The Role of a P1-Type ATPase from *Pseudomonas fluorescens* SBW25 in Copper Homeostasis and Plant Colonization

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The genome of the plant-colonizing bacterium *Pseudomonas fluorescens* SBW25 possesses a putative copper-transporting P1-type ATPase (CueA) that is induced on the plant surfaces. Using a chromosomally-integrated *cueA-lacZ* fusion, we show that transcription of *cueA* can be induced (in vitro) by ions of copper, silver, gold, and mercury. To investigate the biological significance of *cueA*, a nonpolar *cueA* deletion mutant (SBW25 Δ *cueA*) was constructed. This mutant strain displayed a twofold reduction in its tolerance to copper compared with the wild-type strain; however, no change was observed in the sensitivity of the mutant strain to silver, gold, or mercury ions. To obtain insight into the ecological significance of *cueA*, the competitive ability of SBW25 Δ *cueA* was determined relative to wild-type SBW25 in three environments (none contained added copper): minimal M9 medium, the root of sugar beet (*Beta vulgaris*), and the root of pea (*Pisum sativum*). Results showed that the fitness of SBW25 Δ *cueA* was not different from the wild type in laboratory medium but was compromised in the two plant environments. Taken together, these data demonstrate a functional role for CueA in copper homeostasis and reveal an ecologically significant contribution to bacterial fitness in the plant rhizosphere. They also suggest that copper ions accumulate on plant surfaces.

Additional keywords: diaminopimelic acid, IVET, relative fitness.

Copper is an essential trace element that functions as a co-factor for many cellular enzymes (e.g., cytochrome oxidase, superoxide dismutase, and lysyl oxidase). However, excess copper is toxic because of its ability to generate hydroxyl radicals (OH[•]) in a Fenton-type reaction. Thus, most if not all living organisms possess homeostatic mechanisms to maintain optimal levels of intracellular copper. Such homeostatic mechanisms include copper uptake, sequestration, modification, and export (Balamurugan and Schaffner 2006; Rensing and Grass 2003).

Copper compounds have been extensively used in agriculture to control plant disease, because of their relatively high toxicity to plant pathogens (Cha and Cooksey 1991). Recent studies have revealed the impacts of copper accumulation on indigenous soil microbial communities. In general, copper contamination reduces bacterial diversity both in the soil and rhizosphere (the

region of soil surrounding and including the plant root) and increases the frequency of copper-resistant strains (Brandt et al. 2006). Studies on the molecular mechanisms of copper resistance from the recently evolved copper-resistant isolates have led to the discovery of a plasmid-encoded CopABCD system, which binds copper ions in the periplasm and outer membrane of the cell (Cha and Cooksey 1991). A two-component regulatory system (CopRS) that controls the expression of CopABCD genes is part of the same locus (Mills et al. 1993).

The plasmid-borne CopABCD genes usually confer high bacterial resistance to copper with a MIC (minimal inhibition concentration) of more than 800 μ M as measured on MGY (mannitol-glutamate-yeast extract) agar plates (Bender and Cooksey 1986; Cazorla et al. 2002), and conjugative transfer of plasmids encoding copper resistance among pathogenic bacteria is thought to be responsible for the failure of copper-based bactericides to control certain pathogens (Cazorla et al. 2002). Thus, this locus appears to confer a survival advantage to bacteria that inhabit environments contaminated by copper.

Chromosomal genes of copper resistance characterized so far in various bacteria are dominated by homologs of the P1-type copper-transporting ATPase (Rensing and Grass 2003). The P1-type ATPase is also known as the CP_x-type ATPase because of the conserved CP_x ($x = C, H, \text{ or } S$) motif in the sixth transmembrane segment (Solioz and Vulpe 1996). It facilitates export of copper ions out of the cytoplasm, using energy generated from ATP-hydrolysis. Deletion of genes encoding the copper-transporting ATPase results in bacterial sensitivity to copper (Adaikkalam and Swarup 2002; Schwan et al. 2005). The ecological significance of these chromosomal copper transporter genes is therefore predicted for bacteria living in high-copper environments.

Bacteria living in hosts (e.g., plants and animals) grow on nutrients from the host organism but are also challenged by stresses imposed by the host. It is critical for bacteria to continually alter patterns of gene expression in response to environmental changes, in particular, the changes that rapidly occur during colonization of a living host. Genes involved in nutrient acquisition and stress response (e.g., oxidative, acid, and osmotic stress) have been shown to be ecologically important for bacterial survival in hosts (Lugtenberg et al. 2001; Rediers et al. 2005). In terms of copper, niche-specific availabilities in plants and animals are not known. Thus, the ecological significance of copper transporters has not been recognized in the normal life of most bacteria.

