis of an allelic exchange mutant defective in this ORF it was renamed *rexZ* (*r*egulator of *ex*oenzymes) due to its unexpected phenotype (see following section).

The *rexZ* gene is predicted to encode a protein of 262 amino acids with a molecular mass of 29516 Da. Using oligonucleotide primers (Eck1 and Eck2) complementary to the terminal 5′ and 3′ sequences, respectively, of the *E. carotovora* subsp. *carotovora* strain ATTn*10* *rexZ* gene, we also amplified the *rexZ* gene from the Car^{-} *E. carotovora* subsp. *carotovora* strain SCRI193 for comparison. The *E. carotovora* subsp. *carotovora* SCRI193 *rexZ* was almost identical to that of strain ATTn*10* (Fig. 1). Database searches revealed that the *E. carotovora* RexZ protein presents some homology with proteins from *E. chrysanthemi*, *B. subtilis* and *Es-*

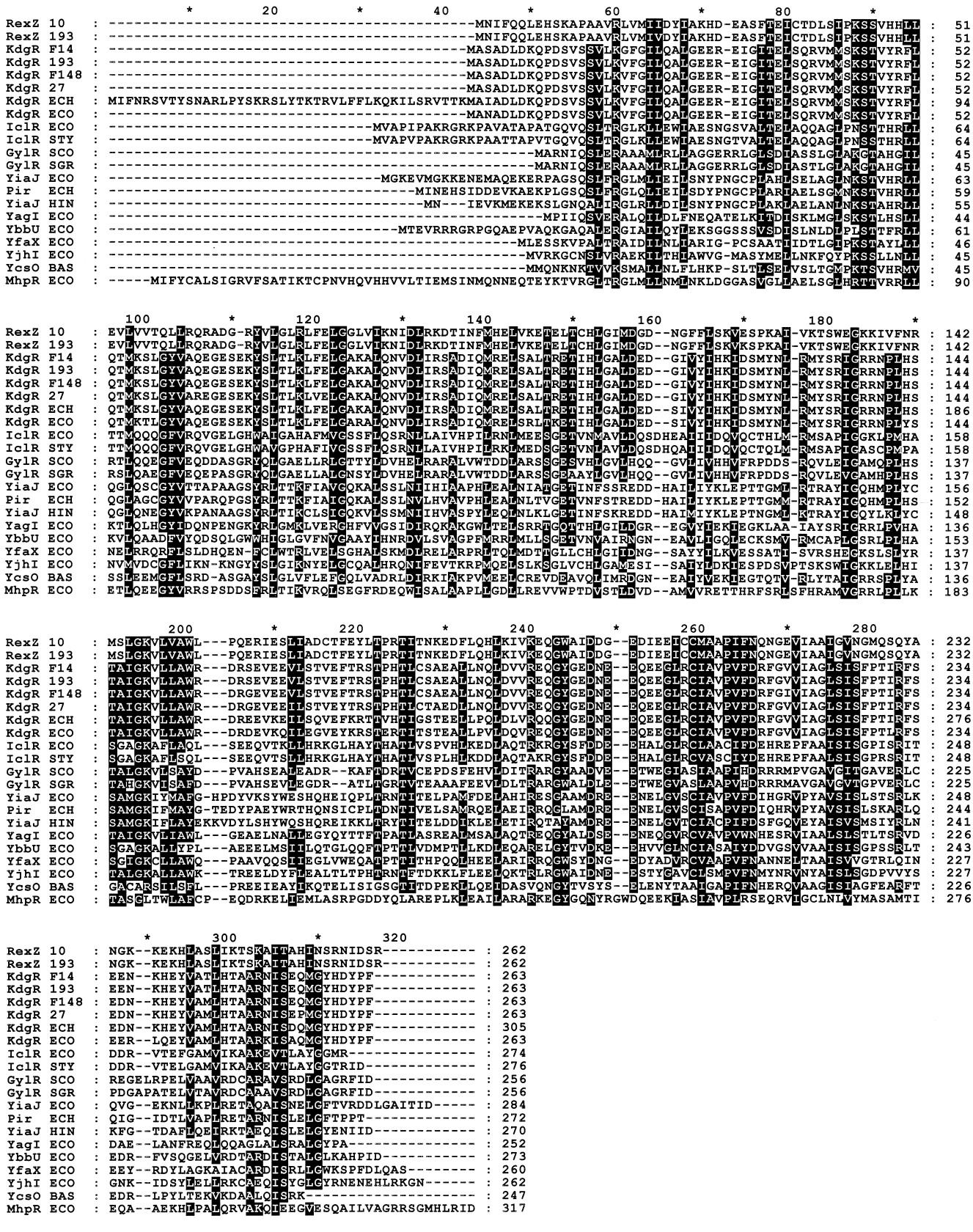

Fig. 1. Alignment of the predicted amino acid sequences of the RexZ and KdgR homologues characterized in this study from the following strains: *E. carotovora* subsp. *carotovora* strains ATTn*10* (**10**; accession number U17224), SCRI193 (**193**; accession numbers AF135394 and AF135787), F14 (**F14**; accession number AF135395) and F148 (**F148**; accession number AF135396); *E. carotovora* subsp. *atroseptica* strain SCRI27 (**27**; accession number AF135397). The above sequences are

