Expression of a *Butyrivibrio fibrisolvens* E14 gene (*cinB*) encoding an enzyme with cinnamoyl ester hydrolase activity is negatively regulated by the product of an adjacent gene (*cinR*)

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A second cinnamoyl ester hydrolase (CEH) encoding gene (*cinB*) has been characterized from the ruminal bacterium *Butyrivibrio fibrisolvens* E14. *CinB* is more similar to *CinA* (previously named *CinI*) (28% amino acid identity), the first CEH described from *B. fibrisolvens* E14, than either of the enzymes are to any other member of the family of hydrolases to which they belong. Upstream of *cinB*, and in the opposite orientation, is a gene (*cinR*) encoding a protein with substantial similarity to members of the MarR family of negative regulators of bacterial gene expression. By alignment of these sequences, a possible helix–turn–helix DNA-binding domain has been identified. *CinR* was expressed at a high level in *Escherichia coli* using the *lac* promoter. In *E. coli* *CinR* repressed the expression of *CinB*, but had no effect on the expression of *CinA*. In gel mobility-shift assays, *CinR* bound specifically to the *cinR*–*cinB* intergenic region. Two identical 16 nucleotide inverted repeats adjacent to the putative *PcinR* and *PcinB* promoters are likely binding sites for *CinR*. The addition of FAXX (O-[5-O-(*trans*-feruloyl)- α -L-arabinofuranosyl]-(1,3)-O- β -D-xylopyranosyl-(1,4)-D-xylopyranose) and Fara [5-O-(*trans*-feruloyl)-arabinofuranose], but not xylobiose, ferulic acid and a number of other soluble components of hemicellulose, inhibited the binding of *CinR* to DNA.

Keywords: *Butyrivibrio fibrisolvens*, cinnamoyl ester hydrolase, negative regulator, signal sequence, xylan

INTRODUCTION

The heterogeneous polysaccharide xylan is one of the major components of plant cell walls. The arabinose side chains of arabinoxylan are sometimes esterified to cinnamic acids (principally ferulic and *p*-coumaric acid). A number of cinnamoyl ester hydrolases (CEHs) able to release cinnamic acids from plant materials have been identified (see Christov & Prior, 1993). The action of the CEH is probably required to allow complete utilization of the esterified arabinose and adjacent xylose residues.

Abbreviations: CAT, chloramphenicol acetyltransferase; CEH, cinnamoyl ester hydrolase; Fara, 5-O-(*trans*-feruloyl)-arabinofuranose; FAXX, O-[5-O-(*trans*-feruloyl)- α -L-arabinofuranosyl]-(1,3)-O- β -D-xylopyranosyl-(1,4)-D-xylopyranose; MUTMAC, 4-methylumbelliferoyl(*p*-trimethylammonium cinnamate chloride).

The GenBank accession number for the nucleotide sequence reported in this paper is U64802.

The cloning of a gene encoding a CEH from the ruminal bacterium *Butyrivibrio fibrisolvens* E14, using the model substrate MUTMAC [4-methylumbelliferoyl(*p*-trimethylammonium cinnamate chloride)] has been described (Dalrymple *et al.*, 1996). Although predicted to be an intracellular enzyme, *CinI* (here renamed *CinA*) exhibited significant cleavage of Fara [5-O-(*trans*-feruloyl)-arabinofuranose] derived from wheat bran, demonstrating the utility of MUTMAC for screening for enzymes with activity against plant-derived cinnamoyl esters. Analysis of the amino acid sequence of *CinA* demonstrated that it was a member of a large and diverse family of serine/aspartic acid/histidine active site hydrolases. However, the nature of the enzyme encoded by the second group of genomic clones isolated using MUTMAC was not investigated.