Various genetic strategies have led to the identification of bacterial genes showing elevated levels of expression during colonization of host plants and animals (Rediers et al. 2005). Homologs of the P1-type copper-transporting ATPase from

Lactobacillus plantarum (Bron et al. 2004) and *Staphylococcus aureus* (Lowe et al. 1998) were found to be induced during colonization and infection of mice. This indicates these bacteria experience relatively high levels of copper during colonization of mouse tissue. Evidence that an ability to deal with these high levels contributes toward fitness within the host environment comes from recent work that showed that a strain of *Pseudomonas aeruginosa* containing a mutation in the P1-type ATPase for copper (CueA) reduced the pathogenicity of this bacterium in a mouse model (Schwan et al. 2005).

Here, we report the identification of *pflu0658* (*cueA*), a putative copper-transporting P1-type ATPase, by an in vivo expression technology (IVET) analysis of plant-induced genes in the genome of *Pseudomonas fluorescens* SBW25. *P. fluorescens* SBW25 is a plant growth-promoting rhizobacterium representative of a group of fluorescent pseudomonads isolated from the field-grown sugar beets at the University of Oxford farm (Wytham, Oxford) in 1989 (Bailey et al. 1995). *P. fluorescens* SBW25 aggressively colonizes a number of crop plant species and can protect seedlings against damping-off disease caused by *Pythium ultimum* (Ellis et al. 2000).

We examined the functionality of *pflu0658* in copper homeostasis by using site-directed mutagenesis combined with a chromosomally integrated 'lacZ' fusion. Moreover, we determined the ecological performance of a mutant strain defective in *pflu0658* during colonization of the roots of sugar beet and pea plants. Our data show that *pflu0658* encodes a copper exporter required for bacterial competitive fitness in planta and suggest that copper ions accumulate on root surfaces.

RESULTS

Identification of *pflu0658* (*cueA*) as a plant-inducible gene.

A previously described *dapB*-based promoter trap (Gal et al. 2003) was employed in a genome-wide identification of SBW25 genes showing elevated levels of expression in the phytosphere of sugar beet. Central to this technique is a *dapB* deletion mutant strain (SBW25 Δ *dapB*) that is unable to grow in the absence of an exogenous source of DAP (diaminopimelic acid) and lysine, and as a result, it is unable to colonize sugar beet seedlings. Thus plant-inducible promoters can be identified by their ability to drive the expression of a promoterless copy of 'dapB' integrated into the genome of SBW25 Δ *dapB*. A primary genome-wide screen identified approximately 200 plant-inducible candidate genes. A full description of these IVET fusions will be published elsewhere. Of particular interest is an IVET fusion to *pflu0658*, which encodes a putative P-type ATPase with predicted roles in copper transport from the cytoplasm. This finding suggested that high copper levels are present in the plant environment and copper homeostasis genes might contribute to bacterial success in planta. Thus, *pflu0658* was subjected to further investigation.

pflu0658 encodes a predicted protein of 733 amino acids. It shows high similarity with several previously characterized

copper-transporting P-type ATPases (GenBank accession number and sequence identity are shown in parenthesis): CueA of *P. putida* PNL-MK25 (AF390440, 84.5%), CopA of *Enterococcus hirae* (P32113, 47%), CopA of *Escherichia coli* (Q59385, 35%), the human Wilson disease-associated protein ATPase 2 (P35670, 32%). Computational hydropathy analysis of Pflu0658 using a hidden Markov model (TMHMM server, version 2.0) predicted eight transmembrane helices. Pflu0658 possesses one heavy metal binding domain (GMTSASC) near the N-terminus. The CPx motif (CPC) is located in the sixth putative transmembrane helix. Other domains conserved in all P-type ATPase are also present in Pflu0658, an SGES phosphatase domain, a DKTGT aspartyl kinase domain, and a GDGINDA ATP-binding domain. These in silico data suggest that *pflu0658* might encode a copper transporter belonging to the P-type ATPase subfamily. Pflu0658 is designated CueA because of the high similarity that it shares with CueA of *P. putida* PNL-MK25, but more importantly, on the basis of the functional analysis described below.

The first step of the investigation was to confirm plant inducibility by reconstructing the IVET fusion to *cueA* and determining whether it could grow in the plant environment. To do this, the promoter region was transcriptionally fused to the promoterless 'dapB' carried in vector pIVETD (Fig. 1 and discussed below). The resulting fusion plasmid (pIVETD12) was integrated into the genome of SBW25 Δ *dapB* in such a way as to ensure that the wild-type CueA function was not affected. The *cueA*-*dapB* fusion strain NR53-1 (SBW25 Δ *dapB*::pIVETD12) was unable to grow on minimal M9 medium in the absence of DAP and lysine, showing that the promoter driving *cueA* expression is inactive on this medium. To test whether this promoter is active in the plant environment, NR53-1 was subjected to a competitive colonization assay on sugar beet seedlings. Approximately 2×10^3 cells were inoculated per seed, and population density on the shoot and rhizosphere of a seedling was determined two weeks after plant sowing. In parallel, seeds were inoculated with IVET fusion strains PBR391 (a positive control with a fusion between a promoterless 'dapB' and a constitutive promoter) and PBR393 (a negative control with fusion to a locus with no detectable promoter activity). Results are shown in Figure 2. The population size of NR53-1 increased in both the rhizosphere and shoot, and the number of bacteria recovered from each environment was similar to that of the positive control strain PBR391. In contrast, the negative control showed little increase in population size. The ability of NR53-1 to grow on sugar beet seedlings but not on minimal M9 medium demonstrates that the promoter driving expression of *cueA* is induced in the plant environment.

