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ORIGINAL PAPER

Identification of a copper-responsive promoter and development of a copper biosensor in the soil bacterium *Achromobacter* sp. AO22

Shee Ping Ng · Enzo A. Palombo · Mrinal Bhave

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Abstract A number of human activities result in environmental contamination with copper compounds that can cause severe detrimental effects on the ecosystem as well as human health. The physico-chemical methods of metal detection have limitations such as inability to distinguish between total versus bio-available metals and differences in metal uptake in different organisms. The heavy metal resistance-encoding genetic systems of certain bacteria provide critical tools for development of biosensors for these purposes. This study reports a copper biosensor utilizing the *cop* operon of the heavy metal resistant bacterial isolate, *Achromobacter* sp. AO22, isolated from a contaminated site in Australia. A section located between the divergently transcribed putative response regulator gene *copR* and multicopper oxidase gene *copA* that included a palindromic *cop* box was identified as a copper-responsive promoter using a *lacZ* reporter construct, pCOPRP, in *E. coli*. The expression was found to be enhanced by inclusion of *copR*. Another engineered strain, AO22(pCOPRP), showed stronger induction, and the *lacZ* expression in both backgrounds was enhanced significantly (250–400 fold) by copper but minimally by other metals. The construct in *Achromobacter* sp. AO22 thus has a high potential as biosensor for detecting copper bioavailability (hence potential toxicity) in a soil bacterial background, while the construct in *E. coli* is ideal for laboratory-based testing.

Keywords Heavy metals · Copper · *Cop* operon · *cop* box · Two-component signal transduction · Biosensors

S. P. Ng · E. A. Palombo · M. Bhave (✉)
Environment and Biotechnology Centre, Faculty of Life and Social Sciences, Swinburne University of Technology, PO Box 218, Melbourne, VIC 3122, Australia
e-mail: mbhave@swin.edu.au

S. P. Ng
e-mail: spng@nilai.edu.my

Present Address:
S. P. Ng
School of Science and Technology, Nilai University College, Persiaran Kolej Bbn, 71800 Putra Nilai, Negeri Sembilan, Malaysia

Introduction

Copper-containing compounds are introduced to the environment through mining activities, metal working industries and common agricultural chemicals such as bactericides, fungicides and animal growth promoters. Copper is an essential trace element, mainly in electron transfer and dioxygen transport and activation, but is also highly toxic when in excess, due to its high redox potential and ability to produce reactive oxygen species harmful to cellular components (Dorsey and Ingerman 2004). Concerns about heavy metal pollution have increased in recent years, due to several well documented cases highlighting its drastic effects on ecosystems and health. Some notable cases of anthropogenic pollution include the copper and mine tailings pollution at the Ok Tedi mine, Papua New Guinea, which affected the livelihood of thousands of villagers and devastated the ecosystems over 2,000 km^2 (Hettler et al. 1997), Mole River, Australia (Cu/As/Ag/Pb/Zn pollution; 60 km^2 area affected) (Ashley and Lottermoser 1999), and soil contamination in Dabaoshan, China (Cd/Cu/Pb/Zn; contaminated food crops) (Zhuang et al. 2009). These cases exemplify the need for early detection of pollutants, before the onset of consequences. The physico-chemical methods of detection are highly sensitive and accurate, but have limited applicability in the biological contexts as they

cannot distinguish between the total amounts in the environment and the bioavailable (thus potentially toxic) levels, or differences in metal uptake in different species/environments due to factors such as physical properties of the soil or differences in cell wall structures. Research efforts are thus now being directed towards developing biosensors for detection of a heavy metal that has been actually taken up by a cell and is potentially toxic if it exceeds the threshold for that species. For this purpose, the genetic systems of certain heavy metal tolerant bacteria are being focussed on, due to their remarkable properties of perception, uptake, and removal, accumulation and/or detoxification of metals. In a typical engineered biosensor, the response of a promoter to an inducer (i.e., a heavy metal) activates the transcription of a reporter gene driven by this promoter, such as β-galactosidase (*lacZ*), luciferase, or green fluorescent protein. Such biosensors are renewable and have the potential to significantly reduce the costs of environmental monitoring (Corbisier et al. 1999; Verma and Singh 2005).