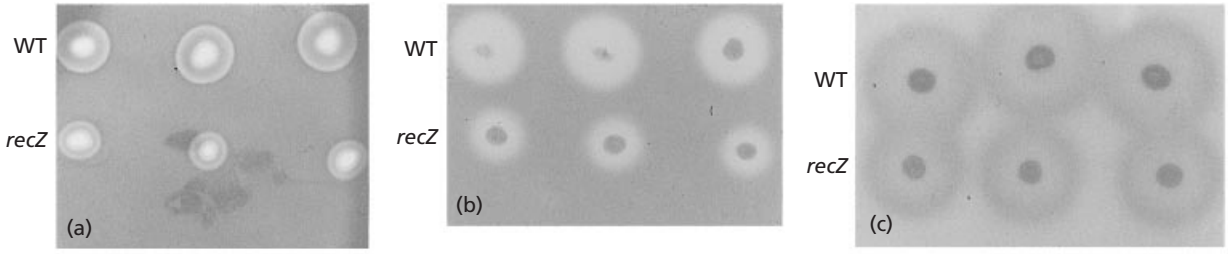

Fig. 2. Phenotypic analysis of the *Erwinia carotovora* RexZ$^-$ marker-exchange mutant. Cultures of the wild-type *Erwinia carotovora* strain ATTn*10* (WT) and *Erwinia carotovora rexZ*::*kan* strain SM2 (*rexZ*) were spotted onto assay plates for the production of cellulase (a), protease (b) and pectate lyase (c). The production of exoenzymes on these assay plates is indicated by the presence of a halo.

cherichia coli, all of which are members of the IclR family of DNA-binding proteins (Fig. 1; Sunnarborg *et al*., 1990). The homologous *E. chrysanthemi* protein is the major repressor of genes involved in pectinolysis, KdgR. As previously reported for *E. chrysanthemi* KdgR, the *E. carotovora* RexZ possesses a possible helix–turn–helix, DNA-binding motif in the N-terminus and a conserved region in the C-terminus that corresponds to the signature pattern for the IclR family of proteins: [GA]-X_3-[DS]-X_2-E-X_6-[CSA]-[LIVM]-[GSA]-X_2-[LIVM]-[FYH]-[DN] (Reverchon *et al*., 1991; Sofia *et al*., 1994). The putative helix–turn–helix and IclR family signature motifs are located in RexZ between residues 33–52 (FTEICTDLSIPKSSVHHLLE) and 192–213 (GWAIDDGEDIEEICCMAAPIFN), respectively. Since the helix–turn–helix motifs are known to be involved in protein–DNA interactions (Harrison & Aggarwal, 1990), it was reasonable to assume that RexZ is a DNA-binding protein.

Construction and characterization of an *E. carotovora rexZ* null mutant strain

In contrast with the homologies between the KduI and KdgT proteins from *E. chrysanthemi* and *E. carotovora*, which are high, the homology between the *E. carotovora* RexZ protein and the *E. chrysanthemi* KdgR repressor is low (26% identity; Fig. 1). Given this low homology, and the wide spectrum of physiological roles that other members of the IclR protein family perform (Reverchon *et al*., 1991; Smith & Chater, 1988; Sunnarborg *et al*., 1990), we considered it imperative to determine the phenotype of a *rexZ* null mutant. Southern blot analysis of subclones of the carbapenem biosynthetic cosmid cWU142 (McGowan *et al*., 1995) indicated that plasmid pMO11 (McGowan *et al*., 1997) carried *rexZ* (data not shown). The 2·8 kb of chromosomal DNA insert carried by pMO11 was cloned in pKNG101 and used to mutate the chromosomal *rexZ* gene by marker exchange (see Methods). Southern blot analysis of the chromosomal DNA of several of the resulting exconjugants was carried out to confirm that marker exchange of the wild-type gene had taken place with fidelity (data not shown). The *rexZ*::*kan* marker-exchange mutant strain SM2 was used for all further analysis.

Comparison of the production of virulence-associated factors in the *E. carotovora rexZ* mutant strain SM2 and in the wild-type strain allowed us to assess the function of *rexZ*. Plate assays revealed that pectate lyase, cellulase and protease activity was reduced in the mutant compared to the parental strain (Fig. 2). These results suggest that the *E. carotovora* RexZ protein acts as an activator of exoenzyme synthesis. The more sensitive liquid enzyme assays revealed that the production of cellulase and protease was reduced by 86% and 63%, respectively, in a RexZ$^-$ mutant when compared to the wild-type *E. carotovora* strain ATTn*10* (data not shown). Liquid enzyme assays also showed that the pectate lyase activity was reduced fourfold in the mutant either in the absence or in the presence of PGA (Table 2) when compared to the wild-type strain ATTn*10*. This unexpected result demonstrated that, although RexZ and the KdgR proteins share sequence

aligned with the other members of the IclR family of regulatory proteins from the following species: *Escherichia coli* (**ECO**) – KdgR (transcriptional regulator of *kdgK* and *kdgT*; accession number P76268), YjhI (hypothetical protein; accession number P39360), YagI (hypothetical protein; accession number P77300), YfaX (hypothetical protein; accession number P77732), IclR (repressor of the acetate operon; accession number P16528), YiaJ (hypothetical protein; accession number P37671), YbbU (hypothetical protein; accession number P77734), MhpR (activator of the 3-hydroxyphenylpropionate degradation pathway; accession number P77569); *Erwinia chrysanthemi* (**ECH**) – KdgR (regulator of pectinolysis; accession number X62072), Pir (regulator of virulence; accession number AB017637); *Bacillus subtilis* (**BAS**) – YcsO (hypothetical protein; accession number P42968); *Salmonella typhimurium* (**STY**) – IclR (repressor of the acetate operon; accession number P17430); *Haemophilus influenzae* (**HIN**) – YiaJ (hypothetical protein; accession number P44996); *Streptomyces griseus* (**SGR**) – GylR (regulator of the glycerol operon; accession number P22866); and *Streptomyces coelicolor* (**SCO**) – GylR (regulator of the glycerol operon; accession number P15360). Black shading has been used to indicate positions at which at least 15 out of the 21 residues are either identical or similar, as assigned by GeneDoc (version 2.2).