The *cueA* is induced by elevated copper, silver, gold, and mercury ions.

Next, we sought the conditions that induce expression of *cueA* in laboratory medium. To determine the *cueA* transcriptional inducers in vitro, wild-type SBW25 carrying a *cueA*-*'lacZ'* fusion was constructed in a similar way as the IVET fusion of *cueA*-*'dapB'* described above.

Previous studies on the transcription of copper-transporting P-type ATPases in gram-negative bacteria showed that they are regulated by a MerR-type activator (CueR) in a copper-responsive manner (Outten et al. 2000; Stoyanov et al. 2001). Notably, the CueA homolog in *E. coli* is also induced by elevated silver (Stoyanov et al. 2001) and gold ions (Stoyanov and Brown 2003), though they are not involved in bacterial resistance to these metal ions (Franke et al. 2001). Therefore, a survey of inducing activities by various metal ions, including copper, was performed in order to gain a better understanding of CueA expression in *P. fluorescens* SBW25.

Fig. 1. Genetic organization of the *pflu0658* (*cueA*) locus of *Pseudomonas fluorescens* SBW25 based on the SBW25 genome sequence. The 868-nt fragment was fused to promoterless 'dapB' and 'lacZ' (orientation is indicated by the arrowed line). The region deleted in SBW25 Δ *cueA* is indicated by gray shading.

The *cueA-lacZ* fusion strain SBW25::pUIC3-17 was subjected to a β -galactosidase assay using cells grown in minimal M9 medium and M9 medium supplemented with different metal ions (concentration shown in parenthesis): CuSO₄ (7.5 μ M), ZnCl₂ (0.5 mM, 1 mM), HgCl₂ (0.5 μ M, 1 μ M), CdCl₂ (50 μ M, 100 μ M), NiCl₂ (50 μ M, 100 μ M), AgNO₃ (2 μ M), and AuCl₃ (2.5 μ M). Results are shown in Table 1. Strong responses of approximately 16-, 33- and 73-fold induction were observed with Cu(II), Ag(I), and Au(III), respectively. A weak but significant induction was found with Hg(II); *cueA* expression was increased 1.6-fold and 7.1-fold in the presence of 0.5 and 1 μ M HgCl₂, respectively. No significant induction was found with Zn(II), Cd(II), and Ni(II) salts. Dynamic response of the *cueA* promoter to copper, silver, gold, and mercury was then tested at a range of concentrations. Results shown in Figure 3 indicate that inductions by the four metal salts were all dose-dependent but showed different induction patterns. Maximal induction was observed with addition of 15 μ M CuSO₄, 2.5 μ M AgNO₃, 5 μ M AuCl₃, or 4 μ M HgCl₂. When metal salts were supplemented at high concentrations, growth was inhibited and a decrease of *cueA-lacZ* activity was observed.

Deletion of *cueA* resulted in sensitivity to copper but not to silver, gold, or mercury ions.

Having demonstrated that *cueA* is induced by elevated amounts of copper, silver, gold, or mercury ions, we next asked whether it is involved in detoxification of copper and other heavy metal ions. To do this, an in-frame deletion mutant was constructed by allelic exchange. If CueA acts as an efflux system as expected from in silico analysis, a mutant strain defective in the expression of CueA should display less tolerance to these metal ions as compared with the wild-type strain.

To test metal sensitivity of the *cueA* deletion mutant (SBW25 Δ *cueA*), MIC were determined on minimal M9 agar plates supplemented with varying concentrations of the following metal salts: CuSO₄, AgNO₃, ZnCl₂, HgCl₂, CdCl₂, NiCl₂, and AuCl₃. The wild-type strain SBW25 and the mutant strain SBW25 Δ *cueA* were tested in parallel by inoculating each strain onto the same agar plates. Each assay was repeated using three

independent cultures. Results showed that deletion of *cueA* resulted in a decrease of the MIC of copper sulphate from 300 μ M for the wild-type strain to 150 μ M for the mutant. No MIC value differences were found with the other metal salts tested.

MIC values may vary a lot for the same bacterial strain when grown in different laboratory media. Previous studies of copper resistance of environmental *Pseudomonas* isolates were mostly performed on MGY medium. Copper-resistant strains were recognized by MIC values greater than 800 μ M of Cu(II) (Bender and Cooksey 1986; Cazorla et al. 2002). To compare SBW25 copper tolerance to published data, the MIC of copper was also determined on MGY agar plates supplemented with CuSO₄, varying from 50 μ M to 1 mM in 50- μ M increments. The results showed that the copper MIC value for wild-type SBW25 was 500 μ M. This value places SBW25 in the group of copper-sensitive strains. The mutant strain SBW25 Δ *cueA* exhibited a MIC of 250 μ M on MGY plates, which is half the copper tolerance of the wild-type strain.