The regulation of expression of CEHs has been studied in the non-ruminal bacterium *Streptomyces olivochro-*

mogenes. Oat spelt xylan, de-starched wheat bran and sugar cane bagasse were demonstrated to induce between 45 and 172-fold increases in the specific activity of a CEH (MacKenzie *et al.*, 1987; Johnson *et al.*, 1988). In a number of non-ruminal fungi, CEH activity was not induced by growth on oat spelt xylan (does not contain cinnamic acid esters), but was induced by growth on meadow fescue grass (Smith *et al.*, 1991). In contrast, CEH production in *Aspergillus oryzae* was not related to the phenolic acid content of the substrates (Tenkanen *et al.*, 1991). In a separate series of experiments, growth of the fungus *Schizophyllum commune* on both Avicel and wheat bran produced more CEH activity than growth on oat spelt xylan (MacKenzie & Bilous, 1988). Clearly the regulation of expression of CEHs is complex and may vary substantially from one organism to another. The precise nature of the compounds responsible for inducing the expression of CEHs is not known.

In this paper we describe the sequence of *cinB*, a second CEH-encoding gene isolated from *B. fibrisolvens* E14, and the role of a putative negative regulator of *cinB* expression that is encoded adjacent to *cinB*. We have also determined the nature of molecules that appear to be involved in inducing *cinB* expression.

METHODS

Bacterial strains and vectors. Host strains *Escherichia coli* XL1-Blue, *E. coli* JM109 and *E. coli* KW251 were used for the cloning and expression of genes. Subcloning of fragments and expression of the recombinant proteins were carried out in plasmids pGEM-T, pGEM3Zf(+) and pGEM7Zf(+). Promoter-probe vector pKK232-8 was obtained from Pharmacia.

Media and chemicals. Bacterial cells were cultivated in L broth (LB) or LB containing 1.5% agar for agar plates and 0.7% agar for top agar (Sambrook *et al.*, 1989). Ampicillin was used at 50 µg ml⁻¹, IPTG at 1 mM and X-Gal at 40 µg ml⁻¹. Chloramphenicol was used at various concentrations between 25 µg ml⁻¹ and 1 mg ml⁻¹ as required. Fara and FAXX [O-[5-O-(*trans*-feruloyl)- α -L-arabinofuranosyl]-(1,3)-O- β -D-xylopyranosyl-(1,4)-D-xylopyranose] were prepared as described previously (Dalrymple *et al.*, 1996). MUTMAC, lactose, cellobiose, methylated pectin, α -D-galacturonic acid, *p*-coumaric acid and L-arabinose were obtained from Sigma. Ferulic acid and chlorogenic acid were obtained from Fluka and alduronic acid and xylobiose were obtained from Megazyme.

Preparation and manipulation of DNA. Bacteriophage particles and DNA were prepared from plate lysates using lambdasorb (Promega) following the manufacturer's recommended procedure. For subcloning, bacteriophage and plasmid DNA was digested with restriction enzymes and fragments were separated by agarose gel electrophoresis following standard techniques (Sambrook *et al.*, 1989). Prior to ligation, DNA samples were purified using the Magic DNA preparation kit (Promega). Colonies containing inserts were screened with MUTMAC overlays as described previously (Dalrymple *et al.*, 1996). The nucleotide sequences of subcloned restriction fragments were determined by the dideoxynucleotide chain-termination procedure using the Sequenase sequencing kit (Amersham). Synthesis was primed using standard SP6 and T7 primers (Promega) or with internal primers synthesized on an

Oligo 1000 DNA Synthesizer (Beckman). Computer analyses of DNA and protein sequences were carried out using MacVector (IBI) and the ANGIS (Australian National Genomic Information Service) facility. Database searches were carried out using the BLAST program (Altschul & Lipman, 1990) at The National Centre for Biotechnology Information and the BLOCKS (Henikoff & Henikoff, 1994) and PROSITE (Bairoch, 1993) databases at ANGIS. The *cinR* gene was amplified by PCR using the primers shown in Fig. 2 for 35 cycles at 94 °C (60 s)/50 °C (60 s)/72 °C (30 s). The 50 µl reaction mix contained approximately 1 ng plasmid DNA (containing the complete cloned *B. fibrisolvens* fragment), 50 mM KCl, 10 mM Tris/HCl, pH 9, 0.1% Triton X-100, 2 mM MgCl₂, 0.1 mM each dNTP and 1 unit *Taq* polymerase. Prior to ligation into pGEM-T the PCR product was purified using the Magic PCR Preps DNA purification system (Promega).