The heavy metal biosensors developed so far include a copper biosensor using *E. coli copA* (in *E. coli* host) (Riether et al. 2001), a cadmium and lead biosensor in *E. coli* (Shetty et al. 2003), a multi-metal (Cd, Pb, Sb) biosensor also in *E. coli* (Liao et al. 2006), a Cd/Zn/Cr/Hg/Pb biosensor using *E. coli zntA* in *E. coli* (Riether et al. 2001), or the *copSRcopA* system of a megaplasmid of *C. metallodurans* in the same host (Leth et al. 2002). While these systems are sensitive and adequate for lab-based detection, it should be noted that a major aim of environmental monitoring is detecting the bioavailability of metal(s) to diverse species in the ecosystem, while the above efforts have focussed on metal uptake and reporter expression in *E. coli*, an enteric bacterium unlikely to occur in natural environments. Only a few examples include biosensor developments using environmental bacteria as hosts, e.g., BIOMET®, which utilises several *Cupriavidus* species including *C. metallidurans* CH34 (Diels et al. 2009), and *P. fluorescens*-based biosensors (Nybroe et al. 2008). Nine heavy metal-specific biosensors in Gram negative (*Escherichia, Pseudomonas*) and Gram positive (*Staphylococcus, Bacillus*) bacteria showed that different genera need to be used in parallel, as the bioavailability to different bacteria may differ (Bondarenko et al. 2008).

The aim of this work was to develop a biosensor system for detecting copper bioavailable to soil bacteria. For this purpose, we exploited a soil bacterium *Achromobacter* sp. AO22, isolated earlier from a contaminated site and found tolerant to lead, copper and other metals (Trajanovska et al. 1997). The strain houses a mercury-resistance operon, *merRTPADEorf2,* on a Tn*21*-related transposon (Ng et al. 2009), as well as a second non-identical operon, *mer2* (*merRTPA*) and a *cop* operon (Ng et al. 2012; Genbank accession number GU929214). The *cop* operon encodes 10 putative genes, *copSRABGOFCDK,* with significant similarity to a *cop* locus in *Delftia acidovorans* SPH-1 (GenBank NC_010002). This work investigates copper inducibility of the *copA* and *copR* promoters, as well as engineered AO22 and *E. coli* as hosts for a copper biosensor.

Materials and methods

Construction of *lacZ* reporter plasmids containing *copA* and *copR* promoters:

The *cop* operon (putative genes *copSRABGOFCDK*) of *Achromobacter* sp. AO22 (Ng et al. 2012; Genbank number GU929214) contains a 255 bp region between the proposed start codons of *copR* and *copA* (Fig. 1). Analysis by BPROM (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) (Ng et al. 2012) had identified two putative promoters in this region, one upstream of *copA* and the other in the complementary strand, upstream of *copR*. The region also contained a 7 bp perfect palindrome ATTACATGAATGTAAT with identity to '*cop* boxes' reported in other copper (Mills et al. 1994; Rouch and Brown 1997) and silver (Gupta et al. 1999) inducible genes. These putative promoter regions were amplified from the genomic DNA of *Achromobacter* sp. AO22 using various primer combinations (Table 1). The cloning vector used was the promoter-less vector pMU2385, which carries a trimethoprim resistance (Tp^r) marker and a multiple cloning site (MCS) upstream of a *galK'-lac'Z* fusion (Praszkier et al. 1992). Restriction sites HindIII and EcoRI were introduced into the forward and reverse primers, respectively, for directional cloning of the putative promoter regions into pMU2385, upstream of the *lacZ* reporter gene. Amplifications were typically carried out at primer annealing temperatures of 60°C. The resultant products were purified using Perfectprep® Gel Clean Up kit (Eppendorf), double-digested with EcoRI + HindIII and ligated to similarly-digested pMU2385. The ligation mixtures were transformed into *E. coli* CB454 (Schneider and Beck 1986) (Table 2), potential clones selected by growth at 37°C on Luria–Bertani (LB) agar plates containing trimethoprim, and subjected to DNA sequencing.