The growth kinetics of SBW25 and SBW25 Δ *cueA* were measured in M9 broth medium. The growth effects of four metal salts (CuSO₄, AgNO₃, HgCl₂, and AuCl₃), which are able to induce *cueA* expression, were tested in 96-well plates using a VersaMax microtiter plate reader. Consistent with the results of the MIC tests, no growth effect was observed with silver, gold, or mercury ions (data not shown), whereas clear effects were found with copper (Fig. 4). Growth of wild-type SBW25 and SBW25 Δ *cueA* was comparable in M9 broth and in M9 with the addition of copper sulphate at concentrations below 0.125 μ M. The maximum growth rate (μ _{max}) was 0.34 ± 0.02 μ _{max} per h under these conditions. Differential growth inhibitions were observed with the addition of copper at concentrations greater than 0.25 μ M. In the presence of 0.5 μ M Cu(II), the growth rates of SBW25 and SBW25 Δ *cueA* were 0.21 ± 0.04 and 0.13 ± 0.01 μ _{max} per h, respectively (Fig. 4B). Growth of SBW25 Δ *cueA* was abolished in the presence of 1 μ M Cu(II), whereas the wild-type strain still showed measurable growth at this concentration with 0.18 ± 0.01 μ _{max} per h (Fig. 4C). Taken together, these data indicate that CueA is required for bacterial resistance to copper but not to the other heavy metal ions tested.

Ecological significance of *cueA* for bacterial colonization in planta.

The fact that SBW25 Δ *cueA* is sensitive to excess copper but not silver, mercury or gold makes this strain useful for deter-

Fig. 2. Expression of *pflu0658* in sugar beet seedlings as demonstrated by growth on the shoot (open column) and rhizosphere (filled column). In vivo expression technology (IVET) fusion strains (to *pflu0658* [NR53-1], to a constitutive promoter [PBR391, positive control] and to a locus without detectable promoter activity [PBR393, negative control]) were inoculated onto sugar beet seeds in direct competition (1:1) with wild-type strain SBW25-Sm. After 14 days, the competitor was present at the similar level as the positive control strain PBR391 across all treatments (data not shown). Data are means and standard errors from three plants. Data from the shoot and rhizosphere were analyzed separately by Tukey's honestly significant difference ($P < 0.05$), and significances are indicated by different upper or lower case letters above the columns.

Table 1. Response of the *cueA* promoter to elevated levels of heavy metal salts

Inducer	β -galactosidase activity (aM 4MU/min/cell) ^a	Fold increase ^b
None	0.90 $\pm$ 0.02	
CuSO ₄ (7.5 μ M)	14.51 $\pm$ 3.01	16.21
ZnCl ₂ (0.5 μ M)	1.13 $\pm$ 0.03	1.23
ZnCl ₂ (1 μ M)	0.81 $\pm$ 0.25	0.90
HgCl ₂ (0.5 μ M)	1.42 $\pm$ 0.12	1.58
HgCl ₂ (1 μ M)	6.35 $\pm$ 0.53	7.10
CdCl ₂ (50 μ M)	1.90 $\pm$ 0.41	2.12
CdCl ₂ (100 μ M)	2.05 $\pm$ 0.62	2.29
NiCl ₂ (50 μ M)	0.97 $\pm$ 0.06	1.09
NiCl ₂ (100 μ M)	1.88 $\pm$ 0.15	2.10
AgCl ₃ (2 μ M)	29.14 $\pm$ 2.42	32.55
AuCl ₃ (2.5 μ M)	65.92 $\pm$ 2.48	73.25

^a β -galactosidase assay was performed by using the *cueA-lacZ* fusion strain of SBW25 for cells growing in minimal M9 medium (none) or M9 medium supplemented with varying concentrations of metal salts. Data are means and standard errors of three independent cultures. 4MU = 7-hydroxy-4-methylcoumarin

^b Fold increase was calculated by dividing the mean of respective β -galactosidase activity by the average value of the uninduced cells (none).

mining whether copper has accumulated in the rhizosphere. If copper has accumulated, then the mutant strain will be competitively inferior to the wild-type strain during the course of rhizosphere colonization; on the other hand, if copper is absent, then the fitness of SBW25 Δ *cueA* should be equivalent to the fitness of the wild type (provided that both strains are equally fit in laboratory medium).