Cell fractionation and analysis. *E. coli* cultures were grown to an OD₆₀₀ of 0.5–0.8 in LB and, if required, protein expression was induced by the addition of IPTG to 1 mM. Uninduced and induced cultures were harvested by centrifugation after a further 16 h incubation at 37 °C. Cells were resuspended in PBS (Sambrook *et al.*, 1989) and lysed by sonication, or fractionated as described by Hsiung *et al.* (1986). Culture supernatants, sucrose fractions, periplasm fractions and sonicated cells were assayed for enzyme activity by spotting samples at the appropriate dilution and overlaying with MUTMAC-containing agarose as previously described (Dalrymple *et al.*, 1996). Proteins were separated by SDS-PAGE by the method of Laemmli (1970). Prestained low-range molecular mass standards were obtained from Bio-Rad. The concentration of protein in samples was determined using the Bio-Rad protein assay kit with bovine serum albumin standards.

The first ten amino-terminal residues of the recombinant CinB protein were determined by automated Edman sequencing with a 471A gas-phase sequencer (Applied Biosystems) using periplasmic fractions separated by SDS-PAGE and transferred to a PVDF membrane by Western blotting.

Assays of CinR-regulated expression of CinB and CinA in *E. coli*. High-titre stocks of bacteriophage genomic clones were diluted to obtain either plate lysates or well-separated plaques on lawns of *E. coli* KW251 cells containing the appropriate plasmid by using standard methods described by Sambrook *et al.* (1989), except that the top agarose contained 10% clarified and autoclaved rumen fluid (see Dalrymple *et al.*, 1996). To recover proteins from the plate lysates, 3 ml of 100 mM NaCl, 17 mM MgCl₂, 50 mM Tris/HCl, pH 7.5, was poured onto the surface and the plates rocked at room temperature for 2–3 h. CEH activity was measured as described previously (Dalrymple *et al.*, 1996). On plates with separated plaques, CinB activity was visualized using MUTMAC as described previously (Dalrymple *et al.*, 1996).

Gel mobility-shift assays. The method described by Tupper *et al.* (1994) was modified as follows. Approximately 20–50 ng DNA was incubated for 15–30 min at room temperature with approximately 1 µg protein from sonicated and centrifuged lysates of *E. coli* JM109. Reaction samples were made to a final volume of 20 µl with a final concentration of 100 mM Tris/HCl, pH 8, 1 mM EDTA and 15 mM KCl. Samples were incubated for 5 min at room temperature with all of the components except the DNA and for a further 30 min after the addition of the DNA prior to electrophoresis. The total sample volume was separated on either 1% DNA-grade agarose (Progen), or 2% Metaphor agarose (FMC) gels in

45 mM Tris/borate, 1 mM EDTA at 5 V cm⁻¹. Gels were stained with ethidium bromide and illuminated on a UV transilluminator to visualize the DNA.

RESULTS AND DISCUSSION

Subcloning and deletion analysis of *cinB*

Subclones of the insert in the genomic clone #11, a representative of the second of the two groups of genomic clones isolated from a library of *B. fibrisolvens* E14 using MUTMAC (Dalrymple *et al.*, 1996), were obtained by partial digestion of the bacteriophage DNA with *Hind*III followed by ligation into the *Hind*III site of the plasmid vector pGEM7Zf(+). All of the subclones that were positive for cleavage of MUTMAC contained a 7.5 kb *Hind*III fragment containing both λ vector and *B. fibrisolvens* E14 DNA (Fig. 1). Deletion analysis of the *B. fibrisolvens* DNA delineated a region between the third *Eco*RV site and the *Bal*I site that contained sequences involved in the expression, or coding, of CinB (Fig. 1). Enzyme expression was increased by the addition of IPTG when transcription of the *lac* promoter was from the *Hind*III to the *Eco*RI site (see Fig. 1), but not in the reverse direction (data not shown).

