Copper-inducible transcriptional regulation at two promoters in the *Escherichia coli* copper resistance determinant *pco*

D. A. Rouch and N. L. Brown

Author for correspondence: N. L. Brown. Tel: +44 121 414 5467. Fax: +44 121 414 5907.
e-mail: N.L.Brown@bham.ac.uk

Microbial Molecular
Genetics and Cell Biology
Research Group, School of
Biological Sciences, The
University of Birmingham,
Edgbaston, Birmingham
B15 2TT, UK

The *pco* determinant of *Escherichia coli* plasmid pRJ1004 encodes inducible resistance to the trace element copper. The identification of two copper-dependent transcriptional initiation regions within *pco* that each contain a similar upstream hyphenated dyad motif is described. Deletion constructs showed that this 'copper box' motif was essential for copper-inducible activity at both *pco* promoters, *PpcoA* and *PpcoE*. The placement of the motif differs in the two promoters, and *PpcoA* contains an extended –10 nonamer typical of promoters for which RNA polymerase does not bind specifically to –35 sequences. *PpcoE* does not contain this motif and is the more strongly expressed promoter. The transcript from *PpcoA* contains the *pcoABCDRS* genes, while *PpcoE* expresses only *pcoE*. The induction profiles for *PpcoA*– and *PpcoE*–*lacZ* fusions were flattened sigmoidal curves with a gradual response to increasing copper concentration. On high-copy-number plasmids, zinc was found also to induce transcription from both promoters *in vivo*. Both promoters showed inducible activity in the absence of *pcoRS*, the plasmid-borne two-component regulatory system, indicating that a second *trans*-acting regulatory system is present on the chromosome. The *pcoR* product showed repressor action in the absence of *pcoS*, while still allowing induction, suggesting the chromosome encoded a similar two-component system to *pco*. *TnphoA* insertion mutagenesis identified chromosomal genes which affected promoter expression, including *ptsH*, *ptsI* (sugar phosphotransferase system) and *cya* (adenylate cyclase). The results support the idea that *pco*-encoded copper resistance is an auxiliary mechanism for handling copper, the regulation of which is integrated with the chromosomal regulation of cellular copper metabolism.

Keywords: copper resistance, plasmid, gene regulation, trace element, homeostasis

INTRODUCTION

Microbial resistance to a wide range of toxic metal ions has been reported. This includes resistance to metals that are purely toxic, with no ascribed biological function, such as mercury and cadmium, and to metals that are toxic in excess but are required in small amounts for biological activities, such as copper and zinc (Silver, 1992; Silver & Ji, 1994). A study of the cellular response to such toxic but essential metals is necessary in

understanding the mechanisms of metal ion homeostasis (Lee *et al.*, 1990; Rouch, 1986).

Plasmid-encoded resistance to copper has been documented in a number of species, the best studied of which are *Escherichia coli* (Tetaz & Luke, 1983), *Pseudomonas syringae* pv. *tomato* (Bender & Cooksey, 1986) and *Xanthomonas campestris* pv. *juglandis* (Stall *et al.*, 1986), and copper resistance has been reviewed (Brown *et al.*, 1993, 1992; Cervantes & Gutierrez-Corona, 1994; Cooksey, 1993, 1994). The conjugative plasmid pRJ1004 confers copper resistance and was isolated from *E. coli* in the gut flora of pigs fed a diet supplemented with copper sulphate as a growth promotant (Tetaz & Luke,

Abbreviation: CAT, chloramphenicol acetyltransferase.

1983). The copper resistance specified by this plasmid involves the *pco* gene cluster, which contains seven genes, *pcoABCDSE* (Brown *et al.*, 1995; Lee *et al.*, 1990). Copper resistance in *P. syringae* pv. *tomato* is specified by the *cop* determinant, which contains six genes, *copABCDSE*, arranged in a single operon, homologous to the equivalent *pco* genes (Brown *et al.*, 1995; Cooksey, 1994; Silver *et al.*, 1993). The sequence of a plasmid-borne resistance determinant from *X. campestris* pv. *juglandis* has also been analysed, and contains genes homologous to both *pcoABCD* and *copABCD* (Lee *et al.*, 1994). In the last two examples resistance is likely to have been selected due to the use of copper-containing antimicrobial formulations on vegetable crops where these phytopathogenic bacteria occur.

In all three cases copper resistance has been shown to be inducible (Mellano & Cooksey, 1988; Rouch *et al.*, 1985; Voloudakis *et al.*, 1993). The *pco* and *cop* determinants specify regulatory systems, *pcoRS* and *copRS*, respectively, which show sequence similarity to other two component systems (Parkinson, 1993; Parkinson & Kofoid, 1992) in which R (receiver) gene products are transcriptional regulators that receive control information from the S (sensor) gene products. An additional sensor gene *pcoS** may be encoded by second-site translation initiation within *pcoS*. Both the *pcoR* and *copR* regulatory gene products are class 2 receivers by the homology typing scheme (Pao *et al.*, 1994): this class also includes OmpR, PhoB, VirG and the *Streptomyces lividans* chromosomal copper-responsive gene CutR (Tseng & Chen, 1991). CopR has been shown to bind to an upstream motif in the *Pseudomonas copABCD* promoter region (Mills *et al.*, 1994). The regulatory genes for the *X. campestris* pv. *vesicatoria* *cop* determinant are not yet known (Lee *et al.*, 1994). We have predicted that bacterial resistance to copper should be expressed in relation to the external concentration of this trace element, to maintain copper ion homeostasis (Lee *et al.*, 1990).