Electroporation of *E. coli* and *Achromobacter* sp. strain AO22

Electroporations of bacterial cells with plasmid constructs were performed with the Gene Pulser Xcell™ Electroporation System (BioRad, Australia). Aliquots of *E. coli*

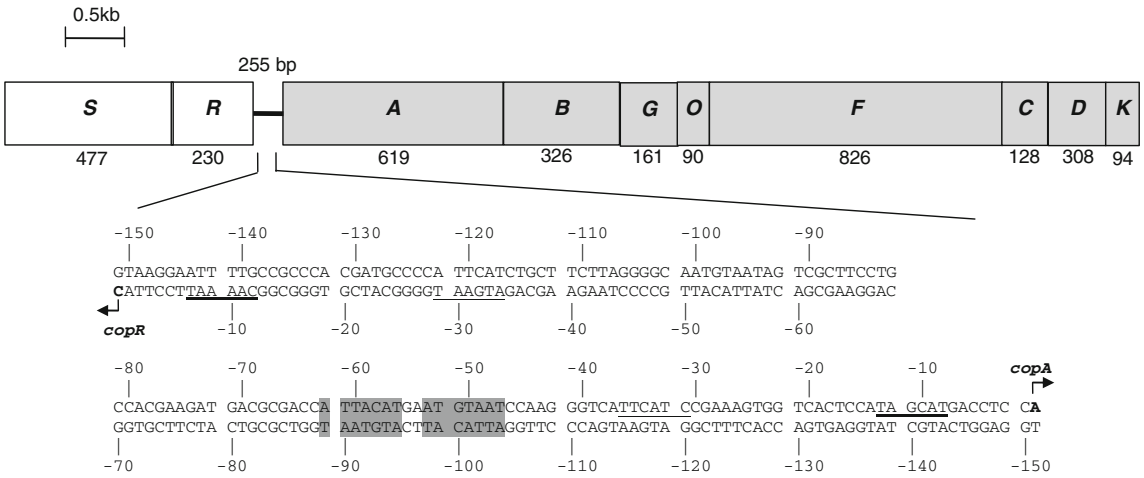


Fig. 1 Putative promoters of the *copA* and *copR* genes of *Achromobacter* sp. AO22 *cop* locus. *Blocks* represent individual genes. *Numbers below blocks* indicate the length (in amino acids) of the putative proteins encoded by each gene. The expanded sequence represents the intergenic region between the putative transcription initiation sites of AO22 *copR* and *copA* genes, indicated by *arrows*. The predicted -10 and -35 hexamers of *copA* and *copR* are shown by *dark* and *light underlines*, respectively. The AT-rich inverted repeat is *shaded*

Table 1 Primers used for amplification of various putative promoter regions for cloning

Constructs	Forward primer[a]	Reverse primer[a]	Coordinates (with respect to gene copA) and length of fragments
pCOPSP	COPSP-F	COPAP-R	−2359 to +61; 2420 bp
	CA**AAGCTT**TTAAGCGATGCGAGGCAG	AT**GAATTC**CCTGAACAAAACGCCG	
pCOPSP2	COPSP-F	COPSP-R	−2359 to −119; 2240 bp
	As above	As above	
pCOPAPF	COPAP-F	COPAP-R	−168 to +61; 229 bp
	TC**AAGCTT**CTGCCACGAAGATGAC	As above	
pCOPAPF1	COPAP-F1	COPAP-R	−132 to +61; 193 bp
	TA**AAGCTT**ATCCAAGGGTCATTCAT	As above	
pCOPAPF2	COPAP-F2	COPAP-R	−113 to +61; 174 bp
	TA**AAGCTT**GAAAGTGGTCACTCC	As above	
pCOPAPF3	COPAP-F3	COPAP-R	−261 to +61; 322 bp
	AA**AAGCTT**CGCATGCCCCATGA	As above	
pCOPRP	COPRP-F	COPAP-R	−998 to +61; 1059 bp
	AC**AAGCTT**CCGTGACCCTCAATGC	As above	
pCOPRP2	COPRP-F	COPSP-R	−998 to −119; 879 bp
	As above	CG**GAATTC**ATGACCCTTGGATT	
pCOPR O/P	COPR O/P–F	COPR O/P-R	+61 to −261; 322 bp
	GT**AAGCTT**GCCTGAAATCCTCCATAG	CC**GAATTC**TCTTCAACGACCAACAG	

[a] The restriction site introduced in each primer is shown in bold and underlined

CB454 cells were thawed on ice, 1–5 μL (approximately 100 ng) of plasmid added, tubes held on ice for 1 min and transferred to a chilled 0.1 cm electroporation cuvette, which was then pulsed using the preset *E. coli* protocol for 4.9–5.0 ms (capacitance 25 μF; external resistance 200 ohm, voltage 1.8 kV). *Achromobacter* sp. AO22 cells were transformed with ~500 ng plasmid DNA, using a 0.2 cm cuvette and the preset protocol for *P. aeruginosa* (25 μF/200 ohm/2.5 kV). The cell suspensions were transferred to a 5 mL tube, shaker-incubated at 37°C for 1 h and plated on LB plates with appropriate antibiotics. All *E. coli* cultures were incubated at 37°C, and *Achromobacter* sp. AO22 cultures at 30°C. Transformants were confirmed by analysis of plasmids by gel electrophoresis. The bacterial strains, plasmids and clones used/constructed in this study are listed in Table 2.