The sequence of the subcloned region was determined between the *Hind*III site and the second *Dra*I site (Fig. 1). One long open reading frame (ORF), *cinB*, encoding a potential protein of 285 amino acids (predicted molecular mass 31 449 Da) and consistent with the results from the deletion analysis, was identified between nucleotides 1182 and 2836 (Fig. 2). The first methionine is preceded by a probable ribosome-binding site (RBS) and is followed by a probable signal peptide with a predicted site of cleavage after amino acid 17. However, an adjacent site also shows similarity to the consensus sequence for the cleavage of lipoprotein signal sequences, L(S,A)(A,G)C(S,G), with the cysteine at residue 19 predicted to be the site of addition of the lipid moiety (Sutcliffe & Russell, 1995). An inverted repeat downstream of *cinB*, between residues 2097 and 2143, may form a transcription termination signal.

The amino acid sequence of CinB exhibits 28% amino acid identity with that of CinA (Fig. 3). CinB is more similar in sequence to CinA than either of the enzymes are to any of the other members of the large and diverse family of serine/aspartic acid/histidine active site hydrolases to which they both belong (see Dalrymple *et al.*, 1996). However, the probable substrate-binding domains of CinA and CinB, between amino acids 177 and 210 in CinB [by analogy with XylF, described by Diaz & Timmis (1995)], are not similar in sequence. It is unlikely that *cinA* and *cinB* have arisen through a recent gene duplication and it is possible that their natural substrates are quite different.

High-level expression of CinB in *E. coli*

Preliminary experiments with plasmid 13BS in *E. coli* strain JM109 identified a protein of approximately 35 kDa in the periplasm fraction that was not present in

control cells. Enzyme activity was enriched in the periplasmic fraction relative to the cell fraction. To increase the level of expression of CinB, the DNA sequence between the *Ecl*136II site downstream of the plasmid *lac* promoter and the third *Eco*RV site in the inserted *B. fibrisolvens* E14 DNA was deleted to generate plasmid 13BEE (Fig. 1). A significant increase in expression of CinB was observed, with a significant proportion of the protein again located in the periplasmic fraction (Fig. 4). However, induction of the cultures with IPTG led to substantial cell lysis during growth, with little additional yield of protein (data not shown). By direct sequencing, the amino-terminus of the recombinant CinB in the periplasmic fraction was determined to be MKSDYTVNTE.

The predicted amino acid sequence preceding the experimentally determined amino-terminus was not the sequence predicted from the location of the putative signal sequence. The sequence immediately preceding the MKSDYTVNTE sequence does not fit with consensus sequences for bacterial signal peptidase cleavage sites, suggesting that CinB was not exported via a signal-sequence-dependent pathway involving cleavage to generate the observed amino-terminus. Inspection of the DNA sequence preceding the methionine codon identified a possible RBS (Fig. 2). In *E. coli* CinB appears to be translated from the second possible AUG in the open reading frame. In *B. fibrisolvens* it is possible that the first start codon is utilized to produce a protein that is secreted via a signal-sequence-dependent pathway, or even that both intra- and extracellular forms of the enzyme may be produced from the one gene.

A putative negative regulator of *cinB* expression is encoded by the *cinR* gene immediately upstream of *cinB*

Located 170 bp upstream of *cinB* is an ORF on the opposite strand (Fig. 1). This ORF is preceded by a probable RBS and it encodes a protein of 142 amino acids with a predicted size of 16 kDa (Fig. 2). The sequence of the encoded protein was used to search all of the protein sequence databases using the BLAST algorithm. A number of proteins with significant similarities were identified (Fig. 5). PecS (Reverchon *et al.*, 1994), Hpr (Perego & Hoch, 1988), HpcR (Roper *et al.*, 1993), EmrR (del Castillo *et al.*, 1991) and MarR (Cohen *et al.*, 1993) have all been shown to be negative regulators of bacterial gene expression. The *B. fibrisolvens* sequence is most similar to HpcR, a negative regulator of the homoprotocatechuate degradative operon of *E. coli* C (Roper *et al.*, 1993), and PecS, a negative regulator of pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi* (Reverchon *et al.*, 1994), with 31% and 26% amino acid identity respectively.