We report here the transcriptional characterization of the regulatory system that controls the inducible expression of *pco*-encoded copper resistance. Two architecturally different promoters with copper-dependent *cis* control elements were identified within the *pco* determinant, which are under *trans* control from both plasmid and chromosome. Transposon mutagenesis showed that mutations in the *ptsHI* and *cya* genes affect expression of the *pco* promoters.

METHODS

Bacterial strains, plasmids and media. Bacterial strains used were *E. coli* K-12 derivatives; TG2 (Δ *lac-pro supE thi hsdD5 recA::Tn10* [*F'* *traD36 proAB⁺ lac^r lacZAM15*]) and SM10 (*thi thr leu tonA lacY supE recA::RP4-2-TC::Mu*) (Taylor *et al.*, 1989). Plasmids used in this study are shown in Table 1, and inserts of recombinant plasmids used are shown in Figs 1 and 2. Plasmid pMU2385 allows promoter sequences to be identified at low copy number by plate assay for *lacZ*

expression, avoiding problems of titration of regulators associated with high-copy-number reporter plasmids. Plasmid pKK232.8 is medium copy number; it was used for ease of genetic manipulation and for more precise assay of promoter activity (by initial rate activity of chloramphenicol acetyl-transferase) than is possible with the *lacZ* reporters.

LB, LA and copper-containing media were made as described elsewhere by Rouch *et al.* (1985). HA contained (l^{-1}): 8 g tryptone, 4 g NaCl and 15 g agar; similarly for soft HA except 6 g agar. Standard additions were (μ g ml^{-1}): carbenicillin, 250; tetracycline, 25; trimethoprim, 25; kanamycin, 50; X-Gal, 40. $CuSO_4$ and $ZnSO_4$ were ultrapure grade (Aldrich); for induction a stock solution of $CuSO_4$ (20.0 mM) in LB, pH 7.5, was diluted as appropriate. In quantitative copper response assays, copper was removed from broth, or agar in molten form, by twofold treatment with 0.1% (w/v) Chelex-100 resin (Bio-Rad) for 1 h prior to the addition of $CuSO_4$ and trace element solution [as for *Neurospora* Minimal Medium (Daggett *et al.*, 1982) except without copper and zinc].

Recombinant DNA techniques. DNA manipulations were performed according to Sambrook *et al.* (1989). Vent DNA polymerase (New England Biolabs) was used according to the supplier's instructions in all PCR experiments, due to its proof-reading capability. The identities of cloned PCR products were verified by DNA sequencing.

Isolation of promoter-*lac* fusions. Fragments of a *Sau3AI* digest of pPA87 were ligated into pMU2385, transformed into TG2(pPA87) and plated on LA containing ampicillin, trimethoprim, X-Gal and 0.8 mM $CuSO_4$. Dark blue colonies were replicated onto LA containing carbenicillin, trimethoprim and X-Gal. Recombinants showing copper-inducible β -galactosidase activity (i.e. dark blue in the presence of copper, light blue or colourless in its absence) were identified. Plasmids from these recombinants were transformed into TG2 to separate the promoter-fusion plasmids from pPA87. Trimethoprim-resistant transformants were selected and their plasmid DNA was isolated for nucleotide sequence analysis.

Construction of full and truncated promoter (-*cat*, -*lacZ*) fusions. Oligonucleotide primers were designed to give PCR products from pPA87, which were cloned into the filled-in *HindIII* site of pKK232.8. The fragments generated are described in the legend to Fig. 2, numbered according to the nucleotide sequence reported in Brown *et al.* (1995). The A(box-35-10) and E(box-35-10) PCR products were also cloned into the *SmaI* site of pMU2385, to give pMUA and pMUE, respectively. Recombinants were chosen with fragments orientated with the -10 ends closest to the reporter genes of the vectors.

Construction of *pco-lux* fusions. The A(box-35-10) PCR product, *pco* 128-291, was cloned into the *SmaI* site of pUCD615, to form pLUXA. pLUXAR was formed by cloning the end-filled 5.1 kb *HindIII*-*BglII* fragment of pR5 into the *SmaI* site of pUC19, and cloning the resulting *HindIII* (filled)-*XbaI* fragment into pUCD615 digested with *SmaI* and *XbaI*. Construction of pLUXBR was initiated with ligation of the end-filled 2.5 kb *SphI*-*BglII* fragment of pR5 (S. R. Barrett & N. L. Brown, unpublished) into the *SmaI* site of pBluescript SK(+), followed by cloning of the resulting *SacI* (filled)-*XbaI* fragment into *SmaI*/*XbaI*-digested pUCD615. Plasmid pR5 is a pPA87 derivative in which the non-unique *RsrII* site in wild-type *pcoR* had been replaced by a unique *BglII* site. pA5, containing the 5' end of *pco*, resulted from ligation of the 0.7 kb *HindIII*-*KpnI* fragment from pPA173 into pSU19: this vector was chosen since it is compatible with pUCD615.