Table 2. Pectate lyase activity under various growth conditions and in different genetic backgrounds of *E. chrysanthemi* (Ech) and *E. carotovora* subsp. *carotovora* (Ecc)

Enzyme assays were performed on cells grown to early stationary phase. Pectate lyase specific activity is expressed as μmol products liberated min^{-1} (mg bacterial dry weight)$^{-1}$.

Inducer	Plasmid	Specific pectate lyase activity in strain:			
		Ecc ATTn*10* (wild-type)	Ecc SM2 (RexZ$^-$)	Ech 3937 (wild-type)	Ech A903 (KdgR$^-$)
–	–	0·15	0·04	0·11	1·80
–	pROU2*	0·04	0·01	0·05	0·30
PGA	–	1·90	0·34	1·60	2·50
PGA	pROU2*	0·76	0·18	0·70	0·85

* pROU2 is a pULB113 derivative harbouring *kdgR* of *E. chrysanthemi*.

identity, they are not functional homologues and that, unlike the *E. chrysanthemi* KdgR, the activity of RexZ is not modulated by pectinolytic intermediates (Table 2; Reverchon *et al.*, 1989; Nasser *et al.*, 1991). Further investigations of the action of RexZ on exoenzyme gene expression were therefore warranted.

Overproduction and purification of RexZ

To investigate whether RexZ indeed interacts with the regulatory regions of the exoenzyme genes, as suggested by the presence of a helix–turn–helix motif in its sequence, we overproduced RexZ using the T7 RNA polymerase system (Tabor & Richardson, 1985). Under the standard conditions, the overproduced RexZ protein precipitated and formed inclusion bodies. Various approaches (chemical treatment, changes in the conditions of growth or induction) used to try to resolubilize the overproduced RexZ protein were unsuccessful. However, the unusual solubility properties of RexZ were exploited to purify the protein on a preparative SDS-PAGE gel. Purified protein was used for the generation of specific anti-RexZ antibodies.

The regulation of expression of *rexZ* by KdgR and CRP

Previous studies have shown that the major regulators of genes involved in pectinolysis in *E. chrysanthemi* are CRP and KdgR (Reverchon *et al.*, 1997; Nasser *et al.*, 1997). Interestingly, computer searches looking for consensus sequences recognized by regulatory proteins characterized in *E. carotovora* or *E. chrysanthemi* revealed the presence of potential binding sites for CRP and KdgR in the promoter region of the *rexZ* gene (Fig. 3a). To establish the precise location of any potential cAMP–CRP and KdgR binding sites in the regulatory region of the *rexZ* gene, DNase I protection experiments were conducted. A single protected region with an average length of approximately 36 and 47 bp was obtained in the presence of cAMP–CRP and KdgR at either subsaturating or saturating concentrations, respectively. These protected regions encompass the predicted CRP-binding site and the KdgR box (Fig. 3a, b). The cAMP–CRP-binding site is centred at position −41·5 with respect to the putative transcription start site of *rexZ*, which is typical for class II CRP-dependent promoters (Fig. 3a). This result suggests that the cAMP–CRP complex could act directly as an activator of the *rexZ* promoter. However, the KdgR-protected region covers the nucleotides between −67 and −110; this makes it unlikely that the binding of KdgR would interfere with the expression of the *rexZ* promoter, and therefore suggests that the *rexZ* promoter is independent of KdgR repressor control. In the absence of a direct effect on *rexZ* expression it was formally possible that, because the binding sites of KdgR and CRP are close together (Fig. 3a), KdgR may affect *rexZ* expression by interfering with the ability of cAMP–CRP to bind DNA and therefore activate gene expression.

To determine whether CRP and KdgR could indeed interact with the regulatory region of *rexZ* in a co-operative, independent or antagonistic way, we performed gel retardation assays using the *E. chrysanthemi* purified CRP and KdgR proteins. Typical results of band shifts obtained in the presence of the KdgR or CRP protein and the *rexZ* regulatory region are shown in Fig. 4(a). Two clearly separate bands, corresponding to free DNA and a DNA–protein complex, were observed when the *rexZ* operator was incubated with either CRP or KdgR, suggesting the existence of a unique CRP-binding site and a unique KdgR box in the *rexZ* regulatory region. The mutual influence of CRP and KdgR on the ability to bind the *rexZ* regulatory region was estimated by using control reactions containing only one of these two proteins. The addition of a subsaturating quantity of KdgR and CRP to a solution containing the *rexZ* promoter fragment resulted in three protein–DNA complexes: two corresponding to the KdgR–DNA and cAMP–CRP–DNA individual complexes and one corresponding to cAMP–CRP–KdgR–DNA complex. At a saturating concentration of