A section of the LexA family of regulatory proteins (see Garriga *et al.*, 1992) was also identified as similar to a small region of CinR and other members of the family. Additional searches carried out using the BLOCKS database identified regions of the DeoR (BL00894A), GntR

Fig. 1. Restriction enzyme map of the region of the subcloned fragment containing *cinB* and immediate flanking *B. fibrisolvens* DNA. Deletions were constructed by restriction enzyme digestion and religation. Clones were assayed for CEH activity by overlay with MUTMAC in agarose. The direction of transcription by the plasmid-borne *lac* promoter is indicated for each plasmid. The extent and direction of translation of ORFs identified from the DNA sequence are indicated by arrows. Vertical arrows, locations of possible promoters and operators; T, location of a possible terminator of transcription. The extent of the *HincII* fragment used in the gel mobility assays is indicated by a dashed line. Restriction enzyme sites: Ba, *BalI*; D, *DraI*; E, *EcoRI*; Ec, *Ecl36II*; H, *HindIII*; Hc, *HincII*; Rv, *EcoRV*; Sp, *SphI*; X, *XbaI*. Restriction enzyme sites contained within the linkers are in parentheses.

(BL00043) and LuxR (BL00622) families of regulatory proteins as similar to small regions of CinR and other members of the family. These regions of similarities covered the proposed helix–turn–helix DNA-binding domains of the LexA, DeoR, GntR and LuxR families. The similarities suggest that amino acids 48 to 69 of CinR, and the equivalent amino acids in the other members of the family, may constitute a helix–turn–helix DNA-binding domain (Fig. 5). The spacing of the hydrophobic residues in this region, which is identical to that conserved in such domains from many proteins (see Suzuki *et al.*, 1995), also supports this proposal.

In *E. coli*, expression of CinB, but not CinA, is reduced by the over-expression of CinR

In order to test the hypothesis that CinR regulates the expression of CinB, over-expression of CinR was required. Using PCR primers flanking *cinR* (see Fig. 2), the gene was amplified and inserted into the vector pGEM-T. Both orientations of insertion of *cinR* were obtained. The constructs had translation stop codons in all three frames preceding the proposed start codon and included only half of a potential operator site. The *cinR* gene in pCINR#7 was sequenced and no differences from the previously determined DNA sequence of the original cloned *B. fibrisolvens* E14 DNA were detected. A protein of less than 22 kDa was observed in extracts of *E. coli* JM109 carrying pCINR#7, but not in cell extracts carrying a plasmid with the gene containing a deletion inserted in the opposite orientation (pCINR#4) (Fig. 4). A small induction in expression of CinR was observed from pCINR#7 in samples with IPTG added (Fig. 4).

Both plasmids were transformed into *E. coli* strain KW251 and each cell line infected with bacteriophage genomic clones containing either *cinA* (#14) or *cinB* (#11) to give well-separated plaques. Overlay with MUTMAC was used to obtain a rough estimate of the level of esterase expression. All samples were positive

for esterase activity, but the plaques that contained genes expressing CinB and CinR exhibited substantially less fluorescence. To obtain a more accurate assessment, plate lysates of the combinations of the two plasmids and the two recombinant bacteriophages were made and assayed for cleavage of FAXX. The presence of CinR had no significant effect on the expression of CinA, but reduced the expression of CinB approximately sixfold.

CinR binds to sequences between *cinR* and *cinB*

Extracts of *E. coli* JM109 containing plasmids pCINR#4 (CinR⁻) and #7 (CinR⁺) were mixed with *HinfI*-digested plasmid 13E1 DNA. Addition of extract #4 had no effect on the mobility of any of the fragments. Addition of extract #7 decreased the mobility of only the fragment that contained the *cinR*–*cinB* intragenic region (Fig. 6). Thus, the mobility-shift was specific for the presence of CinR and for the *cinR*–*cinB* intragenic region of the plasmid. No *EcoRV* fragments exhibited a gel-shift with either extract (data not shown), suggesting that the 16 nucleotide inverted repeats that lie upstream of *cinR* and *cinB* and contain *EcoRV* sites (Fig. 2) are the binding sites for CinR.