To examine the competitive ability of SBW25 Δ *cueA* relative to the wild-type strain, SBW25 Δ *cueA* was mixed 1:1 with a *lacZ*-marked wild-type strain (SBW25-*lacZ*). The mixed bacteria were inoculated onto sugar beet seeds that were subsequently germinated and grown on water-agar plates. One week after inoculation, the sugar beet roots were destructively harvested, and the ratio of mutant to wild-type competitor was determined by enumeration on M9 plates supplemented with X-gal. Results showed that the fitness of SBW25 Δ *cueA* relative to the wild-type strain is significantly less than zero (Fig. 5), indicating that the mutant strain is compromised in its ability to competitively colonize the root of sugar beet seedlings. The fitness of the mutant strain was also determined relative to the wild type in M9 broth. Results showed that the mutant strain and the wild-type competitor (SBW25-*lacZ*) were equally fit in this laboratory medium (Fig. 5). These data suggested that copper accumulates on the surface of sugar beet roots and that optimal ecological performance of SBW25 requires *cueA*.

Previous work by Evans and associates (1992) using chemical analysis showed that copper accumulates in the roots of the pea plant (*Pisum sativum*). This prompted a fitness assay of the mutant strain SBW25 Δ *cueA* on the roots of *Pisum sativum* seedlings. The fitness of SBW25 Δ *cueA* was determined relative to the wild-type strain as described above. The fitness of

SBW25 Δ *cueA* was significantly compromised during the course of colonization of pea roots (Fig. 5).

DISCUSSION

Analysis of bacterial genes induced specifically in planta can provide insight into the biological function and ecological significance of soil-dwelling bacteria and can provide valuable clues as to the nature of the plant environment. Here, we have characterized, from both ecological and genetic perspectives, *cueA* from the plant growth-promoting bacterium *P. fluorescens* SBW25. *cueA* was induced in response to extracellular copper ions, and it was required for bacterial resistance to copper. The genetic data, combined with *in silico* analysis, indicate that *cueA* encodes a functional P1-type ATPase for copper export. Significantly, such a copper export gene was induced on the plant surfaces, as shown by IVET analysis, indicating that this gene plays an important role in plant colonization. Moreover, the mutant strain carrying the *cueA* deletion was compromised in its ability to competitively colonize the roots of sugar beet and pea plants. As a control, the same mutant strain was as fit as the wild-type strain when growing in laboratory medium. The fact that the mutant strain (SBW25 Δ *cueA*) displayed a reduced tolerance to copper and not to any other metal ions tested strongly suggests that copper has accumulated on the plant surfaces.

Our finding that copper accumulates in the rhizosphere is consistent with a number of studies on plant copper homeostasis. Evans and associates (1992) showed that copper accumulates on pea roots and is associated with iron availability. In response to iron deficiency, root-surface copper-chelate reduc-

Fig. 3. Dosage response of the *cueA* promoter to **A**, copper, **B**, silver, **C**, gold, and **D**, mercury ions. Induction of *cueA* in wild-type SBW25 was monitored using an integrated *cueA*-*lacZ* fusion and β -galactosidase activity (aM 7-hydroxy-4-methylcoumarin (4MU)/min/cell) was assayed for cells growing in minimal M9 medium supplemented with varying concentrations of metal salts. Data are means and standard errors of three independent cultures.

tase activity increases (Robinson et al. 1999; Yi and Guerinot 1996), which might facilitate enhanced copper uptake by copper transporters. Subsequently, the excess copper ions are pumped out of plant cells and accumulate on root surfaces. Based on these reports, we suggest that copper accumulation on plant roots is due to copper metabolism in plants growing in certain "soil" conditions and is not a response to bacterial colonization or infection.

The molecular homeostatic mechanisms for copper are poorly understood in *Pseudomonas* spp. Indigenous *Pseudo-*

monas populations are sensitive to the stress imposed by copper and are more severely affected than other populations of heterotrophic soil bacteria (Brandt et al. 2006). Adaikkalam and Swarup (2002, 2005) characterized the copper homeostatic genes carried in the chromosome of *P. putida* PNL-MK25 and identified two transport systems, CueA (a P1-type ATPase) and CopABCD (homologous to the plasmid-encoded CopABCD). Disruption of *cueA* by insertion of a mini-Tn5 transposon resulted in a sixfold reduction of copper tolerance, whereas inactivation of *copABCD* produced only a slight reduction (0.16-fold) (Adaikkalam and Swarup 2005), leading the authors to conclude that CueA is the principle copper exporter. Similar results from SBW25 lead us to a similar conclusion; indeed, it is likely that CueA is the major copper exporter in all *Pseudomonas* species.