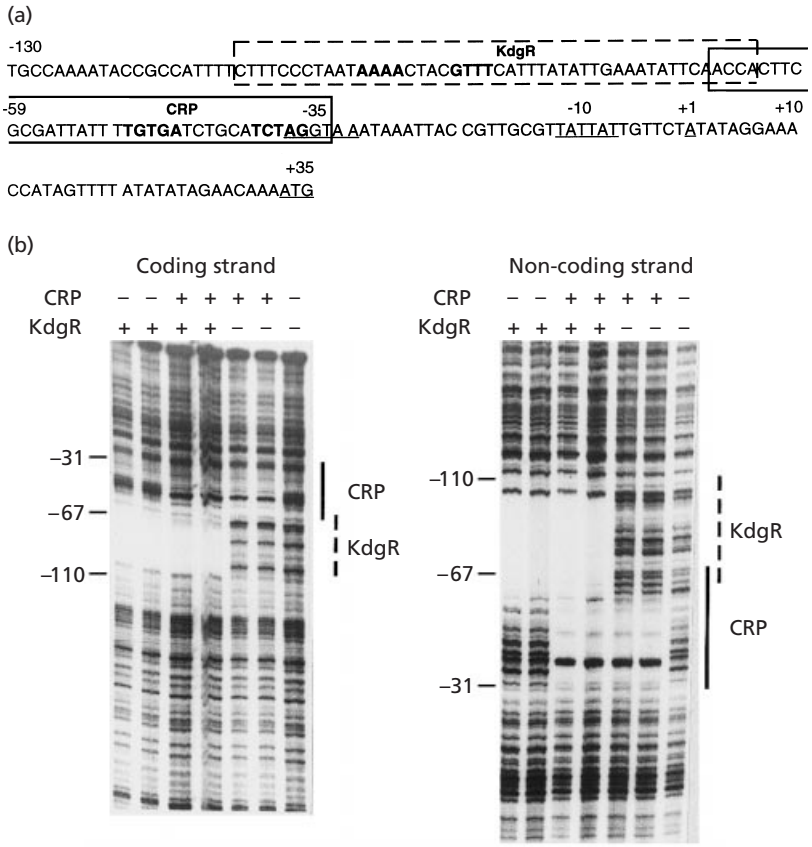

Fig. 3. (a) Organization of the promoter–operator region of *rexZ*. The sequence is numbered from the putative transcription start site, base '**A**', shown underlined at position +1. Regions corresponding to the putative −10 and −35 promoter sites are labelled and underlined. The sequences contained within the broken- and solid-lined boxes represent the binding sites for KdgR and cAMP–CRP, respectively, as defined by DNase I footprinting experiments. The emboldened nucleotides contained within the broken- or solid-lined boxed regions correspond to the sequences that share homology with the consensus binding site for KdgR and cAMP–CRP, which are (AAT[G/A]AAA[C/T])N(N[T/G][G/A]TTT[C/T]A) (Nasser *et al.*, 1994) and AATGTGAN$_6$TCACATT (Kolb *et al.*, 1993), respectively. The putative ATG translation initiation codon has been underlined at position +35. (b) DNase I footprinting of the cAMP–CRP and KdgR binding sites within the promoter–operator region. Purified proteins were used for DNase I footprinting at a final concentration of 100 or 200 nM for KdgR and 50 or 100 mM for CRP.

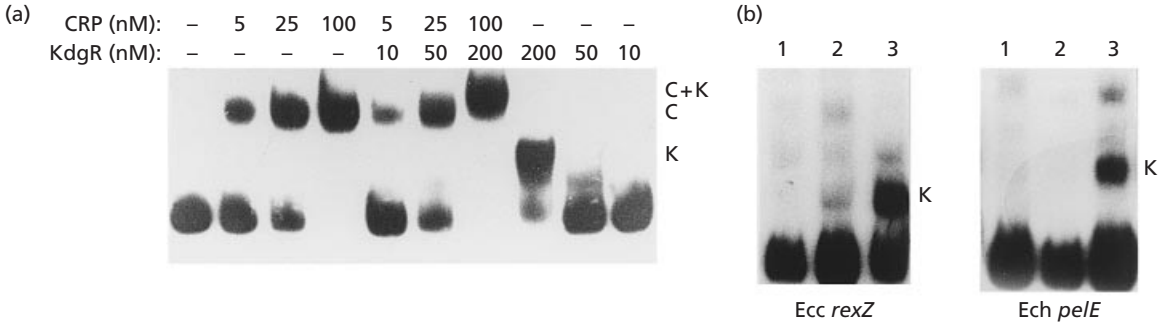

Fig. 4. (a) Analysis of the cAMP–CRP-KdgR (Ech) ternary complex formation with the *rexZ* regulatory region from Ecc. C, K, and C+K correspond to the DNA–protein complexes involving cAMP–CRP, KdgR, and both cAMP–CRP and KdgR, respectively. The concentration of each protein added to the reaction mix is indicated above. (b) Interactions between the Ecc *rexZ* and Ech *pelE* operator-regulatory regions with the partially purified KdgR protein from Ecc strain SCRI193. Twenty micrograms of the Ecc SCRI193 partially purified KdgR protein (contained in the 20–40 % ammonium sulphate saturated fraction) was incubated with the respective operator DNA fragment (lane 3). K corresponds to the DNA–protein complexes involving KdgR. In lanes 1 and 2 the partially purified KdgR protein was first incubated with 4 mM KDG or 1 µl of the anti-KdgR antibodies, respectively, prior to the addition of the operator DNA fragments. Ecc and Ech denote *E. carotovora* subsp. *carotovora* and *E. chrysanthemi*, respectively.

CRP and KdgR proteins, only a ternary complex was observed (Fig. 4a). Simultaneous binding of both regulators did not modify their respective affinity for the *rexZ* operator (data not shown). DNase I footprinting experiments revealed that the KdgR and cAMP–CRP binding sites partially overlap on the *rexZ* promoter–operator region (Fig. 3a, b). This finding suggests that CRP and KdgR are likely to bind to different sides of the DNA helix so that it is possible for them to bind simultaneously on the same stretch of DNA. A similar situation was previously reported for the *E. chrysanthemi pelC* gene (Nasser *et al.*, 1997).