DNA binding has also been demonstrated for Hpr (Kallio *et al.*, 1991), MarR (Martin & Rosner, 1995; Seoane & Levy, 1995), SlyA (Oscarsson *et al.*, 1996) and PecS (Praillet *et al.*, 1996). The MarR-binding sites are also inverted repeats with the two five-base half repeats separated by two bases (Martin & Rosner, 1995). A similar inverted repeat lies between the promoter and start codon of SlyA (see Ludwig *et al.*, 1995). In contrast, the Hpr-binding sites appear to be a somewhat shorter and imperfect inverted repeat (Kallio *et al.*, 1991), whilst the PecS-binding sites appear to be much longer (Praillet *et al.*, 1996). Our identification of a possible DNA-binding helix–turn–helix motif in CinR and the related

proteins suggests that all members of this family may bind directly to DNA via this region.

The effect of addition of various sugars, cinnamic acids, cinnamoyl esters and products of arabinoxylan degradation to the gel mobility-shift assay was investigated. The following compounds had no effect on controls, nor

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CinA	MYIVDDGIKLNAILDMPEG	19
CinB	MKGVTLLTASLCLLAAGTSSNSKEIVMKSDYVWVEVVEIPSGDNTLYGTLYTPET	60
CinA	GAEKPLCLVFGHFTGHIEDHIVAVAKGLNEIGVATLRVDLFG...HGKSEGEFREHNL	76
CinB	.DSKTPLIIMCHGYNGV...GDDFQEEGKYFAQNGIATYTLDFCGGSTRSKSTGETKDMTI	117
CinA	YKWLNNILAVVDYAKKLD...FVTDLYICGHSQGGGLAVTLAAAMERDTIKALMPL.SPAYV	133
CinB	PTEKADLLAAYNYFKTQDNIDNNIFLFGSGQGLVTTLTATEELGDEV.AGMALYFPALC	176
CinA	IIDGAKAGMLLGQPFDPPEHIPDELVSWDGRTLNGYIRVAQSI.DLDAAMKKF...TGPVL	190
CinB	IADNWRET...FPETDMIPKEEFW.GMTLGKNFF...ESIHDFDVFEISGYPNNVL	228
CinA	IVHGDADDIVPVEFAIDASKKFFANCKLELIKDDHCYKGMMDL.MVKAVQEFVRKL	246
CinB	ILHGDKDEIVPLSYSEKAASIYEHAKLIVMEGEGHGFAPFAAKTAREDLVLSFMKENIR	285

Fig. 3. Alignment of CinA and CinB. The amino acid sequence of CinA (formerly CinI) is from Dalrymple *et al.* (1996). Amino-terminal residues of the proteins expressed in *E. coli* that were determined by direct sequencing are underlined. A possible signal sequence in CinB is shown in italics. *, Amino acid identities; ^, amino acids conserved in most members of the family of hydrolases (see Dalrymple *et al.*, 1996); #, proposed active-site amino acids. Dots indicate gaps introduced into the sequences to facilitate alignment.

Fig. 4. Expression of CinB and CinR. (a) Periplasmic fractions of *E. coli* JM109 containing either plasmid 13BEE (lane 2) or pGEM7Zf(+) (lane 1) were prepared and proteins separated on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Molecular mass markers are shown in kDa. (b) Total soluble protein of *E. coli* JM109 containing either plasmid pCINR#7 (CinR⁺) or pCINR#4 (CinR⁻) were prepared and proteins separated on a 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Molecular mass markers are shown in kDa. Addition of IPTG to the growth medium is indicated by +.

and Fara, whilst no effect was seen on controls, a small effect was seen at 30 μ M and with concentrations greater than 300 μ M binding of CinR was almost completely abolished (Fig. 6). Addition of extracts of *E. coli* containing CinB to the assay system reversed the inhibition of binding of CinR by FAXX and Fara (data not shown).

The members of this family of negative regulators are quite diverse in sequence and are involved in regulating a wide range of different processes. However, 4-hydroxyphenylacetic acid (HpcR) (Roper *et al.*, 1993), salicylate (MarR and ErmR) (Martin & Rosner, 1995; Seoane & Levy, 1995; Lomovskaya *et al.*, 1995), 2,4-dinitrophenol (ErmR) (Lomovskaya *et al.*, 1995) and Fara and FAXX (CinR) all contain an aromatic ring, suggesting that the binding sites of the regulators may have a preference for such groups (Sulavik *et al.*, 1995).