Overlapping substrate specificities are often found for heavy metal transport proteins. Genes encoding the copper-exporting P1-type ATPase in *Enterococcus hirae* (named CopB) and in *E. coli* (named CopA) are induced by extracellular copper and silver ions (Odermatt et al. 1993; Stoyanov et al. 2001). The ability to transport both Cu(I) and Ag(I) was demonstrated in vitro for CopB of *Enterococcus hirae* (Solioz and Odermatt 1995). However, the copper-exporting ATPases in these two organisms confer bacterial resistance only to copper and not to silver ions (Franke et al. 2001; Solioz and Odermatt 1995). Resistance to silver ions in *E. coli* is encoded by *ybdE*, which is induced by both silver and copper ions (Franke et al. 2001). Apart from copper and silver, gold is also capable of inducing expression of CopA (copper exporter) in *E. coli* (Stoyanov and Brown 2003). Notably, the *CopA* gene of *E. coli* is regulated by a MerR-type activator (CueR) (Oутten et al. 2000; Stoyanov et al. 2001), whereas the *CopB* gene of *Enterococcus hirae* is regulated by a repressor protein, CopY, and a copper chaperon, CopZ (Solioz and Stoyanov 2003).

Induction of *cueA* by four metal ions, copper, silver, gold, and mercury, is unusual. The fact that the *cueA* deletion of SBW25 resulted in bacterial sensitivity to copper but not to silver, gold, or mercury, suggests that CueA functions (in vivo) solely as a copper exporter. The unusual mechanism of *cueA* induction by metal ions may reside in the specific interactions between the *cueA* regulatory protein and the *cueA* operator site. In a separate study, we have shown that *cueA* is regulated by CueR, a gene product of *pflu0657* located downstream of *cueA* (Fig. 1). CueR also regulates expression of a copper chaperon-like protein Pflu0660 and indirectly regulates ex-

Fig. 4. Copper sensitivity of *Pseudomonas fluorescens* SBW25 (circle) and SBW25Δ*cueA* (square). Growth was measured for cells growing in **A**, minimal M9 medium and M9 medium supplemented with copper sulphate at the following doses (μM): 0.03, 0.06, 0.125, 0.25, **B**, 0.5, **C**, 1 and 2. Data are means and standard errors of six repeats (error bars lie within the symbols).

Fig. 5. Fitness of *Pseudomonas fluorescens* SBW25Δ*cueA* relative to wild-type SBW25 when growing in laboratory medium (M9) and in the rhizosphere of sugar beet and pea (*Pisum sativum*). Data are means and standard errors of eight plants inoculated with independent cultures. Analysis of variance showed that means from sugar beet and pea are significantly less than zero ($P < 0.05$).

pression of the CopCD homologs (X. X. Zhang, *unpublished data*). Details of the CueR regulon will be published elsewhere.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 2. *P. fluorescens* and *E. coli* strains were grown routinely in Luria-Bertani (LB) medium at 28°C and 37°C, respectively. *P. fluorescens* strains were also cultivated in minimal M9 medium (Sambrook et al. 1989) with glucose and ammonia as the sole carbon and nitrogen sources. M9 plates contained half-strength CFC (cetrimide, fucidin, and cephalosporin) supplement from Oxoid (Hampshire, U.K.) were used to select for *P. fluorescens* recovered from the plant. When necessary, antibiotics were added at the following concentrations (μg/ml): tetracycline (Tc), 10; kanamycin, 50; spectinomycin, 100; streptomycin (Sm), 100; nitrofurantoin (NF), 100. Nutritional supplements of DAP and lysine were added as required at concentrations of 800 and 60 μg/ml, respectively.

Genetic techniques.

Standard DNA manipulation techniques (Sambrook et al. 1989) were used. Plasmid DNA was prepared using the Qiagen miniprep extraction kit (Qiagen, Victoria, Australia). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Hitchin, U.K.). Polymerase chain reactions (PCR) using *Taq* DNA polymerase (Invitrogen, Auckland, New Zealand) were performed according to the manufacturer's protocol with an annealing temperature of 56°C. Oligonucleotide primers were purchased from Invitrogen. DNA was sequenced using the BigDye terminator sequencing kit (Applied Biosystems, Warrington, U.K.) on an automated DNA Sequencer, model 310 (Perkin Elmer, Warrington, U.K.).

Construction of *cueA* transcriptional fusions.

To monitor *cueA* expression in the plant environment and in laboratory medium, transcriptional fusions were made to pro-

moterless '*dapB*' and '*lacZ*', respectively. According to the SBW25 genome sequence (Sanger Institute website), a pair of primers, PcueA1 and PcueA2, was designed to amplify an 868-bp DNA fragment that contains the putative *cueA* promoter region (Fig. 1). Restriction sites *Bgl*III and *Bam*HI were incorporated into the primers to facilitate subsequent gene cloning (Table 2). The PCR product was first cloned into pCR8/GW/TOPO (Invitrogen), and the fragment was sequenced to ensure that it was error-free. Next, the 868-bp fragment was retrieved by *Bgl*III/*Bam*HI digestion and was cloned into the *Bgl*III site of integration vector pIVETD or pUIC3, which carries the reporter genes of '*dapB*' and '*lacZ*', respectively. Correct insertion orientation was determined by PCR analysis using primers PcueA2 and Pbla (a forward primer located in the *bla* gene of the vector). The resulting *cueA*-'*dapB*' fusion and *cueA*-'*lacZ*' fusion plasmids were designated pIVETD-12 and pUIC3-17, respectively.