To obtain additional evidence for the possible role of cAMP–CRP as a regulator of *rexZ* expression *in vivo*, quantitative analysis of the RexZ protein was performed

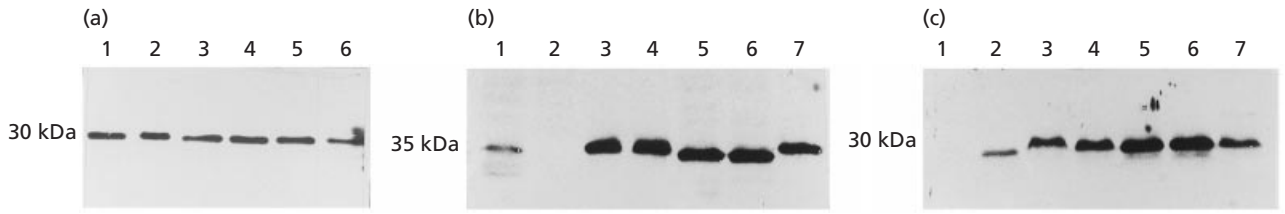

Fig. 5. (a) Quantification of RexZ produced in different genetic backgrounds or from bacteria grown under differing conditions; (b, c) occurrence of KdgR (b) and RexZ (c) homologues in different strains of *Erwinia* spp. Cultures were grown to a cell density of 4×10^8 (a, c) or 1×10^8 (b) cells ml^{-1}. Crude protein extracts, made from these cultures, were immunoblotted, after SDS-PAGE separation, and incubated with KdgR- (b) or RexZ- (a, c) specific antibodies. (a) Lanes 1, 2 and 3, *E. carotovora* subsp. *carotovora* strains SCRI193, ATTn*10* and PNP22 (*carI*$^-$), respectively; lanes 4, 5 and 6, *E. carotovora* subsp. *carotovora* strain ATTn*10* grown in the presence of galacturonate, polygalacturonate and glucose, respectively. (b) Lanes 1 to 7, *E. amylovora* strain SCRI449, *kdgR* mutant *E. chrysanthemi* strain A903, *E. chrysanthemi* strain EC16, *E. chrysanthemi* strain 3937, *E. carotovora* subsp. *carotovora* strain ATTn*10*, *E. carotovora* subsp. *atroseptica* strain SCRI31 and *E. herbicola* strain SCRI424, respectively. (c) Lanes 1 to 7, *rexZ* mutant *E. carotovora* subsp. *carotovora* strain SM2, *E. amylovora* strain SCRI449, *E. chrysanthemi* strain EC16, *E. chrysanthemi* strain 3937, *E. carotovora* subsp. *carotovora* strain ATTn*10*, *E. carotovora* subsp. *atroseptica* strain SCRI31 and *E. herbicola* strain SCRI424, respectively. Cultures were grown at 30 °C in LB medium supplemented as indicated. The approximate molecular mass of the detected proteins is indicated on the left.

in *E. carotovora* cells grown in the presence or absence of glucose, using anti-RexZ antibodies. This experiment revealed a significant reduction in the amount of RexZ in cells grown in the presence of glucose (Fig. 5a). However, immunoblotting experiments performed using cell extracts from cultures grown in the presence or absence of PGA or galacturonate did not show any significant variation in the quantity of the RexZ protein (Fig. 5a). These data, combined with the *in vitro* binding of cAMP–CRP to the *rexZ* promoter region, suggest that, although cAMP–CRP acts directly to control *rexZ* expression, KdgR is not a repressor of the expression of this gene since its binding on the *rexZ* regulatory region does not interfere either with RNA polymerase or with the capacity of cAMP–CRP to bind in this region.

Involvement of CarI in the synthesis of the RexZ protein

In *E. carotovora* subsp. *carotovora*, degradative exoenzyme synthesis is regulated by quorum sensing, via the production of OHHL, which is directed by CarI/ExpI/HslI (Jones *et al.*, 1993; Pirhonen *et al.*, 1993; Chatterjee *et al.*, 1995). In an attempt to determine whether RexZ production is controlled by OHHL, we performed Western blot experiments with protein extracts from *E. carotovora* PNP22 (CarI$^-$). No differences were noted in the quantity of RexZ in the parental strain and the *carI* mutant (Fig. 5a). Thus, it is unlikely that RexZ synthesis is regulated by OHHL. These data may also suggest that RexZ and CarI are involved in two distinct activation mechanisms of exoenzyme synthesis.

Occurrence of RexZ and KdgR in *Erwinia* spp.

Immunoblotting experiments conducted with antibodies raised against the *E. chrysanthemi* KdgR or *E. carotovora* subsp. *carotovora* RexZ proteins were used to detect these proteins in cell extracts of different strains: *E. carotovora* subsp. *carotovora* strains SCRI193 and ATTn*10*, *E. carotovora* subsp. *atroseptica* strain SCRI31, *E. chrysanthemi* strains EC16, 3937 and A903, *E. amylovora* strain SCRI449, and *E. herbicola* strain SCRI429. Using anti-KdgR antibodies, a band corresponding to approximately 35 kDa was detected for all the *E. chrysanthemi* strains (with the exception of the *E. chrysanthemi kdgR* mutant strain A903), in addition to *E. herbicola* and *E. amylovora* (Fig. 5b). A smaller band was observed for *E. carotovora* subsp. *carotovora* strain ATTn*10* and strain SM2 (*rexZ*; data not shown) as well as *E. carotovora* subsp. *atroseptica*. These data showed that the anti-KdgR and anti-RexZ antibodies were specific, having no significant cross-reactivity. Moreover, this result confirmed that the KdgR and RexZ proteins are distinct and that *E. carotovora* also contains another KdgR homologue, in addition to RexZ.