Identification of promoters in the *cinR*–*cinB* intergenic region

The promoters of RNA transcription of *cinB* and *cinR* are expected to lie between the two genes. In the absence of information concerning the nature of promoters in *B. fibrisolvens*, the intergenic region was searched for sequences homologous to the *E. coli* sigma-70 promoter consensus sequence TTGACA (–35) and TATAAT (–10). Two overlapping and divergent candidate pro-

motors were identified (Fig. 2). To test the ability of these sequences to direct transcription in *E. coli*, the 76 bp *EcoRV* fragment containing the two potential promoters was cloned into the *SmaI* site of the promoter-probe plasmid pKK232-8. *E. coli* JM109 clones containing the *EcoRV* fragment inserted in either orientation were very resistant to chloramphenicol. With *PcinB* transcribing the CAT (chloramphenicol acetyltransferase) gene, colonies were formed on plates containing 1 mg chloramphenicol ml^{–1}. With *PcinR* driving the CAT gene, colonies were visible up to 0.8 mg chloramphenicol ml^{–1}. With no promoter driving the CAT gene no colonies were visible on 25 μ g chloramphenicol ml^{–1}.

A single copy of the 16 nucleotide inverted repeat to which CinR probably binds lies between each of the two proposed divergent promoters, *PcinR* and *PcinB* (Fig. 2), and the corresponding ORF.

A hypothesis for the regulation of CinB expression by CinR

The location of the probable CinR-binding sites suggests that the expression of both CinB and CinR is regulated by CinR. Autoregulation has been observed for MarR (Martin & Rosner, 1995) and EmrR (del Castillo *et al.*, 1991) and may be a common feature of this family of negative regulators.

	30	40	50	60	70
Bf CinR	LTEAQPKILYILGFN---	EGIVQKDFAKLC--	AIK--	PSTMTVQLARLEKDLIRR	
	*** * * *	* *	*	• * * * *	*
Ec HpcR	LTEQQWRIVRILAES---	PSMDFHDLAYRA--	CIL--	RPSLTGILTRMERDGLVLR	
Er PecS	LSAREFDILATLRRRGAPYAYSPSQIVNAL--	MIN--	NSTLTSRLDRLEQAGWLRR		
Bs YybA	LDLTRGQYLYLVRIYEN--	PGIIQEKLAEI--	KVD--	RTTAARAIAKKLEMQGFIQK	
Bs Hpr	LNINEHHILWIAIYQL--	NGASISEIAKFG--	VMH--	VSTAFNFSKKLEERGYYLRF	
Ec prs	INHVSVMVLTTLFTAES--	HCLSPSEISQKL--	QFT--	RTNITRITDFLEKTGYVKR	
Ec EmrR	INETLFMALITLESQEN--	HSIQPSELSICAL--	GSS--	RTNATRIADELEKRGWIER	
Ec MarR	ITAAQFKVLCISIRCA---	ACITPVELKKVL--	SVD--	LGALTRMLDRLVCKGWVER	
Pa MexR	LTPPDVHVKLKIDEQ---	RGLNLQDLGRQM--	CRD--	KALTTRKTRELEGRNLVRR	
Ms AcaO	LKIEDWRVLDLDELAGR---	RTVPMSDLAQAT--	LTT--	GPTLTTRTVDRLVSGQIIYR	
Rc PetP	YGRAHHRRAIHFINRE---	PGLTVTTLISVL--	GVT--	KQSLNRVLRRLTLDGLVES	
Bs TyrSO	LSWTAFSILYDLWVW---	GALETRKIAELS--	GIS--	TATASNVIKTLEKKSFCRK	
St SlyA	LTQTHWVTLHNIHQLP--	PDQSQIQLAKAI--	GIE--	QPSLVRTLQDLQLEKGLISR	
Pd IorABO	LTAQFIVLATLLES---	GEASQSDLVRRQT--	AID--	QATIRGITDRKLTGLVTT	
Cf ExoO	PSARVRRVAHVAVRCP--	GLTARIKELGEGS--	LLT--	QPSLSRMVERLEAAGLVSR	
Bs MotAO		/LQQIGDKI--	LLA--	SGTITYVVDKLEQKELLIR	
Bp GlrO	FHNIQFVALQWLEEL---	GDITIGDLSNRL--	YLA--	FSTTTDLVDRMEKNELVKR	
con	L	hL L	L h h	ThTR hD LE Ghh R	
con GntR		/G..LPSE.EL...	F--GVS--R	Thr.AL..L...Ghh.R/	
con DeoR	/RQ..Ih..L---	...L...EL.E.L--	VS--	ThRRDL..L...Ghh.R/	
con LexA	/LT.RQ..IL.hI....	G.PP...EIA..h--	Gh.S...	h...HL..L.RKGhh..	
con LuxR	/L..RE..VL.hh..G-----	N..IA..L--	hS--	TV..H..N...KL/	
			+	+	+
			+	+	+
			helix	turn	helix

