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

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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 that 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, 1983).
1983). The copper resistance specified by this plasmid involves the pco gene cluster, which contains seven genes, pcoABCDRSE (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, copABCDRS, 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-respondive 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 pslH 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 (Δlac-pro supE thi hsdS recA::Ts10 [F' traD36 proAB lactA lacZAM153]) and SM10 (thi tir 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 acetyltransferase) 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 (1 l): 8 g tryptone, 4 g NaCl and 15 g agar; similarly for soft HA except 6 g agar. Standard additions were (µg ml⁻¹): carbenicillin, 250; tetracycline, 25; trimethoprim, 25; kanamycin, 50; X-Gal, 40. CuSO₄ and ZnSO₄ were ultrapure grade (Aldrich); for induction a stock solution of CuSO₄ (200 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 CuSO₄ 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 CuSO₄. 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 Smal site of pMU2385, to give pMU 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 Smal site of pUCD615, to form pLUXA. pLUXA was formed by cloning the end-filled 5·1 kb HindIII–BglII fragment of pR5 into the Smal site of pUC19, and cloning the resulting HindIII (filled)–XbaI fragment into pUCD615 digested with Smal 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 Smal site of pBluescript SK (+), followed by cloning of the resulting SacI (filled)–XbaI fragment into Smal/XbaI-digested pUCD615. Plasmid pR5 is a pPA87 derivative in which the non-unique RsII 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th><strong>pcO</strong> region</th>
<th>Relevant features†</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK232.8</td>
<td>–</td>
<td>bla, cat reporter; medium copy number</td>
<td>Brosius &amp; Holy (1984)</td>
</tr>
<tr>
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<td>galK–lacZ reporter, Tp; low copy number</td>
<td>Praszker et al. (1992)</td>
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<td>pRT733</td>
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<td>bla, TnphoA suicide vector</td>
<td>Taylor et al. (1989)</td>
</tr>
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<td>pBluescript SK(+)</td>
<td>–</td>
<td>bla, cloning vector</td>
<td>Stratagene</td>
</tr>
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<td>pUC19</td>
<td>–</td>
<td>bla, cloning vector</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pSU19</td>
<td>–</td>
<td>cat, cloning vector</td>
<td>Bartolome et al. (1991)</td>
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<td>Rouch (1986)</td>
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<td>9 kb <em>BglII–HindIII</em> fragment from pPA87 in pMU530</td>
<td>S. R. Barrett &amp; N. L. Brown, unpublished</td>
</tr>
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<td>pR5</td>
<td><strong>pcOABCDRSE</strong></td>
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<td>PpcOe</td>
<td>0.24 kb <em>Sau3AI</em> insert in pMU2385</td>
<td>This study</td>
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<td>pA</td>
<td>PpcOA</td>
<td>PCR product in pKK232.8</td>
<td>This study</td>
</tr>
<tr>
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<td>PpcOA</td>
<td>PCR product in pKK232.8</td>
<td>This study</td>
</tr>
<tr>
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<td>Truncated PpcOA</td>
<td>PCR product in pKK232.8</td>
<td>This study</td>
</tr>
<tr>
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</tr>
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<td>This study</td>
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<td>pUCD615</td>
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<td>Rogowsky et al. (1987)</td>
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<td>This study</td>
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<td>pLUXA</td>
<td>PpcOA</td>
<td>PCR product in pUCD615</td>
<td>This study</td>
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<tr>
<td>pA5</td>
<td><em>pcO S′</em> region</td>
<td>0.7 kb <em>HindIII–KpnI</em> fragment from pPA87 in pSU19</td>
<td>This study</td>
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<td>pMUA</td>
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<td>PCR product in pMU2385</td>
<td>This study</td>
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</tr>
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<td>pPA256</td>
<td><strong>pcoRSS′E</strong></td>
<td>3.5 kb <em>EcoRV</em> fragment from pPA87 pBR322</td>
<td>Rouch (1986)</td>
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† 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 CuSO₄, and grown at 37 °C with shaking for 30 min prior to addition of chloramphenicol to 25 μg ml⁻¹. Incubation was continued and OD₆₅₀ was monitored at 30 min intervals for at least 3 h. Growth rates were calculated as Δlog(OD₆₅₀)/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 10 ml LB, with or without 0.6 mM CuSO₄, and grown to an OD₆₅₀ 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).

(iii) Enzymic assay. Overnight cultures in LB were diluted 1/100 in 10 ml LB, with or without 0.6 mM CuSO₄, and grown to an OD₆₅₀ 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 CuSO₄, 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 1/30 in the same media, with or without 0.6 mM CuSO₄, 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[pK7733], 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, tetra-cycline 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 pMUE23 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-TTACTGCACCCGGGCCGT) 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:

\[ Y_p = \left( \frac{[Cu]_i H}{([Cu]_1^n + [Cu]_2^n)} \right) \times Y_{max} + Y_b \]

where \( Y_p \) is the predicted β-galactosidase activity at copper concentration \( i \), \([Cu]_i \) is copper concentration, \( H \) is the Hill coefficient, \([Cu]_1 \) 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 (TTTCGTCGAGGCTTTTCCA) and D3007 (ACCCAA-ACAATCATGTC) 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 Sat3AI 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 start of pcoA, 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 TTTGACA, ATGAAAT (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 pcoABCDRS(S*)E. p, Promoters 5' to pcoA (PpcoA) and pcoE (PpcoE); t, a putative p-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)ΔEcoRI, 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 (<>). In the sequence of PpcoA pco box, a 25 bp region extending from the −35 hexamer to the −10 hexamer is indicated by a vertical bar. This 25 bp region is conserved in the E. coli cop box.

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 AxxTxAxACAxxATTG−TxATxATxxG. 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, 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 (capA) and chromosomal (capH) 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< >AxTGTaATxaG (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
Regulation of copper resistance

**Fig. 4.** Assays of wild-type and truncated promoters linked to the CAT reporter gene in pKK232.8. (a), (c) and (e) are \( \text{PpcoA} \); (b), (d) and (f) are \( \text{PpcoE} \), 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.

\text{EcoRI} \text{ site. This 105 bp DNA, A(box − 35–10)ΔEcoRI, retained copper-inducible transcription activity (data not shown).} \text{ 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}
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 HindIII 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 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
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 $\beta$-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 $45 \pm 0.8$ and PpcoE $41 \pm 3$ units, but at 4 mM copper they were $212 \pm 14$ and $3256 \pm 324$ units, respectively. The relative differences in strength of PpcoE and PpcoA, as detected using the $\beta$-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. $\beta$-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 pcoABCD genes. The pcoABCD promoters 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 (Ttgemeyer, 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.

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