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A Two-Component Regulatory System Required for Copper-Inducible Expression of the Copper Resistance Operon of *Pseudomonas syringae*

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Specific induction of the copper resistance operon (*cop*) promoter from *Pseudomonas syringae* was measured by β-galactosidase production from a *cop* promoter-*lacZ* fusion. Induction of the *cop* promoter in *P. syringae* pv. syringae required *trans*-acting factors from copper resistance plasmid pPT23D, from which *cop* was originally cloned. Tn*5* mutagenesis of pPT23D was used to localize two complementation groups immediately downstream from *copABCD*. Cloning and sequencing of the DNA in this region revealed two genes, *copR* and *copS*, expressed in the same orientation as the *cop* operon but from a separate constitutive promoter. The amino acid sequence deduced from these genes showed distinct similarities to known two-component regulatory systems, including PhoB-PhoR and OmpR-EnvZ. In addition, CopR showed strong similarity to copper resistance activator protein PcoR from *Escherichia coli*. Functional chromosomal homologs to *copRS* activated the *cop* promoter, in a copper-inducible manner, in copper-resistant or -sensitive strains of *P. syringae* pv. tomato and other *Pseudomonas* species. This implies that copper-inducible gene regulation is associated with a common chromosomally encoded function, as well as plasmid-borne copper resistance, in *Pseudomonas* spp.

Copper compounds are commonly used in agriculture as antimicrobial agents for control of bacterial and fungal diseases of crop plants and as growth enhancers in pigs. Subsequently, copper resistance has been described in several bacterial species that are exposed to high levels of Cu^{2+} ions in agricultural environments (12, 45). In addition to its toxic effects at high concentrations, copper is required in trace amounts as an essential component of several bacterial enzymes. Copper-resistant bacteria must therefore balance their resistance mechanisms with the need to acquire copper at low levels. In *Escherichia coli* isolates from pigs, a plasmid-encoded efflux mechanism encoded by the *pco* genes from plasmid pRJ1004 (35, 37) is tightly coupled to chromosomal copper transport functions (34, 35, 41). In the phytopathogen *Pseudomonas syringae* carrying the plasmid-borne *cop* operon (3, 27), copper is excluded from the cytoplasm by proteins that bind copper in the periplasm and outer membrane (7, 14). This copper exclusion mechanism may be balanced by components of the *cop* operon that function in copper uptake, since expression of *copC* and *copD*, which produce a periplasmic copper-binding protein and a probable inner membrane protein, causes copper hypersensitivity and hyperaccumulation (8).

In addition to transport functions that may balance copper exclusion mechanisms, copper resistance genes are tightly regulated and induced only by high levels of copper. Copper inducibility of the *pco* genes of *E. coli* was first shown in growth studies in which a lag phase observed upon addition of copper to the growth medium could be reduced by preinduction with copper sulfate (36). Copper induction is dependent on two genes, *pcoR* and *pcoS*, that are linked to the structural *pcoABCD* genes (6, 40). A predicted amino acid sequence derived from the nucleotide sequence of *pcoR* has been published (6, 21), and it shows strong similarities to several activator components of two-component regulatory systems. Unpublished sequence information also indicates that *pcoS* is related to the sensor components of the two-component systems (40).

Copper resistance in *P. syringae* is also specifically induced by copper, and sequencing of the *copABCD* genes showed that they are organized as an operon under the control of a single copper-inducible promoter (27, 28). Regulation of *cop* operon expression was shown to be at the transcriptional level by analysis of *cop*-specific mRNA induction and by fusion of the *cop* promoter region to *lacZ* as a reporter of transcriptional activation by copper (28).