Table 2 Bacterial strains and clones used and/or created in this work

Bacterial strains	Relevant characteristics
Achromobacter sp. strain AO22[a]	Wild type, heavy metal resistant; experimental organism
AO22 (pCOPRP)	Tp^r; Biosensor experiments
E. coli CB454[b]	F^-, Δ*lacZ*$^-$, *lacY*$^+$, *galK*, *rpsL, thi*, *recA56*, Sm^r; host strain for *lacZ* reporter plasmid constructs
E. coli CB454 (pCOPRP)	Tp^r; study of copper responsive promoter
E. coli CB454 (pCOPAP, pGEM-TCOPSP)	Tp^r, Amp^r; study of copper responsive promoter

[a] Trajanovska et al. (1997);
[b] Schneider and Beck (1986)

Copper and other heavy metal challenge

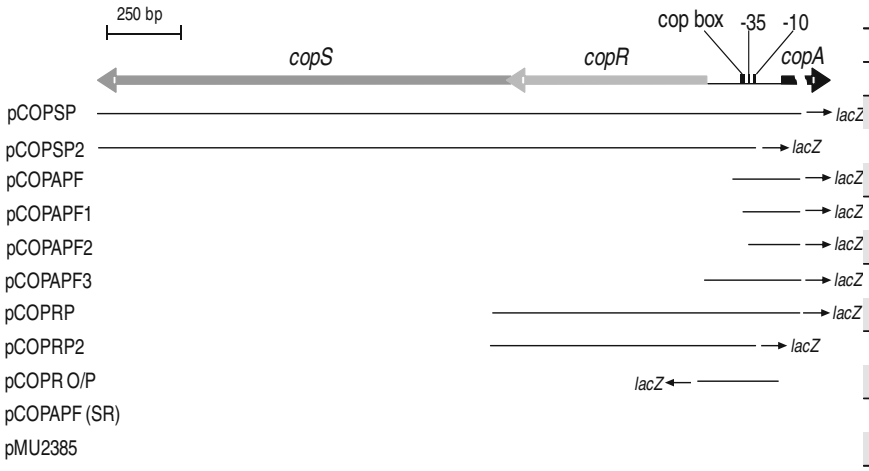
A single colony of *E. coli* CB454 containing a reporter construct was inoculated into 2 mL LB broth supplemented with trimethoprim and incubated at 37°C with shaking at 150 rpm. Cu(II) challenges were performed as per Rouch and Brown (1997) with slight modifications. Overnight cultures were diluted (1:20) in LB with trimethoprim and grown at 37°C under shaking until $OD_{600} = 0.3$. $CuSO_4$ (1 M stock) was then added to various final concentrations. For time course studies, 20 mL of cell suspension was exposed to 0.8 and 2.0 mM Cu, and 0.5 mL fractions collected at 30 min intervals over 5 h and used for β-galactosidase activity assays, indicative of expression of the *lacZ* reporter gene. Cultures without metals and blank (no inoculum) media were tested in parallel as controls, and *E. coli* CB454 with pMU2385 (no insert) was tested to assess background levels of *lacZ* expression. The β-galactosidase activities were assayed as per Miller (1972). The reactions were transferred to 96-well plates, absorbance measured at 420 and 550 nm using an iMark™ Microplate Absorbance Reader (BioRad, Australia) and enzyme activities calculated as Miller units = $1000 \times [(OD_{420} - 1.75 \times OD_{550})]/(T \times V \times OD_{600})$ (where T = time of assay reaction in minutes, V = volume of culture used for assay in mL). At least three separate experiments, each with duplicates, were performed per assay and the data used to calculate mean and standard deviation (SD). Select strains carrying the putative P*copA* were then challenged with $AgNO_3$, $CdSO_4$, $Pb(NO_3)_2$ and $ZnSO_4$ at 0.2, 1.0 and 5.0 μM for Ag(I), 0.02, 0.05 and 0.1 mM for Cd(II) and 0.1, 0.8 and 2.0 mM for Pb(II) and Zn(II), and the enzyme activities assayed as above.