Table 1. Plasmids used in this study

Plasmid	<i>pco</i> region	Relevant features†	Source or reference
pKK232.8	—	<i>bla</i> , <i>cat</i> reporter; medium copy number	Brosius & Holy (1984)
pMU2385	—	<i>galK-lacZ</i> reporter, Tp^r ; low copy number	Praszkier <i>et al.</i> (1992)
pRT733	—	<i>bla</i> , <i>TnphoA</i> suicide vector	Taylor <i>et al.</i> (1989)
pBluescript SK(+)	—	<i>bla</i> , cloning vector	Stratagene
pUC19	—	<i>bla</i> , cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pSU19	—	<i>cat</i> , cloning vector	Bartolomé <i>et al.</i> (1991)
pPA87	<i>pcoABCDRSE</i>	12.5 kb <i>Hind</i> III fragment from pRJ1004 in 2 × pBR322	Rouch (1986)
pPA173	<i>pcoABCDRSE</i>	9 kb <i>Bgl</i> II– <i>Hind</i> III fragment from pPA87 in pMU530	Rouch (1986)
pR5	<i>pcoABCDRSE</i>	9 kb <i>Bgl</i> II– <i>Hind</i> III fragment from pPA173 in pACYC184	S. R. Barrett & N. L. Brown, unpublished
pMUE23	<i>PpcoE</i>	0.24 kb <i>Sau</i> 3AI insert in pMU2385	This study
pA	<i>PpcoA</i>	PCR product in pKK232.8	This study
pA(box–35–10)	<i>PpcoA</i>	PCR product in pKK232.8	This study
pA(–35–10)	Truncated <i>PpcoA</i>	PCR product in pKK232.8	This study
pA(–10)	Truncated <i>PpcoA</i>	PCR product in pKK232.8	This study
pE	<i>PpcoE</i>	PCR product in pKK232.8	This study
pE(box–35–10)	<i>PpcoE</i>	PCR product in pKK232.8	This study
pE(–35–10)	Truncated <i>PpcoE</i>	PCR product in pKK232.8	This study
pE(–10)	Truncated <i>PpcoE</i>	PCR product in pKK232.8	This study
pUCD615	—	<i>luxCDABE</i> , <i>bla</i> , Km^r ; high copy number	Rogowsky <i>et al.</i> (1987)
pLUXAR	<i>pcoABCDR</i>	5.1 kb <i>Hind</i> III– <i>Bgl</i> II fragment from pR5 in pUCD615	This study
pLUXBR	<i>pcoBCDR</i>	2.5 kb <i>Sph</i> I– <i>Bgl</i> II fragment from pR5 in pUCD615	This study
pLUXA	<i>PpcoA</i>	PCR product in pUCD615	This study
pA5	<i>pco</i> 5' region	0.7 kb <i>Hind</i> III– <i>Kpn</i> I fragment from pPA87 in pSU19	This study
pMUA	<i>PpcoA</i>	PCR product in pMU2385	This study
pMUE	<i>PpcoE</i>	PCR product in pMU2385	This study
pPA256	<i>pcoRSS*E</i>	3.5 kb <i>Eco</i> RV fragment from pPA87 pBR322	Rouch (1986)

† Km , kanamycin; Tp , trimethoprim.

Reporter activities. Chloramphenicol acetyltransferase (CAT) activities from pKK232.8 and its derivatives were assessed in three ways.

(i) **Growth rate assay.** Overnight cultures in LB were diluted 1/100 in the same media, with or without the addition of 0.6 mM $CuSO_4$, and grown at 37 °C with shaking for 30 min prior to addition of chloramphenicol to 25 $\mu g\ ml^{-1}$. Incubation was continued and OD_{600} was monitored at 30 min intervals for at least 3 h. Growth rates were calculated as $\Delta \log(OD_{600})\ min^{-1}$, standardized as percentages of the standard growth rate in LB without chloramphenicol.

(ii) **MIC assay.** Overnight cultures in LB were diluted 1/100 in 0.85% saline, 1.0% glycerol. Duplicate aliquots were placed in flat-bottomed 96-well plates and replicated onto solid media using a steel-pin replicator (Sigma). The media were LA containing chloramphenicol from 0 to 400 $\mu g\ ml^{-1}$ in twofold steps, with or without the addition of 0.6 mM $CuSO_4$ or $ZnSO_4$. The presence or absence of growth was measured after 16 and 36 h. Assays were performed three times and the means calculated.

(iii) **Enzymic assay.** Overnight cultures in LB were diluted 1/100 in 10 ml LB, with or without 0.6 mM $CuSO_4$, and grown to an OD_{600} of approximately 0.5. Cultures were then centrifuged and resuspended in 1.5 ml assay buffer (0.1 M Tris/HCl, pH 7.8), sonicated and centrifuged to remove cellular debris. CAT activities of cell extracts were determined in triplicate by a spectrophotometric method (Shaw, 1975).

β -Galactosidase activity from pMU2385 and derivatives was determined according to (Miller, 1972). Overnight cultures in LB were diluted 1/100 in the same media with various concentrations of added $CuSO_4$, grown for 2 h at 37 °C and assayed in triplicate.

Light output from pUCD615 and derivatives was measured directly with a Biocounter M 1500 P (Lumac) at room temperature (22 °C). Overnight cultures in LB were diluted 1/50 in the same media, with or without 0.6 mM $CuSO_4$ and grown for 1.5 h at 37 °C. Samples (0.1 ml) were taken and placed on ice for 10 min, warmed to room temperature for 5 min, then assayed in triplicate.

TnphoA mutagenesis. Overnight cultures (0.5 ml) of the donor, SM10(pRT733), and recipient, TG2(pMUE23), were centrifuged, resuspended together in 0.3 ml LB with 0.2% glucose, plated on LA and incubated at 30 °C for 2–5 h. Cells were resuspended with three × 1 ml LB and 0.1 ml aliquots were plated on HA with 0.2% glucose, trimethoprim, tetracycline and kanamycin, with or without 0.4 mM CuSO₄, and incubated at 37 °C overnight. The plates, with 200–300 colonies, were overlaid with soft HA containing X-Gal, with or without 0.4 mM CuSO₄. Plates without copper were examined for derepressed blue colonies at 4–6 h and plates with copper were examined for super-repressed white colonies overnight. This procedure was performed a number of times. Putative positive colonies were purified and the pMU23 reporter plasmids were checked for integrity by restriction enzyme digestion; plasmids were retransformed into TG2 to test for wild-type inducibility.