Despite the observed differences between the physiological functions of *pco* and *cop* genes, recent unpublished sequence information indicates that the two systems are structurally related (40). However, there may be additional regulatory differences between the two systems, since cloned copper resistance genes from *P. syringae* or the subcloned *cop* promoter did not function in *E. coli* (3, 29). The objective of the present study was to characterize further the regulation of the *cop* operon by cloning and characterizing regulatory genes required for activation of the *cop* promoter.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. *E. coli* strains were cultured at 37°C on LB agar or LB broth supplemented with the appropriate antibiotics (31). LB agar or broth was supplemented with antibiotics at the following concentrations: tetracycline, 10 μg/ml; streptomycin, 20 μg/ml; kanamycin, 20 μg/ml; ampicillin, 50 μg/ml. *Pseudomonas* strains were grown at 28°C on mannitol-glutamate medium (18) supplemented with yeast extract (0.25 g/liter) (MGY) or in MGY broth. *Xanthomonas campestris* was

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TABLE 1. Strains and plasmids

Strain or plasmid	Description[a]	Source or reference
E. coli		
DH5α	F⁻ *recA1* Δ(*lacZYA-argF*)U169 *hsdR17 thi-1 gyrA66 supE44 endA1 relA1* φ80d*lac* Δ(*lacZ*) M15	38
HB101	F⁻ *hsdS20* (r⁻ m⁻) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ⁻	5
S17-1	F⁻ *pro recA1* (r⁻ m⁻) RP4-2, integrated (Tc::Mu) (Km::Tn7 [Sm^r Tp^r]), used for conjugation without helper plasmid	42
C600	F⁻ *thr-1 leuB6 thi-1 lacY1 supE44 rfbD1 fhuA21 mcrA1*	38
XL1-Blue	*supE*⁺ *lac hsdR17 recA1* F' *proAB*⁺ *lacI*^{q} *lacZ*ΔM15, used to propagate bacteriophage M13 vectors	38
P. syringae pv. syringae		
PS51	Rif^r Cam^r Cu^s	2
PS61	Rif^r Cam^r Cu^s	2
P. syringae pv. tomato		
PT12.2	Rif^r Cam^r Cu^s	2
PT23.2	Rif^r Cam^r Cu^r, contains pPT23D	2
PT23.3	Rif^r Cam^r, PT23.2 cured of pPT23D	This study
PT25.1	Rif^r mutant of PT25 (2), Cu^r	This study
PT16.1	Rif^r mutant of PT16 (2), Cu^r	This study
PT11.1	Rif^r mutant of PT11 (2), Cu^s	This study
PT17.3	Rif^r Cam^r Cu^s	2
PT26.1	Rif^r mutant of PT26 (2), Cu^s	This study
PT27.1	Rif^r mutant of PT27 (2), Cu^s	This study
PT28.1	Rif^r mutant of PT28 (2), Cu^s	This study
PT29.1	Rif^r mutant of PT29 (2), Cu^s	This study
P. fluorescens strain 078517	Rif^r Cu^s	This study
P. cichorii strain 07881	Rif^r Cu^r	15
Pseudomonas sp. strain 07888	Sm^r Cu^r	15
X. campestris pv. vesicatoria		
108313	Cm^r Ks^r Cu^s	This study
07882	Sm^r Ks^r Cu^r	15
Plasmids		
pUC119	Ap^r, plasmid cloning vector	48
pUC128	Ap^r, plasmid cloning vector	19
pUC129	Ap^r, plasmid cloning vector	19
pRK415	Tc^r, RK2-derived broad-host-range cloning vector	19
pRK2013	Km^r, mobilization helper	17
pGS9::Tn5	Km^r Cm^r, Tn*5* donor plasmid, IncN	39
pMP190	Sm^r Cm^r, IncQ promoter probe vector with promoterless *lacZ* gene	43
pCOP38	Sm^r Cm^r, pMP190 with *cop* promoter cloned in front of *lacZ*	28
pDAC101	Tc^r, pRK415 carrying an 8.8-kb *Hin*dIII-*Hin*dIII fragment from pPT23D containing *copABCDRS* expressed from *lac* promoter	This study
pDAC102	Tc^r, pRK415 carrying *copABCDRS* opposite in orientation to pDAC101	This study
pDAC103	Tc^r, pRK415 carrying a 6.4-kb *Hin*dIII-*Bam*HI fragment from pPT23D containing *copABCDR* and expressed from *lac* promoter	This study
pDAC104	Tc^r, pRK415 carrying *copABCDR* opposite in orientation to pDAC103	This study
pDAC105	Tc^r, pRK415 carrying a 3.6-kb *Pst*I-*Pst*I fragment from pPT23D containing *copS* expressed from the *lac* promoter	This study
pDAC106	Tc^r, pRK415 carrying *copS* opposite in orientation to pDAC105	This study
pDAC138	Cm^r Sm^r, pMP190 carrying *copRS* promoter region in correct orientation to express promoterless *lacZ*	This study
pDAC139	Cm^r Sm^r, pMP190 carrying *copRS* promoter region opposite in orientation to pDAC138	This study
pPT23D	Cu^r, wild-type plasmid carrying *cop* operon and regulatory genes	2, 3
pPT23D::Tn*5*-A	Cu^s Km^r Tn*5* inserted in *copR*	This study
pPT23D::Tn*5*-B	Cu^s Km^r, Tn*5* inserted in *copR*	This study
pPT23D::Tn*5*-C	Cu^s Km^r, Tn*5* inserted in *copS*	This study