Results

The putative AO22 *copA* '*cop box*' is copper responsive

Analysis of the 255 bp region between the proposed start codons of AO22 *copR* and *copA* by BPROM had previously identified one promoter upstream of *copA* (PcopA) and another in the complementary strand, upstream of *copR* (PcopR) (Ng et al. 2012) (Fig. 1). The predicted -35 and -10 boxes of P*copA* were separated by 17 bp, and those of P*copR* were separated by 16 bp, as typical for sigma-70 promoters (16–18 bp spacing) (Harley and Reynolds 1987). Additionally, a 7 bp perfect palindrome (ATTACATGAATGTAAT) was noted upstream of the -35 box of P*copA*, with significant similarity to '*cop* boxes' of other bacterial copper (Mills et al. 1994; Rouch and Brown 1997) and silver (Gupta et al. 1999) responsive promoters, including a 2-bp spacer between the half-repeats and the distance between the 3′ end of the palindrome and the P*copA* -35 or -10 boxes (10 and 33 bp) being very similar to that (11 and 33 bp) in P*pcoE* (Rouch and Brown 1997) and P*cusC* (Yamamoto and Ishihama 2005). This promoter was thus targeted for functional identification by study of inducibility by copper and other metals. In case of P*copR*, the distance between the palindrome and -35 box was much longer (55 bp), hence its potential as a specific promoter was unclear.

Various *lacZ* reporter constructs were generated containing clones of complete or truncated fragments of the putative P*copA* promoter region and its adjacent genes, in the promoterless vector pMU2385 (Fig. 2). Sequencing data confirmed integrity of all inserts. *E. coli* CB454 cells containing the various constructs, as well as empty vector, were exposed for 2 h to 0.0, 0.8 and 2.0 mM $CuSO_4$ (Fig. 2). The construct pCOPAPF3, which contained the entire 255 bp region, showed 35-fold and 110-fold increases in β-galactosidase activities under 0.8 and 2.0 mM copper, respectively, indicating inclusion of a Cu-responsive promoter. Similarly, high activities from pCOPAPF (229 bp insert), which contained the *cop* box and -35 and -10 boxes but lacked the 93 bp region upstream of the *cop* box, suggested this additional region did not have a notable effect on induction. In contrast, pCOPAPF1 (193 bp insert), with only the -35 and -10 hexamers but no *cop* box, did not exhibit such activity, and neither did pCOPAPF2 (174 bp insert) which lacked both the *cop* box and the -35 element. Thus, the predicted *cop* box, and the -35 and -10 hexamers, were together essential and probably also sufficient for copper-responsive expression of the putative P*copA*.



	β -galactosidase activity (Miller units)*					
	No $CuSO_4$		0.8 mM$CuSO_4$		2.0 mM $CuSO_4$	
	Mean	SD	Mean	SD	Mean	SD
pCOPSP	2.7	1.2	41.6	9.5	77.3	35.2
pCOPSP2	2.4	1.5	2.2	1.6	8.3	2.4
pCOPAPF	0.5	0.3	18.1	4.6	53.6	5.3
pCOPAPF1	0.7	0.9	0.3	0.5	1.0	0.8
pCOPAPF2	1.0	0.8	0.7	0.5	2.4	1.5
pCOPAPF3	0.5	0.4	17.5	13.1	57.5	12.3
pCOPRP	1.2	1.4	309.7	13.6	521.1	78.7
pCOPRP2	0.3	0.5	1.0	0.8	8.8	1.7
pCOPR O/P	6.6	2.7	79.3	49.0	167.1	22.7
pCOPAPF (SR)	0.6	0.2	17.5	13.1	145.8	57.2
pMU2385	2.8	1.4	2.7	1.8	3.8	2.9

Fig. 2 *Achromobacter* sp. AO22 PcopA-*lacZ* constructs and their induction in response to copper. *Thick arrows* indicate the direction of transcription of each gene. Various constructs made to test for copper inducibility are indicated on the *left*. *Lines indicate* the relative position of each insert to this region. *Small arrows* indicate the directions of insertion with respect to the promoterless *lacZ* gene in pMU2385. *β*-galactosidase activities of various constructs in *E. coli* CB454 in response to $CuSO_4$ are shown