Immunoblotting with anti-RexZ antibodies (Fig. 5c) gave rise to a signal for all strains except for the *E. carotovora rexZ* mutant strain SM2. The detected proteins all have a molecular mass of approximately 30 kDa (Fig. 5c). These data showed that the RexZ protein is well conserved in pectinolytic *Erwinia* species. The presence of a possible homologue of RexZ in *E. chrysanthemi* is especially interesting because the majority of the proteins that are known to control exoenzyme production in this genetic background are negative regulators.

The bands corresponding to the RexZ proteins of the non-pectinolytic *Erwinia* strains (*E. amylovora* and *E. herbicola*) were of lower intensity. This may indicate that RexZ is less abundant in non-pectinolytic species, or perhaps these species contain a more distantly related protein.

Isolation and analysis of the *E. carotovora* subsp. *carotovora kdgR* homologue

Immunoblotting with anti-KdgR (*E. chrysanthemi*) and anti-RexZ antibodies revealed that, in addition to RexZ,

E. carotovora possessed another KdgR homologue. To isolate the corresponding *kdgR* gene we first used PCR to amplify a 915 bp *kdgR*-specific gene probe from chromosomal DNA of *E. chrysanthemi* 3937. This 'DIG-11-dUTP'-labelled gene probe was used in a Southern blot to probe the chromosomal DNA of *E. carotovora* subsp. *carotovora*, which had been digested with various restriction enzymes. The results indicated that the *E. carotovora* subsp. *carotovora kdgR* homologue was located on a 6 kb *Eco*RV DNA fragment (data not shown). Based on this result, an *E. carotovora* subsp. *carotovora* chromosomal DNA mini-library was constructed in pACYC184 (Chang & Cohen, 1978) and probed with the *kdgR*-specific gene probe. Of the 300 library clones screened one, pREP1, was identified by Southern blotting to carry the putative *kdgR* gene. Restriction analysis of pREP1 revealed that it carried two simultaneously cloned 6 kb chromosomally derived DNA inserts. Subclones of pREP1 were made in pUC19 and probed with the *kdgR* gene probe in a Southern blot. The putative *E. carotovora* subsp. *carotovora kdgR* gene was mapped to a 2·1 kb *Eco*RV–*Sph*I fragment carried on a subclone denoted pREP4. The insert of pREP4 was sequenced and a total of 2173 bp of nucleotide sequence was generated (accession number AF135787).

The predicted translation of this sequence revealed one complete and one partial ORF. The first partial ORF, denoted *′ogl*, was predicted to encode the C-terminal 153 amino acids of a protein which shared a high level of homology with Ogl from *E. chrysanthemi* and *E. carotovora* subsp. *atroseptica*. Following restriction mapping of pREP4, the contiguous *Sph*I–*Eco*RV fragment was identified and sequenced, completing the sequence of the *E. carotovora* subsp. *carotovora ogl* ORF (accession number AF135787). The *ogl* ORF was predicted to encode a 44426 Da protein of 388 amino acids. Database searches showed that the predicted product of this gene shared 99% and 87% sequence identity with the *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi* Ogl proteins, respectively.

The second ORF on pREP4, denoted *kdgR* (based on sequence homology), was predicted to encode a 29700 Da protein of 263 amino acids. Homology searches showed that the predicted product of this ORF shared a high level of homology with *Escherichia coli* KdgR (88% identity) and also with 263 amino acids out of the 305 amino acids that constitute the *E. chrysanthemi* KdgR protein (95% identity; Fig. 1). The predicted *E. carotovora* subsp. *carotovora* KdgR lacked the N-terminal 42 amino acids present in the KdgR protein from the related species, *E. chrysanthemi*.

To further characterize KdgR homologues from subspecies of *E. carotovora*, additional *kdgR* genes were isolated by PCR amplification, using primers KD1, KD3 and KD5. The purified PCR-amplified products from *E. carotovora* subsp. *atroseptica* strain SCRI27, and from *E. carotovora* subsp. *carotovora* strains F14 and F148, were cloned into pBluescript (Stratagene) cut with *Sma*I, generating pREP27, pREP14 and pREP148, respectively. Sequence analysis of these putative *kdgR* clones revealed that they each carried a single ORF, the predicted products of which shared high homology with the previously described KdgR proteins (Fig. 1). The predicted KdgR proteins of these strains of *Erwinia* spp. also lack the N-terminal 42 amino acids displayed by the homologous *E. chrysanthemi* protein.

N-terminal sequence analysis of the *E. carotovora* subsp. *carotovora* SCRI193 KdgR protein

To definitively identify the translational start site of the *E. carotovora* subsp. *carotovora* SCRI193 KdgR protein, a recombinant protein was purified by virtue of six histidine residues (6-His tag) which had been added on to the C-terminus of the protein (see Methods). The construct carried a total of 1557 bp of PCR-amplified *E. carotovora* subsp. *carotovora* insert DNA, including 677 bp of DNA upstream of the putative *E. carotovora kdgR* ORF, assumed to include the *kdgR* promoter region. A protein of approximately 30 kDa was observed, which was consistent with the predicted size of the *E. carotovora* KdgR. The resultant N-terminal amino acid sequence generated from this clone agreed with that previously predicted from the nucleotide sequence of *kdgR* (Fig. 1). The *E. carotovora* KdgR protein is therefore analogous to the KdgR protein predicted for *Escherichia coli* and all the other KdgR homologues identified in this study. It is obvious from these data that although the gene order of *ogl* and *kdgR* is retained and the *ogl* genes themselves are highly conserved between the *Erwinia* spp., there are significant differences between the N-terminal regions of the predicted KdgR proteins of the two *E. carotovora* subspecies compared to that previously reported for *E. chrysanthemi*.