[a] Str^r, chromosomal streptomycin resistance; Sm^r, streptomycin resistance; Tp^r, trimethoprim resistance; Rif^r, chromosomal rifampin resistance; Cam^r, chromosomal chloramphenicol resistance; Cu^r, copper resistance; Cu^s, copper sensitivity; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Cm^r, choramphenicol resistance; Ks^r, kasugamycin resistance.

grown on yeast-glucose-calcium carbonate agar (47) and inoculated into MGY broth supplemented with Casamino Acids (200 μg/ml) and glucose (1 g/liter) for β-galactosidase assays. MGY was supplemented with the following antibiotics: streptomycin at 5 μg/ml, kanamycin at 10 μg/ml, chloramphenicol at 15 μg/ml, tetracycline at 20 μg/ml, and rifampin at 50 μg/ml for *Pseudomonas* strains and streptomycin at 50 μg/ml, chloramphenicol at 50 μg/ml, and kasugamycin at 25 μg/ml for *Xanthomonas* strains. All strains were stored in 40% glycerol at −70°C and inoculated into the above-described media as needed.

Plasmid isolation, transformation, electroporation, and conjugation. Plasmids were isolated by the method of Zhou et al. (49) and used as crude preparations or further purified on cesium chloride-ethidium bromide gradients.

E. coli competent cells were prepared and transformed by the calcium chloride-heat shock method (38). *P. syringae* cells were transformed with pPT23D by electroporation and selected on MGY supplemented with 1.2 mM $CuSO_4$ as previously described (13). *P. syringae* PT23.2 was cured of pPT23D by electroporation under the conditions used for introduction of plasmids, but without added DNA. Colonies that grew on MGY after electroporation were screened on MGY and MGY with $CuSO_4$ (300 μg/ml), and plasmid isolations and Southern blot hybridizations were performed to confirm loss of pPT23D. Constructed plasmids were conjugated from *E. coli* to *Pseudomonas* or *Xanthomonas* cells by using strain S17-1, as previously described (42), or in triparental matings with pRK2013 as a helper plasmid, and transconjugants were selected on media containing the appropriate antibiotics.

β-Galactosidase assays for induction of the *cop* promoter in pCOP38. *Pseudomonas* strains containing pCOP38 and appropriate plasmid clones were grown in MGY broth to the mid-log phase and subcultured into 5 ml of fresh MGY or MGY with either 0.05 or 0.1 mM $CuSO_4$. These subcultures were then grown for either 6 or 12 h. The cells were lysed and assayed for β-galactosidase activity as described by Miller (31) with *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Both quantitative and qualitative measurements of β-galactosidase activity were recorded.