P*copA* activity is enhanced by CopR

It was then hypothesised that the products of the putative two-component regulatory genes, AO22 *copR* and *copS*, may have a role in the regulation of *copA* expression. A construct, pCOPRP (1,059 bp insert) was, therefore, made to include *copR* in addition to the region covered in pCOPAPF3. The increase in *β*-galactosidase activities of pCOPRP in the presence copper were much higher compared to pCOPAPF3 (Fig. 2). The construct pCOPSP which contained the *copR, copS* and the P*copA* region, was also tested, however, contrary to the expectation, it showed decreased expression compared to pCOPRP. A possible explanation could be that the large insert of pCOPSP (2,420 bp) rendered the plasmid unstable. The results showed that *copR* enhanced the P*copA* activity, while the effect of a functional CopS on expression from P*copA* could not be deduced. Further, pCOPRP2, which contained the same 5′ end as pCOPRP but lacked the -35 and -10 hexamers showed negligible activation.

In a separate experiment, *copRS* was provided in *trans* to pCOPAPF by introducing a pGEM®T Easy clone containing the same insert as pCOPSP (by electroporation) to obtain *E. coli* COPAPF(SR). While there was no difference in expression from PcopA at 0.8 mM Cu, the expression at 2.0 mM Cu was nearly 3 times higher in *E. coli* COPAPF(SR) than in *E. coli* COPAPF, indicating the *trans*-acting CopRS had regulatory effects on P*copA*. Inducibility of P*copR* was also tested in a construct (pCOPR O/P) which contained the same insert as pCOPAPF3, but in the opposite direction. The activity of pCOPR O/P without copper was about six times higher than that of pCOPAPF3 and pCOPRP, suggesting constitutive expression from a putative promoter in pCOPR O/P. In the presence of copper, the increase was 12-fold and 25-fold, respectively, much lower compared to pCOPAPF3 and pCOPRP.

Achromobacter sp. AO22 versus *E. coli* as a host for biosensor plasmid constructs

Based on above results, the plasmid pCOPRP was introduced into *E. coli* to assess its potential as a copper biosensor. In order to develop a more environmentally competent strain than the enteric *E. coli*, plasmid pCOPRP was also introduced into *Achromobacter* sp. strain AO22 to obtain AO22 (pCOPRP), and both the *E. coli* and *Achromobacter* sp. transformants were compared for response kinetics, sensitivity and specificity to copper. Under copper exposure, the *β*-galactosidase assays of half-hourly samples over 5 h showed AO22 (pCOPRP) reached highest expression at 4.5 h while *E. coli* (pCOPRP) reached highest expressions at 1–2 h (Fig. 3a). This could be due to the initial slower growth rate of AO22 (pCOPRP) compared to *E. coli* (pCOPRP) under stress, indicated by OD_{600nm} of cultures (Fig. 3a), due to differences in their endogenous genetic systems. However, the maximum level achieved by AO22 (pCOPRP), about 1,200 Miller units, was double that of *E. coli* (pCOPRP).

Further experiments were thus conducted with induction times of 2 and 4.5 h for *E. coli* (pCOPRP) and *Achromobacter* sp. AO22 (pCOPRP), respectively, at a range of concentrations of $CuSO_4$, and enzyme activity assayed (Fig. 3b). For *E. coli* (pCOPRP), the activity increased from 0.1 mM to reach the maximum of 581.0 ± 72.8 Miller units at 2.0 mM Cu, but the signal fell sharply at 3.0 mM Cu and continued to decrease. In contrast, AO22

(pCOPRP) showed a more rapid increase to reach a maximum of 1,209.5 ± 118.4 Miller units at 3.0 mM Cu, then decreasing at 4.0 mM Cu, to half the activity at 6.0 mM (Fig. 3b). Both strains did not show increases in activity at lower Cu concentrations (0.01 and 0.05 mM) compared to background (no Cu) (data not shown). The results show that, although the detection limit for Cu for both strains was about 0.1 mM, AO22 (pCOPRP) had a larger detection range as well as stronger response to copper compared to *E. coli* (pCOPRP). The drastic fall at 3.0 and 6.0 mM Cu, respectively, could be due to copper toxicity effects on cells, as reported for *Staphylococcus aureus* RN4220 (pTOO24) which showed an abrupt fall in luminescence under Cd and Pb (Tauriainen et al. 1998). Both strains were then exposed to Ag(I), and to the divalent cations Cd(II), Pb(II) and Zn(II), as they may share similar uptake mechanisms. Marginally higher values than the control were noted in AO22 (pCOPRP) compared to *E. coli* (pCOPRP) only at the highest concentrations of metals.