The sequence location of TnPhoA insertions was determined from templates derived from inverse PCR of chromosomal DNA (Ochman *et al.*, 1988), using primers JL37 (ATA-TTACTGCACCCGGCGGT) and JL07 (GTAATATCGCC-CTGAGCAGC) which match to positions 82–101 and the complement of 59–88 in the TnphoA sequence (Manoil & Beckwith, 1985), respectively.

Copper response profile analysis. Fitted lines were calculated from the data, after subtracting the basal level of activity (at zero added CuSO₄), with the aid of a Hill plot (Hill, 1913). For a sigmoidal response, the data points in the mid-response range lie on a straight line, the apparent Hill coefficient (*H*) being equal to the slope of this line. The fitted curves were derived from the equation:

$$Y_{p_i} = ([Cu_i]H)/([Cu_i]^H + [Cu_{0.5}]^H) \times Y_{max} + Y_b$$

where Y_{p_i} is the predicted β -galactosidase activity at copper concentration i , $[Cu_i]$ is copper concentration, H is the Hill coefficient, $[Cu_{0.5}]$ is the copper concentration at the midpoint of the response curve, calculated from the Hill plot, Y_{max} is the maximum β -galactosidase activity (in this case found by trial and error from determining which value gave the maximum correlation coefficient in a linear regression analysis) and Y_b is the basal level of activity.

DNA sequence determination and analysis. DNA nucleotide sequence was determined with the Sequenase/dideoxynucleotide method for dsDNA plasmid templates (USB) or with an ABI Cycle Sequencer for PCR-derived dsDNA templates, both according to the manufacturers' instructions. Sequence analysis was performed using the programs of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Transcription start points. *In vivo* transcription start points were detected by extension of the oligonucleotides D3006 (TTCGTCGAGACGTTTTCAAC) and D3007 (ACCCAA-ACAATCAATGGC) which are complementary to positions 272–291 and 6643–6660 of the *pco* sequence, respectively, hybridized against total cellular RNA (Debarbouille & Raibaud, 1983). RNA was extracted by the hot acid phenol method (Aiba *et al.*, 1981).

RESULTS

Promoter regions and transcription start points

Copper-dependent promoter activity in *pco* was identified by cloning a *Sau*3AI digest of pPA87 (Table 1) upstream of the promoterless *lacZ* gene in the low-copy-

number plasmid pMU2385 and seeking copper-inducible *lacZ*⁺ colonies in *E. coli* TG2. Five such recombinants were found and DNA sequence analysis showed that they contained overlapping fragments from the region immediately upstream of *pcoE* (Brown *et al.*, 1995). Features of the *pco* sequence are summarized in Fig. 1. One of these clones, designated pMUE23 was used in later experiments.

The *pcoABCD* genes are known to be required for copper-inducible copper resistance (Brown *et al.*, 1995) and the above results showed that the region upstream of *pcoE* contained a copper-inducible promoter. Therefore PCR products of regions 5' to both *pcoA* and *pcoE*, of 291 and 272 bp, respectively, were cloned upstream of the promoterless *cat* gene in the medium-copy-number vector pKK232.8, giving plasmids pA and pE (Fig. 2a, b) and these conferred copper-inducible chloramphenicol resistance. For both the pMU2385 and pKK232.8 derivatives copper-inducible expression of the reporter genes was found whether or not pPA87 was present *in trans*, indicating that chromosomal genes also regulate the *pco* promoters.

Primer extension analysis was carried out to determine the transcriptional start points 5' to both *pcoA* and *pcoE*. RNA samples isolated from TG2 harbouring pPA173 (i.e. a complete *pco* determinant, Fig. 1) or one of the two PCR clones, pA (*PpcoA*) and pE (*PpcoE*), were used with the appropriate oligonucleotide primers. Fig. 3 shows that with copper induction, a single weak band was seen for *PpcoA* RNA from pA, while RNA from pPA173 gave a stronger band in the same position. This corresponds to nt A184 in the *pco* sequence (Brown *et al.*, 1995), 82 bp upstream from the initiation codon of the *pcoA* gene. With copper-depleted growth medium pPA173 showed a much weaker band at this point, showing transcription from this point was copper-inducible. In the presence of copper pE RNA gave a strong signal, with two strong bands at nt A6556 and A6557, 127–128 bp upstream from the start of *pcoE*; these were preceded by four weaker bands at nt 6552–6555. With exposure to the same copper concentration the *PpcoE* promoter gave a much stronger signal than *PpcoA*, suggesting a higher level of transcription initiation. Quantification of the relative levels of RNA was not attempted, as residual copper ions in the preparations may have catalysed some RNA degradation.