Selection and screening for regulatory mutants by Tn*5* mutagenesis. To locate the region of pPT23D that encodes the regulatory gene(s) required for induction of the *cop* promoter, PS61 containing pCOP38 and pPT23D was mated with *E. coli* C600(pGS9::Tn*5*) as described by Selvaraj and Iyer (39) to obtain random Tn*5* mutants. Kanamycin-resistant colonies of PS61(pCOP38, pPT23D) were then screened on MGY agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 0.05 mM $CuSO_4$ to identify mutants that were negative for copper-inducible production of β-galactosidase from the *cop* promoter in pCOP38. β-Galactosidase-negative mutants were then screened for copper resistance on MGY agar plates containing 1.0 mM $CuSO_4$; this second screening was done to identify and exclude mutations in pCOP38 that would result in loss of β-galactosidase production but still maintain copper resistance owing to the presence of a wild-type pPT23D plasmid. Mutations in the activator gene should yield copper-sensitive mutants unable to activate expression of the *cop* operon.

A second method for obtaining mutations in the activator gene(s) was to saturate pPT23D in PS61 with Tn*5* insertions and electroporate pooled pPT23D::Tn*5* mutant plasmids into PS61(pCOP38) for screening. In this revised method, PS61(pPT23D) was mated with *E. coli* C600(pGS9::Tn*5*), and kanamycin-resistant mutants of PS61(pPT23D) were pooled (about 2,000 colonies per pool). Plasmid DNA was purified from the pooled mutants and electroporated into PS61(pCOP38) for screening as described above. The screening process for activator mutations in this revised method was much more efficient, since all kanamycin-resistant transformant colonies had Tn*5* inserted into pPT23D.

Mapping of transposon insertions. Tn*5* insertions in pPT23D were localized on the physical map of the plasmid (11) by digestion with *Pst*I and *Bam*HI, followed by agarose gel electrophoresis and Southern blot analysis with Tn*5* as the probe. Southern blot analysis was carried out with the nonradioactive Genius kit (Boehringer Mannheim) in accordance with the manufacturer's instructions.

Plasmid construction for complementation of Tn*5* mutants. Regions corresponding to the Tn*5* insertions in the β-galactosidase-negative, copper-sensitive regulatory mutants were cloned for complementation experiments. These clones included an 8.8-kb *Hin*dIII-*Hin*dIII fragment (pDAC101 and pDAC102), a 6.4-kb *Hin*dIII-*Bam*HI fragment (pDAC103 and pDAC104), and a 3.7-kb *Pst*I-*Pst*I fragment (pDAC105 and pDAC106). All restriction endonuclease fragments were cloned into vector pUC119 or pUC18 and subsequently cloned into broad-host-range plasmid pRK415 in both orientations with respect to the *lac* promoter. DNA fragment cloning was accomplished by restriction endonuclease digestion of pPT23D with the appropriate enzymes, resolution by 0.7 to 1.2% agarose gel electrophoresis, and recovery on DEAE membranes (NA45; Schleicher & Schuell) in accordance with the manufacturer's instructions. The resulting plasmids were conjugated into β-galactosidase-negative, copper-sensitive mutants [PS61(pCOP38, pPT23D::Tn*5*-A, -B, -C) and assayed for restoration of β-galactosidase activity and copper resistance.

DNA sequencing and analysis. Overlapping restriction endonuclease fragments corresponding to the regions of pPT23D that complemented regulatory mutants were cloned into pUC128 and pUC129 and sequenced with Sequenase II (U.S. Biochemical) in accordance with the manufacturer's instructions for double-stranded plasmid sequencing. Small DNA fragment cloning for sequencing was accomplished by restriction endonuclease digestion of the appropriate larger clones described above, resolution on 8 to 10% polyacrylamide gel electrophoresis, and recovery by band excision and elution into a 0.5 M ammonium acetate–1 mM EDTA solution (1). In addition, 7-deaza-dGTP and dITP nucleotide mixtures (U.S. Biochemical) were used in the sequencing reactions to reduce compression artifacts in the gel. In some cases, it was necessary to prepare single-stranded DNA (38) and reduce the concentration of nucleotides in the labeling mixture to sequence difficult G-C-rich areas of the template. Increasing the concentration of the Sequenase enzyme and running the termination reactions at 45°C also reduced band compression on sequencing gels.

Sequence-derived primers (18-mers) were synthesized by the Biotechnology Instrumentation Facility at the University of California, Riverside, and were purified with Cruachem oligonucleotide purification cartridges in accordance with the manufacturer's instructions. These primers were used to sequence within one *Pst*I-*Sac*II clone and to sequence across restriction sites of adjacent clones that had no overlapping DNA sequences.