Fig. 5. Alignment of the proposed helix-turn-helix region of members of the MarR family of regulators. Bf CinR, *B. fibrisolvens* CinR; Ec HpcR, *E. coli* negative regulator of homoprotocachuate degradative operon; Er PecS, *Erwinia chrysanthemi* negative regulator of pectinase and cellulase expression; Bs YybA, *Bacillus subtilis* unknown function NCBI gi: 467356; Bs Hpr, *B. subtilis* negative regulator of protease expression; Ec prs, *E. coli* unknown function; Ec EmrR, *E. coli* negative regulator of multidrug resistance pump, originally MprA; Ec MarR, *E. coli* regulator of multiple antibiotic resistance; Pa MexR, *Pseudomonas aeruginosa* regulator of multidrug resistance; Rc PetP, *Rhodopseudomonas capsulata* unknown function; St SlyA, *Salmonella* regulator of cytolysin expression; Ms AcaO, *Mycobacterium smegmatis* unknown function; Cf ExoO, *Cellulomonas fimi* adjacent to exoglucanase gene; Bs MotAO, *B. subtilis* unknown function. For references see text, Sulavik *et al.* (1995) or Miller & Sulavik (1996). Dashes indicate gaps introduced to aid alignment. The amino acid consensus sequences of the DNA-binding motifs and immediate flanking regions of the GntR, DeoR, LexA and LuxR families of DNA-binding proteins were taken from the PROSITE (Bairoch, 1993) and BLOCKS databases. /, Extent of the sequences shown; *, amino acid identities between CinR and HpcR; +, positions that contain hydrophobic residues in most known helix-turn-helix DNA-binding domains (Suzuki *et al.*, 1995). In the consensus sequences, amino acids conserved in at least half of the sequences are shown. h, A hydrophobic amino acid (Ala, Leu, Ile, Val, Met, Phe, Tyr, Trp, or Cys); dots indicate no conserved amino acid.

Fig. 6. Gel mobility-shift assays. (a) *Hin*I-digested plasmid 13E1 DNA incubated with 0, 0.5 µg or 1 µg total soluble protein of *E. coli* JM109 containing plasmid pCINR#7 (+) or pCINR#4 (-). Samples were separated on a 2% Metaphor agarose gel. The arrow indicates the *Hin*I fragment that contains the *cinR-cinB* intergenic region. Size markers are shown in bp. (b) *Hin*II-digested plasmid 13E DNA incubated with 1 µg total soluble protein of *E. coli* JM109 containing plasmid pCINR#7 (which expresses CinR). In addition, varying concentrations of FAXX were added to the reaction mixture. Samples were separated on a 1% agarose gel. Arrow indicates the *Hin*II fragment that contains the *cinR-cinB* intergenic region.

The binding of CinR to DNA is specifically inhibited by cinnamic acid sugar esters and not by cinnamic acids, sugars, or chlorogenic acid (a non-sugar cinnamic acid ester). Thus, both the sugar and the cinnamic acid

moieties of the inducer are required for induction. It is probable that the inducers bind to CinR, thereby altering its conformation and reducing its ability to bind to DNA. The role of Fara and FAXX in induction suggests

they are indeed among the natural substrates for CinB, and that CinB is likely to be involved in the breakdown of plant materials by *B. fibrisolvens* E14.

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