Suitable –10 promoter elements were identified at the appropriate locations upstream of both transcription starts. These were similar to the *E. coli* consensus hexamer, being TAGATT (*PpcoA*) and a perfect consensus TATAAT (*PpcoE*). There were relatively weak fits to the –35 hexamer TTGACA, ATGAAT (*PpcoA*) and GCGATA (*PpcoE*) at 18 and 16 bp spacing from their respective –10 elements (Fig. 2a, b). *PpcoA* contains an extended –10 element TGCTAGATT. Such a TGN sequence immediately before the –10 sequence is found in promoters which do not require specific RNA polymerase–DNA contacts in the –35

Fig. 1. Map of the *pco* determinant showing the location of large DNA fragments used in this work. The map is derived from the sequence (EMBL accession number X83541; Brown *et al.*, 1995) and shows the structural genes *pcoABCDERS(S*)E*. p, Promoters 5' to *pcoA* (*PpcoA*) and *pcoE* (*PpcoE*); t, a putative ρ -independent transcription terminator.

Fig. 2. *PpcoA* and *PpcoE* promoter regions. The locations of transcription start points, -10 and -35 promoter regions and copper boxes are shown, with the adjacent structural genes marked. Numbers given at the ends of the sequence maps (and in parentheses below) are *pco* sequence co-ordinates and correspond to the map in Fig. 1 (Brown *et al.*, 1995). The copper-inducible expression of individual constructs is marked; details are shown in Fig. 4. (a) *PpcoA* region and constructs. pA, complete *pcoA* promoter with upstream sequences (1-291); pA(box-35-10), *pcoA* promoter containing copper box, -35 and -10 sequences (128-291); pA(-35-10), *pcoA* promoter region lacking copper box (145-291); pA(-10), *pcoA* promoter region lacking copper box and -35 sequence (163-291); pA(box-35-10) Δ EcoRI, *pcoA* promoter containing copper box, -35 and -10 sequences truncated at the 3' end (128-232). (b) *PpcoE* region and constructs. pE, complete *pcoE* promoter with upstream sequences (6388-6660); pE(box-35-10), *pcoE* promoter containing copper box, -35 and -10 sequences (6486-6660); pE(-35-10), *pcoE* promoter region lacking copper box (6514-6660); pE(-10), *pcoE* promoter region lacking copper box and -35 sequence (6533-6660). (c) Alignment of nucleotide sequences in the upstream region of *pco* and *cop* promoters. The *E. coli* copper box (this study) and *Pseudomonas cop* box (Mills *et al.*, 1994) regions are boxed. For each sequence the bases forming the inverted repeat are underlined and the common dyad centre is shown by arrowheads (< >) below the sequences. The minimum essential regions for copper-inducible transcription for *PpcoA* and *PpcoE* identified from analysis of truncated promoters are shaded. The two vertical bars in the *PcopA* and *PcopH* sequences mark the extent of the DNase I footprint for CopR (Mills *et al.*, 1994).

region (Kumar *et al.*, 1993; Minchin & Busby, 1993). In contrast, *PpcoE* does not contain such a sequence.

Inspection of the promoter regions for further similarities which might correspond to *cis*-acting copper

control regions revealed a conserved 25 bp sequence containing a hyphenated dyad AxxTxACaxxATTG-TxATxAxTxG. It overlapped the -35 hexamer region in *PpcoA* (dyad centre at -42.5 relative to the start of transcription) and was 5' to the -35 region in *PpcoE*

Fig. 3. Primer extension analysis for *PpcoA* and *PpcoE* RNA from cells grown with (+) or without (–) 0.8 mM copper. Lanes 1–8, with primer D3006 (for *PpcoA*): 1, pKK232.8(+); 2, pA(+); 3–6, *PpcoA* sequence (TGCA); 7, pPA173(+); 8, pPA173(–). Lanes 9–14, with primer D3007 (for *PpcoE*): 9, pKK232.8(+); 10, pE(+); 11–14, *PpcoE* sequence (TGCA). Exact transcriptional start points were deduced from shorter exposures than that shown here.

(dyad centre at –54.5), bp 132–156 and 6492–6516, respectively (Fig. 2c). This motif is similar to sequences seen in the regions of plasmid (*copA*) and chromosomal (*copH*) genes from *P. syringae* pv. *tomato* to which the CopR protein binds (Mills *et al.*, 1994; Fig. 2c). The consensus of all four sequences is the 25 bp motif AgxTtACaxa <> AxTGTaATxaxxxxG (where <> is the dyad centre). The motif is referred to as a ‘copper box’.

Are copper box sequences required for copper-inducible transcription?

The function of the copper boxes was tested by cloning PCR products into the CAT reporter vector pKK232.8, to give transcriptional fusions. Three types of PCR product were used for each promoter region (Fig. 2

legend), having identical 3′ ends but varying in their 5′ ends, so that they contained (i) the copper box and –35, –10 elements, (ii) only the –35 and –10 elements or (iii) only the –10 element (Fig. 2a, b). The various recombinant plasmids were assayed *in vivo* for metal-inducible transcriptional activity by measurement of (a) the growth rate of transformants when challenged with chloramphenicol, (b) their MIC values for chloramphenicol and (c) CAT activity of cell extracts (Fig. 4). CAT assays provided the most direct measure of reporter activity, but the two growth assay methods proved to be more sensitive at low levels of induction.

Only plasmids containing intact copper boxes gave significant copper inducibility, while the (–35–10) and (–10) fragments gave weak constitutive responses (Fig. 4). Both *PpcoE*(–35–10) and (–10) fragments gave near background constitutive responses, while the *PpcoA*(–10) fragment (which contained the extended –10 sequence) gave a higher constitutive response, close to the value for the uninduced complete promoter region. The *PpcoA*(–35–10) construct also gave a constitutive response significantly above background in the MIC and growth rate tests. The CAT assays had relatively high errors at very low enzyme activities. The higher activities of the *PpcoA*(–10) and *PpcoA*(–35–10) constructs is consistent with the extended –10 motif in this promoter; similar elevated levels of transcription are observed in the *E. coli gal* operon P1 promoter (Chan & Busby, 1989; Minchin & Busby, 1993). Truncated promoters were also tested in MIC assays with the presence of plasmid-expressed PcoR and gave qualitatively similar results to those when it was absent (data not shown).