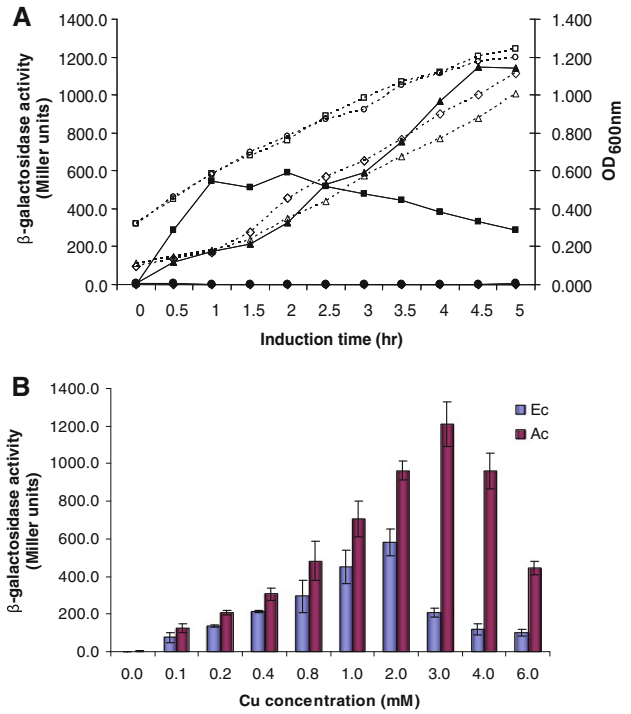


Fig. 3 Response of *E. coli* (pCOPRP) and *Achromobacter* sp. AO22 (pCOPRP) to copper. **a** Time course kinetics after induction with 2.0 mM Cu induction. *Solid squares*, *E. coli*(pCOPRP) with 2.0 mM Cu; *solid circles*, *E. coli* (pCOPRP) without Cu; *solid triangles*, AO22 (pCOPRP) with 2.0 mM Cu and *solid diamonds*, AO22(pCOPRP) without Cu. Growth of various cultures measured as optical density at 600 nm (OD_{600nm}) is indicated by the corresponding open shapes. Data represent mean values from three replicates and *error bars* indicate standard deviation from the mean. **b** *β*-galactosidase activities at various Cu concentrations. The induction time was 2 and 4.5 h respectively for the two strains. Data represent mean values from three replicates and *error bars* indicate standard deviation from the mean

However, the expression was negligible in both hosts compared to that induced by copper (Table 3).

Discussion

Regulation of *Achromobacter* sp. AO22 *copA* expression

The putative sequences of AO22 CopS and CopR exhibit features of histidine kinases and response regulators, respectively, of two-component regulatory systems. By analogy with other two-component systems, it is likely that AO22 CopS may sense the copper in the external environment and relay the signal to CopR, which may, in turn, regulate the expression of the structural genes *copA* and *copB*. The promoter elements predicted in the spacer between *copR a*nd *copA* represent possible binding sites for CopR. The essential roles of cop box and CopR for copper induction of AO22 PcopA were clearly demonstrated in this work. Previous reports of cop boxes involve either phytopathogens or enteric isolates, e.g., promoters of *Pseudomonas syringae copABCDRS* (Mills et al. 1994), *Xanthomonas campestris* pv. vesicatoria (Basim et al. 2005), *E. coli pcoABCDRS* (Rouch and Brown 1997) or *E. coli cusSRCFBA* (Munson et al. 2000; Yamamoto and Ishihama 2005). In *P. syringae* and *E. coli*, purified CopR and CusR have been shown to bind at the cop box of the

Table 3 *β*-galactosidase activities of *E. coli* (pCOPRP) and AO22 (pCOPRP) upon exposure to heavy metals

Challenge[a]	*β*-galactosidase activity (Miller units)			
	E. coli (pCOPRP)		AO22 (pCOPRP)	
	Mean[b]	SD	Mean	SD
No metal	1.9	0.1	3.4	0.3
Cu 2.0	529.6	38.7	849.5	38.9
Zn 0.1	2.1	0.2	3.1	0.1
Zn 0.8	3.1	0.5	4.4	0.1
Zn 2.0	3.1	0.1	8.9	0.3
Pb 0.1	2.3	0.1	3.7	0.5
Pb 0.8	2.4	0.1	3.8	0.7
Pb 2.0	2.4	0.1	3.4	0.5
Cd 0.05	2.0	0.0	12.3	0.3
Cd 0.1	2.1	0.0	13.8	0.2
Ag 0.0005	1.8	0.1	2.9	0.1
Ag 0.001	1.8	0.0	3.4	0.1
Ag 0.005	1.7	0.1	10.9	0.2
Hg 0.005	1.6	0.2	3.6	0.2