To construct an IVET fusion strain for *cueA*, plasmid pIVETD12 was mobilized into *P. fluorescens* SBW25Δ*dapB* by conjugation with the help of pRK2013 (Tra⁺). Integration by single homologous recombination was selected for on LB plates supplemented with lysine, DAP, NF, and Tc. NF was used to inhibit the growth of the *E. coli* parental strains. To generate a strain carrying the *cueA*-'*lacZ*' fusion, plasmid pUIC3-17 was conjugated into the wild-type strain SBW25 and integrants were selected for on LB plates supplemented with NF and Tc.

Assay for β-galactosidase activity.

To establish the assay, cells from overnight M9 cultures were diluted with fresh M9 broth to an optical density at 600 nm (OD₆₀₀) of 0.1. For each treatment, 3 ml of the diluted cell suspension was transferred into a 20-ml plastic tube and metal salt was then added as an inducing factor. The induction was performed by incubating the cells at 28°C for 4 h with shaking (150 rpm) or until a cell density of OD₆₀₀ equivalent to 0.6 was reached. To minimize the possible effects of the metal ions on the β-galactosidase activity, 1 ml of the induced cells was spun

Table 2. Bacterial strains, plasmids and oligonucleotide primers used in this work

Strain, plasmid, or primer	Relevant properties ^a	Source/reference
<i>Pseudomonas fluorescens</i>		
SBW25	Wild-type strain isolated from sugar beet	Bailey et al. 1995
SBW25-Sm	Spontaneous Sm ^r derivative of SBW25	Gal et al. 2003
SBW25Δ <i>dapB</i>	Diaminopimelic acid (DAP)/lysine auxotroph of SBW25	Gal et al. 2003
NR53-1	SBW25Δ <i>dapB</i> carrying the <i>cueA</i> -' <i>dapB</i> ' in vivo expression technology (IVET) fusion	This work
PBR391	SBW25Δ <i>dapB</i> with constitutively expressed promoter-' <i>dapB</i> ' fusion	Zhang et al. 2004
PBR393	SBW25Δ <i>dapB</i> with null expression promoter-' <i>dapB</i> ' fusion	Zhang et al. 2004
SBW25-' <i>lacZ</i> '	SBW25 marked with ' <i>lacZ</i> ' in a phage locus	X. X. Zhang, <i>unpublished data</i>
SBW25Δ <i>cueA</i>	SBW25 with a nonpolar deletion of <i>cueA</i>	This work
SBW25::pUIC3-17	SBW25 carrying a <i>cueA</i> -' <i>lacZ</i> ' fusion	This work
<i>Escherichia coli</i>		
DH5αλpir	<i>supE44 ΔlacU169 hsdR17, recA1 endA1 gyrA96 thi-1 relA1, λpir</i>	Hanahan 1983
Plasmid		
pRK2013	Helper plasmid, Tra ⁺ , Km ^r	Ditta et al. 1980
pUIC3	Integration vector with promoterless ' <i>lacZ</i> ', Mob ⁺ , Tc ^r	Rainey 1999
pIVETD	DAP-based IVET vector, pUIC3 with promoterless ' <i>dapB</i> ', Tc ^r	Gal et al. 2003
pIVETD-12	pIVETD carrying <i>cueA</i> -' <i>dapB</i> ' fusion, Tc ^r	This work
pUIC3-17	pUIC3 carrying <i>cueA</i> -' <i>lacZ</i> ' fusion, Tc ^r	This work
pUIC3-38	pUIC3 containing 1.7-kb <i>cueA</i> deletion fragment, Tc ^r	This work
Primer ^b		
PcueA1	5'-GAAGATCTGGCCGAGGTCATTCCTT-3'	This work
PcueA2	5'-CGGGATCCGTCGGTGGTTCGATTCGCTT-3'	This work
PcueA3	5'-gaatcgaaccacgacgacgacccgTGCAGCGATGGCCTTGTCCA-3'	This work
pcueA4	5'-GAAGATCTCAAGTACGCCAAGGAATG-3'	This work
Pbla	5'-CAGGGTTATTGTCTCATGAGCG-3'	Gal et al. 2003

^a Sm^r Tc^r = streptomycin- and tetracycline-resistant, respectively.

^b Restriction sites incorporated into the primers are underlined. Complementary sequence designed for the SOE-PCR (splicing by overlapping extension polymerase chain reaction) is shown in small letters.

down and resuspended in the same amount of sterile water, and then, 40 µl of the cell suspension was used for β-galactosidase assay by using 4MUG (4-methylumbelliferyl-β-D-galactoside) as the enzymatic substrate. The product (7-hydroxy-4-methylcoumarin, 4MU) was detected in Hoefer DyNA Quant 200 fluorometer (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), following the manufacturer's instructions. The reaction was monitored at 460 nm with an excitation wavelength of 365 nm. The enzyme activity was expressed as "aM 4MU/min/cell."

Construction of a *cueA* deletion mutant and assays for metal-sensitivity.