In the *PpcoA*(–35–10) construct 12 bp of the copper box motif remains due to the overlap of the copper box and –35 regions. However, this construct still failed to exhibit inducible activity. This indicated that the 17 bp region from bp 128 to 145, which contained the 5′ half of the copper box motif in *PpcoA*, was necessary for induction. Correspondingly, for *PpcoE* the 28 bp region from 6486 to 6514, which contained 22 bp of the 25 bp copper box, was necessary for induction.

All three methods confirmed *PpcoE* to be stronger than *PpcoA*, with plasmids carrying both full-length and (box–35–10) fragments, with up to a 25-fold difference in induced CAT activity. *PpcoE* also provided a higher level of constitutive activity in the absence of added copper sulphate. Zinc sulphate was also tested for regulatory activity in the MIC test and was found to give a small level of induction for both *PpcoA* and *PpcoE* in both complete and (box–35–10) form, but was only statistically significant for *PpcoE* (Fig. 4c, d). An attempt was made to test induction with silver ions, since Ag(I) is similar to Cu(I), but their toxicity at the necessary concentrations prevented cell growth.

The *PpcoA* minimal promoter fragment A (box–35–10) was further truncated at the 3′ end by removing DNA downstream of bp 232, 47 bp downstream of the transcription start, using the internal

Fig. 4. Assays of wild-type and truncated promoters linked to the CAT reporter gene in pKK232.8. (a), (c) and (e) are *PpcoA*; (b), (d) and (f) are *PpcoE*, with (+) and without (-) induction with copper; error bars are one SD. (a, b) Relative growth rates; (c, d) MIC measurements; (e, f) CAT assays (compared to the host TG2 strain in the absence of plasmid as zero). The constructs used are summarized in the legend to Fig. 2. In all cases the data bars are: hatched, uninduced; black, following induction with copper sulphate; white (c and d only), following induction with zinc sulphate.

EcoRI site. This 105 bp DNA, A(box-35-10) Δ *EcoRI*, retained copper-inducible transcription activity (data not shown).

Insertion of the A PCR product, *pco* bp 1-291, in pKK232.8 in the reverse orientation to pA revealed strong constitutive promoter activity divergently from

Fig. 5. Assays of *pco::lux* fusions in various plasmids as described in the text. Lux activity was measured in RLU output (Relative Light Units) per OD₆₀₀ unit (cell density factor) per second per litre (sample volume); error bars are one SD. Activities were measured in the presence (black bars) or absence (hatched bars) of 0.6 mM CuSO₄.

PpcoA. This may allow expression of an ORF of unknown function (see Fig. 2a).

Expression of the *pcoRS* region

While inducible control of the structural genes for copper resistance, initiated from *PpcoA* and *PpcoE*, was consistent with their function, it was unclear if the *trans*-acting regulatory genes would be under similar control. The regulatory genes *pcoRSS** form a distinct block within the *pco* determinant, with significant gaps between them and the flanking resistance genes (Fig. 1; Brown *et al.*, 1995), which might allow separate expression. To assess transcription through *pcoR* two *pco-lux* fusions were constructed by cloning into the *lux* vector pUCD615 (i) the segment from the *Hind*III site 5' to *PpcoA* to mid-*pcoR* (bp 4807), to form pLUXAR, and (ii) the segment from mid-*pcoB* to mid-*pcoR* (bp 2339–4807) to form pLUXBR. The *lux* system was used because of its simplicity and great sensitivity in detecting low levels of transcription, and because of its potential use in biotechnology. Fig. 5 shows the activities of these plasmids; pLUXAR exhibited strongly inducible lux activity, similar to that shown by pLUXA which contains the *PpcoA*(box–35–10) fragment. In contrast, pLUXBR lacks *PpcoA* and showed a low degree of constitutive activity, which was significantly greater than pUCD615 vector alone. Whether this is a result of a specific promoter or non-specific promotion is unclear. Either way, the result suggested that *pcoRSS** could be transcribed at a low level in the absence of

Fig. 6. Promoter response profiles across a range of copper concentrations. (a) *PpcoA* (pMUA); (b) *PpcoE* (pMUE). Both profiles have curves for Hill coefficients of 0.67 (full line) and 1.0 (dashed line) fitted.

induction at *PpcoA*. In addition, the detection of inducible transcription at *pcoR*, in pLUXAR, indicates that *pcoRSS** can also be expressed from the *PpcoA* promoter; i.e. *pcoABCDRSS** can be transcribed as a unit. The idea that the *pcoRSS** genes can be transcribed separately while a block transcript can arise from *PpcoA* is supported by complementation analysis of Tn1000 insertion mutants (Brown *et al.*, 1995; Rouch *et al.*, 1989). This showed that *pcoABCD* form a single complementation group separate from the regulatory genes.