Functional interchangeability of the *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* KdgR proteins

Functional interchangeability has been demonstrated for the *Escherichia coli* and *E. chrysanthemi* KdgR proteins, where the *E. coli* homologue was shown to be able to repress *pelD* transcription (James & Hugovieux-Cotte-Pattat, 1996). To test whether the KdgR proteins of *E. carotovora* and *E. chrysanthemi* were interchangeable, we performed band-shift experiments by using protein extracts from *E. carotovora* subsp. *carotovora* and the regulatory regions of both the *E. carotovora rexZ* gene and the *E. chrysanthemi pelE* gene, encoding a major pectate lyase. Partially purified KdgR protein from *E. carotovora* subsp. *carotovora* SCRI193, obtained after fractionated precipitation with ammonium sulphate, was used in gel retardation assays. One major DNA–protein complex was observed when KdgR was incubated with either the *pelE* or *rexZ* operator regions (Fig. 4b). The addition of KdgR-specific antibodies or KDG (the actual inducer of the pectinolysis genes) to the reaction mix, prior to the incubation of

KdgR with the DNA operator regions, inhibited the formation of both complexes (Fig. 4b). Thus the *E. carotovora* KdgR protein appears to be able to bind to a DNA fragment containing a KdgR-box. Consequently, it is reasonable to assume that the *E. chrysanthemi* and *E. carotovora* KdgR proteins are interchangeable. This was confirmed by the fact that the introduction of an R-prime plasmid (pROU2) containing the *E. chrysanthemi* wild-type *kdgR* gene (Reverchon *et al.*, 1991) in *E. carotovora* decreased the pectate lyase activity by two- to fivefold in both ATTn*10* and SM2 strains (Table 2).

DISCUSSION

This study was initiated by the fortuitous discovery that *E. carotovora* possessed a gene, *rexZ*, encoding a homologue of the major repressor of pectinolysis (KdgR) in *E. chrysanthemi*. Because sequence identity between the two proteins was limited, we thought it prudent to further investigate the relationship between the *E. chrysanthemi kdgR* and *E. carotovora rexZ* gene products. In order to determine whether RexZ was a functional homologue of KdgR, we constructed a *rexZ* null mutant. The *E. carotovora* RexZ$^-$ mutant exhibited a reduced level of pectate lyase, cellulase and protease production and therefore RexZ, in contrast to KdgR, was likely to be an activator rather than a repressor of exoenzyme production. In addition, functional studies revealed that, unlike in the *E. chrysanthemi kdgR* mutant (where the KdgR$^-$ phenotype can be phenotypically suppressed by the presence of PGA), the activity of RexZ protein is not modulated by pectic compounds (Table 2). This was unexpected, and confirmed that the *E. carotovora* RexZ regulatory protein is not a functional homologue of the *E. chrysanthemi* KdgR repressor, but acts in an antagonistic fashion as a novel activator of virulence in *E. carotovora*.

Sequence analysis of the *rexZ* promoter region revealed the presence of consensus binding sites for KdgR and CRP. In an attempt to determine the relative position that RexZ occupies in the already complex regulatory network that governs exoenzyme production in *E. carotovora*, we looked at the involvement of CRP, KdgR and the bacterial pheromone, OHHL, on the expression of *rexZ*. It was evident from the *in vitro* DNA-binding assays that cAMP–CRP bound strongly to the *rexZ* promoter region, indicating that RexZ is catabolite repressed. Additional supporting evidence for this notion came from *in vivo* quantification of the RexZ protein in the presence and absence of glucose. Therefore, in *E. carotovora*, there appears to be a hierarchical regulatory cascade controlling degradative exoenzyme synthesis which involves a global cellular regulator (CRP) and a virulence-associated regulator (RexZ). A similar system of control was recently reported in *Pseudomonas aeruginosa*, where Vfr, a CRP homologue, controls the expression of the LasR activator which is involved in quorum sensing and expression of virulence factor genes (protease and exotoxin A) (Albus *et al.*, 1997). Such a cascade can allow a rapid up- and down-regulation of the genes controlling the virulence functions in these bacteria in response to change in environmental conditions.

Distinct from the cAMP–CRP binding site, KdgR (*E. chrysanthemi*) bound to a site upstream of the *rexZ* promoter at a position which would not interfere with the binding of RNA polymerase. However, it was formally possible that, because the binding sites of KdgR and cAMP–CRP overlapped, KdgR could indirectly modulate *rexZ* expression by antagonistically binding to this region. This was shown not to be the case, as both KdgR and cAMP–CRP could simultaneously occupy their respective binding sites in the *rexZ* operator region. Some evidence to support this finding came from *in vivo* quantification of the RexZ protein, which showed that the amount of RexZ detected remained unchanged in presence of pectic compounds in the growth medium (Fig. 5a). However, we cannot rule out the possibility that KdgR could act as repressor on a secondary *rexZ* promoter, expression from which would be regulated by different environmental conditions. The cellular amount of RexZ was also shown to be unaffected in *E. carotovora* strains carrying mutations in the *carI* gene, responsible for the production of the bacterial pheromone OHHL, suggesting that the RexZ activator operates independently of the quorum-sensing system.