Deletion of *cueA* was achieved by a standard protocol of splicing by overlapping extension PCR (Horton et al. 1989) in conjunction with a two-step allelic exchange strategy (Rainey 1999). Briefly, two pairs of primers (PcueA1 and PcueA2 and PcueA3 and PcueA4) were designed to amplify two DNA fragments of similar sizes flanking the *cueA* locus. Primer PcueA3 contains additional sequence complementary to the sequence of primer PcueA2 (Table 2). This made it possible to join the two DNA fragments together via a PCR reaction with primers PcueA1 and PcueA4. The resulting 1.7-kb fragment carrying the *cueA* deletion was cloned into pCR8/GW/TOPO by using the TA cloning kit from Invitrogen. After the sequence identity was confirmed by DNA sequencing, the fragment was sub-cloned into the *Bgl*III site of integration vector pUIC3 to generate pUIC3-38.

To delete *cueA*, pUIC3-38 was mobilized into *P. fluorescens* SBW25 by conjugation with the help of pRK2013. Integration by the single event of homologous recombination was selected for on LB plates supplemented with NF, Tc, and X-gal. To select for allelic exchange mutants, purified single blue-colored transconjugants were grown in 200 ml of LB broth for two successive 24-h periods. Bacterial cells were plated on LB plates containing X-gal. Allelic exchange mutants produced white-colored and Tc-sensitive colonies. They are either wild type or the desired *cueA* deletion mutants, which were then determined by PCR amplification using primers PcueA1 and PcueA4; wild-type strains produced a 3.6-kb fragment, whereas the mutant strains produced a 1.7-kb fragment.

Metal sensitivities of the *P. fluorescens* strains (wild type and Δ *cueA*) were determined for cells growing in M9 plates supplemented with varying concentrations of metal salts. Overnight cultures were diluted by 10⁵ times, and 20 µl of the diluted cell suspension was spotted onto the surface of the M9 agar plate. The MIC of each of the metal salts tested was considered to be the minimum concentration tested at which no colony formation was observed after 72 h. The MIC of copper sulphate was also determined on agar plates of MGY medium (Bender and Cooksey 1986).

Sensitivity of the *P. fluorescens* strains to copper, silver, gold, and mercury salts was also determined for cells growing in M9 broth. The growth curves were measured in microtiter plates, using a VersaMax microtiter plate reader with SOFTmax PRO software (Molecular Devices Corp., Sunnyvale, CA, U.S.A.). For the growth experiments, 200 µl of M9 broth supplemented with or without metal salt was added into each well and then inoculated with 2 µl of each strain grown overnight in M9 culture. The absorbance at the wavelength of 450 nm was determined every 5 min over a period of 48 h.

Plant experiments.

A competitive colonization assay of the *cueA* IVET fusion strain against the competitor strain of SBW25-Sm was performed as previously described by Rainey (1999). Coated sugar beet seeds (*Beta vulgaris* var. Amethyst) were germi-

nated and cultivated in 15-ml plastic tubes containing nonsterile vermiculite as a growth substrate. Bacteria were inoculated at a ratio of 1:1 (approximately 2,000 cells per seed) and were recovered from the shoot and rhizosphere (roots with attached vermiculite) after 14 days and then were plated on selective media to enumerate each of the bacterial populations present in the shoot and rhizosphere. The fusion strain was counted on M9 plates supplemented with lysine, DAP, CFC, Tc, and X-gal, while the competitor strain was counted on M9 plates supplemented with CFC and Sm.

To determine the ecological significance of *cueA* for bacterial colonization in planta, SBW25 Δ *cueA* was competed against *lacZ*-marked wild-type strain *P. fluorescens* SBW25-*lacZ*, in the rhizosphere of sugar beet and pea plants. Seeds were sterilized by soaking in 70% ethanol (5 min), sterile water (rinsed twice), 7.5% sodium hypochlorite containing 5% Triton X-100 (15 min) and washing five times in sterile water. Bacterial inoculants were prepared by mixing equally overnight M9 cultures of the mutant strain and the wild-type competitor strain. Then, 10 µl of diluted bacterial suspension (OD₆₀₀ = 0.1) was inoculated onto each sterilized seed. Initial densities of the competing strains were determined on M9 plus X-gal plates. The inoculated seeds were germinated (in the dark for 3 days) and grown on 1% water agar plates. Ten days after inoculation, bacteria in the roots were recovered on M9 plates supplemented with X-gal, and colonies of the mutant and wild-type competitor were counted. Population densities (*N_i*) determined at time *t* = 0 and at *t* = *T* were used to calculate the Malthusian parameter (Lenski et al. 1991), which is the average rate of increase and was calculated for both competitors as follows: $m_i = \ln[N_i(T)/N_i(0)]$. Relative fitness is expressed here as the selection rate constant: $r_{ij} = m_i \cdot m_j$, resulting in a fitness of zero when competing organisms are equally fit.

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