Response profiles of *pco* promoters

The response profiles of *PpcoA* and *PpcoE* to a range of copper sulphate concentrations were assessed, with transcription from the minimal promoters (box–35–10) fragments used to drive β -galactosidase reporter activity from the low-copy-number vector pMU2385 (Fig. 6). Curves were fitted to the data using the Hill equation, which assumes a response profile to be sigmoidal in shape. A standard Michaelis–Menten response gives a sigmoid with a Hill coefficient, *H*, of 1.0, while steeper and flatter responses have *H* values greater or less than 1.0, respectively. Since no relative flattening of the responses for both *PpcoA* and *PpcoE*

Fig. 7. (a) β -Galactosidase reporter activities of *TnphoA* mutants. Strains containing the reporter plasmid pMUE23 (*PpcoE*::*lacZ*) are shown for each copper concentration in the order (top to bottom): mutants 1, 12, 13, 14, 16, 17 and parental strain TG2; each assay is the mean of two independent measurements. (b) Location of *TnphoA* insertions in the *E. coli* *pts* and *cya* chromosomal regions (from EMBL database files *ecphosys* and *ecyaa01*, respectively). Half arrows show the gene orientations; arrows mark *TnphoA* insertions in the orientations shown.

was seen at the higher copper concentrations used, as expected for saturation of a sigmoidal response, it was assumed that these copper levels were not high enough to show flattening. Copper toxicity precluded the use of higher copper levels. Nevertheless, the calculated curves gave an acceptable fit to the data. Fig. 6(a) shows the response for *PpcoA*, fitted with a curve of $H = 0.67$. A curve with $H = 1.0$ is given for comparison, demonstrating that *PpcoA* gives a shallower response than a standard Michaelis-Menten curve. Little change in promoter activity was seen for copper levels up to 0.02 mM, while above 0.1 mM activity increased markedly with rising copper level.

As reported in separate experiments with *PpcoE* on a different DNA fragment (Mills *et al.*, 1994), the data for *PpcoE* fitted with a H value of 0.67, thus giving a response similar in shape to *PpcoA*. However, the actual β -galactosidase activities for *PpcoE* were an order of magnitude higher than for *PpcoA*. Both promoters gave similar basal levels in the absence of added copper,

PpcoA 45 ± 0.8 and *PpcoE* 41 ± 3 units, but at 4 mM copper they were 212 ± 14 and 3256 ± 324 units, respectively. The relative differences in strength of *PpcoE* and *PpcoA*, as detected using the β -galactosidase reporter plasmid pMU2385, was similar to that seen with the CAT reporter plasmid pKK232.8 (Fig. 4).

Investigation of chromosome-directed regulation

To identify chromosomal genes affecting the regulation of *pco*, *TnphoA* mutagenesis was performed with pMUE23 acting as the reporter plasmid. Mutants were screened for either a derepressed or super-repressed response, as judged by plate tests. From this initial screening of over 40000 transconjugants, six mutants were chosen for further study: numbers 1, 12, 13, 14, 16 and 17. β -Galactosidase activity of the mutant strains containing pMUE23 was determined across a range of copper sulphate concentrations (Fig. 7a). Each mutant retained copper inducibility, but showed an altered response compared to wild-type. Mutants 1 and 12 were slightly derepressed in the absence of added copper and showed increased activity at all copper concentrations. Mutants 13, 14 and 17 showed increased reporter gene activity at low (0.0 and 0.2 mM) added CuSO_4 , while 13 and 14 showed greater induction than wild-type at high (1.0 and 2.0 mM) added CuSO_4 . Mutant 16 alone showed a super-repressed phenotype. Furthermore, the additional presence of pPA256, which bears the *pco* regulatory genes *pcoRSS**, failed to restore wild-type reporter activity (data not shown). The six mutants all showed significantly enhanced copper tolerance, with MICs of 2.0 mM CuSO_4 compared to 1.2 mM for the parental strain and other *TnphoA* mutants.

The sites of *TnphoA* insertion were examined by sequencing of PCR products obtained by inverse PCR. Chromosomal sequence flanking the 5' end of *TnphoA* was obtained for five mutants and used to search the EMBL and GenBank DNA sequence databases for similarities. Chromosomal DNA from four mutants, 1, 12, 13 and 16, showed near identity to known *E. coli* DNA sequences, while the 316 bp sequence from mutant 17 remained unidentified. Sequences from mutant 14 could not be obtained. Three of the mutants, 1, 12 and 13, had resulted from insertions in two genes specifying parts of the *pts* sugar phosphotransferase system, *ptsH* and *ptsI*, while mutant 16 contained an insertion in the adenylate cyclase gene, *cya*.

DISCUSSION

The two copper-dependent promoters found within the *pco* determinant, *PpcoA* and *PpcoE*, differ both in structure and relative activity. Both contain a conserved 25 bp copper box motif, of which at least the 5' half was necessary for copper-dependent activity. The motif is located differently in each case: that for *PpcoA* is centred at -42.5 and that for *PpcoE* at -54.5 relative to the start of transcription. The related *cop* boxes identified in the plasmid-encoded *cop* operon and its

chromosomal homologue from *Pseudomonas syringae* pv. *tomato* as binding sites for the regulatory protein CopR (Mills *et al.*, 1994) are at position -42.5 . The CopR protein has a 28 bp DNase I footprint on the top strand of the two *Pseudomonas* promoters, *in vitro* (Mills *et al.*, 1994). The PcoR protein and its putative chromosomal homologue, CutR, are expected to recognize the copper box motifs in both *PpcoA* and *PpcoE*. The *Pseudomonas* promoter regions are otherwise different from the two *E. coli* promoters, possibly due to species differences in RNA polymerase action.