[a] Numbers indicate metal concentration in mM

[b] From three independent experiments. *SD* standard deviation

copper-inducible promoters PcopA and PcusC, respectively (Mills et al. 1994; Yamamoto and Ishihama 2005). The fact that AO22 P*copA* responded to copper in the absence of CopR suggests that a related regulatory protein from the *E. coli* host may have acted on P*copA*. Also, copper-responsive expression from AO22 P*copA* increased in the presence of CopR even without its partner CopS. In the present case, the host *E. coli* RR and HK which may interact with AO22 PcopA, are likely to be CusR and CusS, respectively. Our results suggest that copper homeostasis by two-component systems may be a common theme among phytopathogens, enteric isolates and soil isolates; this hypothesis needs to be investigated experimentally. Though the copper-induced expression of *copA* was clearly demonstrated, it is possible that other genes downstream of *copA* may also be under the control of the same promoter, and/or that other promoters also occur at this locus.

Achromobacter sp. AO22(pCOPRP) as a potential copper biosensor

Achromobacter sp. AO22(pCOPRP) exhibited a dose-dependent response to copper with a linear range of (0.1–3.0 mM) and an apparent specificity to copper. Direct comparison between the response profiles of AO22(p-COPRP) and *E. coli* (pCOPRP) to copper and other metals may not be appropriate, as differences in endogenous systems may account for some of the observed differences, such as the initial slower growth rate (longer lag phase) of AO22 in its adaptive stage (Fig. 3a), as typically observed in some bacteria upon changes to nutrients or application of stress. However, due to a larger linear range and stronger response compared to *E. coli* (pCOPRP), the strain AO22(pCOPRP) seems a better candidate as a biosensor. Further, as a soil isolate, *Achromobacter* sp. AO22 may be better suited for applications to environmental samples compared to *E. coli*. The copper concentrations are typically 14–29 ppm dried weight for surface soils and <50 ppm for sediments (Dorsey and Ingerman 2004). The guidelines for copper levels that may be unsafe for vegetables are >200 ppm and unsafe for gardens or contact by children are >500 ppm (A&L Eastern Laboratories, USA; http://www.aleastern.com/forms/Heavy%20Metal%20Interpretation.pdf; accessed 22 February 2012). The European safe limit for copper in drinking water is 4 mg/L (4 ppm) (European Copper Institute; http://www.eurocopper.org/vra/documents/en/VRA_Summary.pdf; accessed 22 February 2012), and the Maximum Contaminant Level Goal (MCLG) and hence action limit for copper are set at 1.3 ppm by the US Environmental Protection Agency (http://water.epa.gov/drink/contaminants/basicinformation/copper.cfm; accessed 22 February 2012). The linear detection range of AO22(pCORP) (0.1–3.0 mM, or 6–191 ppm) thus appears adequate for detection of contaminations at and exceeding these safety levels. In comparison, the biosensor constructed in *C. metallidurans* CH34 has a Cu(II) detection range of 0.1–10 μM (Leth et al. 2002) while an *E. coli*-based biosensor detects 0.3–300 μM (Hakkila et al. 2004). Both of our biosensor strains using PpcoA also have the advantage of being highly specific to copper, whereas other *E. coli*-based biosensors with similar copper-responsive promoters also respond to silver (Hakkila et al. 2004). As with other biosensors, there are challenges to be addressed before the application of AO22(pCOPRP) into the environment, e.g., stability of the strain, reproducibility and linearity of the detection, and ease of use. Future experiments should also investigate the effects of factors such as growth phase, cell density, pH and enzyme substrate. As concluded in a recent review, the prospect for whole-cell bacterial biosensors is encouraging (van der Meer and Belkin 2010).

Conclusion

Timely detection of exposure of organisms to potential copper toxicity remains a challenge due to the complexity of the biochemical processes utilising copper as well as sensitivity to its overload. This work has functionally developed two potential biosensor strains for monitoring of environmental copper pollution. With further optimisation, AO22(pCOPRP) has the potential to be developed into a copper biosensor for environmental samples. The major advantage of a whole-cell bacterial biosensor is to complement the well accepted physico-chemical methods by detecting the bioavailable fraction of the total heavy metal concentration, which is critical to evaluating the potential biological impact of the contaminant.

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