Immunoblotting experiments showed that RexZ homologues are widely distributed in the erwinias and revealed that *E. carotovora* possessed a protein which bound the KdgR-specific antibodies, yet was distinct from RexZ. This observation was consistent with Southern blot experiments revealing different genes for *rexZ* and *kdgR* (data not shown). We isolated the *kdgR* homologues from three strains of *E. carotovora* subsp. *carotovora* and one strain of *E. carotovora* subsp. *atroseptica*. Sequence analysis showed that the *E. chrysanthemi* KdgR protein had an N-terminal extension of 42 amino acids compared with the *E. carotovora* homologues. However, if the DNA upstream of the *E. carotovora* subsp. *carotovora kdgR* ORF is artificially translated, in-frame with the predicted ATG start site, then 26 of these predicted amino acids match exactly the corresponding amino acids in the *E. chrysanthemi* KdgR N-terminus (data not shown). Nevertheless, the *E. carotovora* subsp. *carotovora* sequence possesses an in-frame stop codon and lacks a suitable translational start site in this region. This result was taken to suggest that the previously designated translational start codon for the *E. chrysanthemi kdgR* ORF might be incorrect and that the true start codon was at a position corresponding to that of the *Escherichia coli* and *E. carotovora* subsp. *carotovora kdgR* ORFs. However, immunoblotting experiments suggested that the *E. chrysanthemi* KdgR protein may well be larger than the *E. carotovora* protein (Fig. 5b), consistent with the possibility that the KdgR proteins from these related bacteria do differ in the N-terminus, due to the position of their translational start codons.

In light of the possible differences between the KdgR proteins of *E. carotovora* and *E. chrysanthemi* and the

obvious difference in function of RexZ, it was important to determine whether this *E. carotovora* KdgR protein was functionally similar to the *E. chrysanthemi* homologue. We demonstrated that partially purified *E. carotovora* KdgR protein was able to bind specifically to sequences carrying the consensus KdgR-box, and that binding was abolished by the addition of KdgR-specific antibodies and pectin compounds. Thus it would appear that the 'true' *E. carotovora* KdgR homologue is very similar to that of *E. chrysanthemi* in both sequence and function. However, that the *E. carotovora* KdgR protein exerts its control on target genes by identical mechanisms to those already established for the *E. chrysanthemi* KdgR protein (Nasser *et al*., 1997) may require confirmation. Preliminary experiments indicate that the *in vitro* binding affinities of the *E. chrysanthemi* KdgR protein for the *E. carotovora pelC*, *pelD* and *aepH* (*rsmB*) promoter regions are low (data not shown), which is in agreement with the significant degeneration of the 'KdgR-box' sequence identified in the regulatory regions of *E. carotovora pel* genes versus the KdgR consensus defined in the *E. chrysanthemi* system (Nasser *et al*., 1994; W. Nasser & G. P. C. Salmond, unpublished results). Given the N-terminal sequence differences of these proteins, these data support the notion that the mechanisms of these closely related proteins may be subtly different. Work is currently in progress to compare directly the activity of the KdgR proteins from *E. carotovora* and *E. chrysanthemi*.

We have shown that *E. carotovora* possesses two previously uncharacterized proteins, both of which belong to the IclR family of transcriptional regulators. These proteins, KdgR and RexZ, represent two new regulatory inputs in the already complex regulatory network that governs virulence in the phytopathogen *E. carotovora*. The presence of multiple positive regulators controlling exoenzyme production in *E. carotovora* generally contrasts with the situation in *E. chrysanthemi*, where only two true activators, represented by the CRP and Pir proteins, had been previously discovered (Reverchon *et al*., 1997; Nomura *et al*., 1998). However, this study has revealed that *E. chrysanthemi* expresses a protein homologue of the positively acting regulatory protein RexZ. It would be interesting to discover whether this, as yet uncharacterized, homologue of RexZ also acts in a similar manner in this background. In *E. chrysanthemi*, synthesis of exoenzymes, particularly of the pectinases, is fundamentally governed by repression mediated by *pecS*, *pecT* and *kdgR* (Reverchon *et al*., 1991, 1994; Surgey *et al*., 1996). Analysis of the distribution of these different regulators in bacteria of the genus *Erwinia* has revealed the presence of *pecT*/*hexA* (Castillo & Reverchon, 1997; Harris *et al*., 1998), *expI*/*carI* (Nasser *et al*., 1998; Reverchon *et al*., 1998; Jones *et al*., 1993; Pirhonen *et al*., 1993), and *rexZ* and *kdgR* homologues (this work) in both *E. chrysanthemi* and *E. carotovora*. However, *in vivo* comparative studies for many of these proteins, such as PecT/HexA and proteins encoded by the quorum-sensing locus, suggest that the mechanism by which they are involved in *pel* gene expression is different in these two *Erwinia* species (Nasser *et al*., 1998; Reverchon *et al*., 1998; Harris *et al*., 1998). This is reinforced by the fact that, until this study, all the screening for mutations affecting exoenzyme production, particularly production of Pel, failed to identify a common regulator in both *E. chrysanthemi* and *E. carotovora*. However, this work has shown that, not only is the *E. carotovora* KdgR protein highly similar to that of *E. chrysanthemi*, it is also functionally interchangeable. Therefore, this represents one of the few regulators of exoenzyme production that is both common to and interchangeable between *E. chrysanthemi* and *E. carotovora*.

NOTE ADDED IN PROOF

We have recently sequenced the N-terminus of the *E. chrysanthemi* KdgR protein and confirmed the true translational start to be the methionine at position 43 of the published sequence (and therefore coincident with the start sites of all other KdgR homologues). The relevant GenBank entry, X62072, will be updated accordingly.

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