The centre of binding of the cAMP receptor protein (CRP) transcriptional activator at class II sites is at a similar position (-41.5) to that for the copper box at *PpcoA*. Furthermore, *PpcoA* (though not *PpcoE*) has an extended -10 promoter element, which is also found in the CRP-dependent *gal* operon P1 promoter (Chan & Busby, 1989; Minchin & Busby, 1993). This extended promoter element may account for the low-level transcriptional activity seen with the *PpcoA*(-10) and ($-35-10$) constructs, since specific -35 contacts for RNA polymerase may not be required. *PpcoE* does not show such an extended -10 sequence and the *PpcoE*($-35-10$) and *PpcoE*(-10) promoter constructs failed to show significant constitutive activity. These data support the importance of sequences upstream of the -35 region in *pco* promoter activity, in addition to the -10 elements.

PpcoE provides much stronger copper-dependent activation of transcription than *PpcoA*, as judged by both the CAT and β -galactoside reporter studies, as well as by primer extension analysis. This may be accounted for in part by the different placement of the copper box in *PpcoE*, at -54.5 . A placement this far 5' to the start of transcription may allow for direct contact by PcoR/CutR with RNA polymerase as the means of activation. The best studied class 2 receiver OmpR, a positive as well as negative regulator, also binds far upstream, at multiple sites for the *ompF* promoter, though its exact mode of regulation is unclear (Rampersaud *et al.*, 1994, 1989). In contrast, sequences upstream of the copper box in both *PpcoA* and *PpcoE* appear unnecessary for induction of promoter activity.

The sequence differences between the *PpcoA* and *PpcoE* promoters may reflect the requirements for differential expression of the resistance genes. The *pcoABCD* genes are expressed on a polycistronic message from *PpcoA*, which allows for stoichiometric production of the four components. The separate expression from *PpcoE* may allow for higher, non-stoichiometric production of PcoE compared to the other components, or enables its expression at lower copper concentrations. Consistent with this, the *pcoE* gene has a codon usage typical of a highly expressed protein, in contrast to *pcoABCD* which do not (Brown *et al.*, 1995). Thus, PcoE may act separately from PcoABCD at high copper concentrations. PcoE is a periplasmic protein (C. J. D. Yates & N. L. Brown, unpublished) and it may be responsible for sequestering copper at low toxic copper concentrations before the *pcoABCD* operon is fully induced.

Two important requirements of a system for managing exposure of a cell to a trace element that is also toxic, such as copper, are: (1) the system is relatively specific for the particular trace element, since lack of specificity risks poor control of that and other similar metals; and (2) the system is regulated in such a way as to maintain cellular homeostasis for the trace element, as a deficiency would reduce growth and an excess is toxic. The specificity requirement was examined at the regulatory level by testing for inducibility of *PpcoAE* by zinc ions: Zn(II) is the most similar trace element to Cu(II). Zinc did cause some activation but at a very low level compared with that caused by copper at a similar concentration. In this case regulation was provided by the chromosomal system, so the presumptive CutR-dependent regulatory mechanism has good specificity for copper.

The shape of the induction curve, derived from measuring promoter activities for a range of copper concentrations, has been predicted to be influenced by the requirement for homeostasis (Lee *et al.*, 1990). For a metal that is required for growth, such as copper, very high or very low metal concentrations may be disadvantageous to cell growth, and a promoter expressing genes for metal resistance proteins is expected to be expressed proportionally to the concentration of the metal. The response of *PpcoA*- and *PpcoE*-*lacZ* fusions to copper under chromosomal regulation by CutR do show gradual response profiles. These are flattened sigmoidal curves with apparent Hill coefficients of 0.67, which give a relatively wide range over which an approximately proportional response to copper occurs, compared with the normal Michaelis-Menten response. The observation that the level of expression of pRJ1004 (*pco*)-mediated copper resistance is proportional to the external copper concentration (Rouch *et al.*, 1985) is consistent with these reporter gene studies, although detailed analyses of the current data are complicated by the presence of both chromosomal and plasmid-borne regulatory systems.

In an attempt to inactivate the *cutR* gene, to allow PcoR activity to be examined alone, a number of insertion mutants were obtained that derepressed or hyper-repressed *pco* promoter activity. Interestingly, no mutations were identified in putative two-component regulatory genes, but in four other genes, *ptsH* (PTS phospho-carrier protein), *ptsI* (PTS enzyme I) and *cya* (adenylate cyclase, catalysing cAMP production) and one unknown gene. The PTS system may therefore mediate the effects seen. The *ptsHI* gene products are thought to interact with the CheA/CheY chemotaxis regulators (Titgemeyer, 1993), and there may be cross-talk with the presumptive CutR/CutS system. All mutants showed some inducibility and the presumptive primary response regulator, CutR, was not identified.

The dual control of the *pco* promoters by chromosomal and plasmid regulators indicates integration of *pco* expression with that of the cellular copper control systems. Furthermore, the shapes of the promoter

response curves support the idea that expression of *pco* is regulated in such a way as to help maintain cellular copper homeostasis. Thus, the *pco*-encoded copper resistance system can be seen as an auxiliary copper control mechanism for the cell, which co-operates with the host cell copper management systems, to extend the range of environmental copper concentrations over which the cell can survive.

ACKNOWLEDGEMENTS

We are grateful to Barry Lee for discussion and to Carol Bender for communicating results prior to publication. This work was supported by the Medical Research Council, UK (grant G.8921751CB) and the Royal Society Small Grants Scheme. Automated DNA sequencing done by Alta Bioscience, The University of Birmingham, was on an ABI machine provided by the Wellcome Trust and the MRC. For computer support we thank staff at both the Academic Computing Service, the University of Birmingham, and SEQNET, Daresbury, UK.

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Received 13 September 1996; revised 2 December 1996; accepted 5 December 1996.