Sequence reactions were electrophoresed on 4.5 or 6% polyacrylamide gels (1) on a Bethesda Research Laboratories S2 sequencing apparatus in accordance with the manufacturer's instructions. DNA and protein sequences were

analyzed with the University of Wisconsin Genetics Computer Group program (16).

Nucleotide sequence accession number. The DNA sequence containing the *P. syringae copR* and *copS* genes has been assigned GenBank accession no. L05176.

RESULTS

Requirement of *trans*-acting regulatory factors supplied by pPT23D for induction of the *cop* promoter. Reporter vector pCOP38 was constructed as a transcriptional fusion between the *cop* operon promoter and the promoterless *lacZ* gene in vector pMP190 (28). Vector pMP190 alone showed a low background level of β-galactosidase activity in the strains tested (Table 2). Copper-inducible expression of the *cop* promoter was observed in copper-resistant (PT23.2) and copper-sensitive (PT12.2) strains of *P. syringae* pv. tomato. However, when pCOP38 was placed in strain PT23.3 (PT23.2 cured of plasmid pPT23D, carrying the *cop* operon), no expression from the *cop* promoter was observed. No expression from the *cop* operon promoter was observed when pCOP38 alone was introduced into copper-sensitive strains PS61 and PS51 of *P. syringae* pv. syringae grown in the presence or absence of 0.05 mM $CuSO_4$. However, copper-inducible expression of β-galactosidase from the *cop* promoter was observed when pPT23D and pCOP38 were both introduced into these strains (Table 2).

Most other *Pseudomonas* species that were previously shown to contain chromosomal *cop* homologs (15) supported copper-inducible expression of β-galactosidase from the cop promoter in pCOP38 (Table 2). However, no copper-inducible expression from pCOP38 was observed in a strain of *X. campestris* containing a plasmid-borne *cop* homolog that functions in copper resistance (15) or in a copper-sensitive strain of *X. campestris* (Table 2).

The region of pPT23D that encodes the regulatory gene(s) required for induction of the *cop* promoter was located by Tn*5* mutagenesis. Two copper-sensitive, β-galactosidase-negative mutants were obtained with the first mutagenesis strategy, and seven were obtained with the second strategy (see Materials and Methods). The second method was much more efficient, since all of the kanamycin-resistant transformant colonies had Tn*5* inserted into pPT23D.

Restriction endonuclease digestion and Southern blot analyses showed that the Tn*5* insertions in all of the β-galactosidase-negative, copper-sensitive mutants mapped to three general sites, all in a 2-kb region immediately 3′ to the *cop* operon (Fig. 1A). We chose one mutant from each of the three general sites of insertion, PS61(pPT23D::Tn*5*-A), PS61(pPT23D::Tn*5*-B), and PS61(pPT23D::Tn*5*-C) (Table 1), for further investigation. In this study, we discovered a previously unmapped 312-bp *Pst*I-*Pst*I fragment immediately 3′ to the *cop* operon where the Tn*5* insertion in PS61(pPT23D::Tn*5*-A) was localized (Fig. 1A). The Tn*5* insertion in PS61(pPT23D::Tn*5*-B) was located 3′ to the PS61(pPT23D::Tn*5*-A) insertion in the adjacent 3.7-kb *Pst*I-*Pst*I fragment. The PS61(pPT23D::Tn*5*-C) insertion was also located in the 3.7-kb *Pst*I-*Pst*I fragment approximately 0.7 kb 5′ to the *Bam*HI site (Fig. 1A).

Two genes required for activation of the *cop* promoter. The 8.8-kb *Hin*dIII-*Hin*dIII fragment from pPT23D cloned in pRK415 (pDAC101 and pDAC102) was able to complement the Tn*5* mutations in all of the β-galactosidase-negative, copper-sensitive mutants for β-galactosidase activity and copper resistance, regardless of its orientation with respect to the *lac* promoter (Fig. 1B). The 6.4-kb *Hin*dIII-*Bam*HI fragment in pRK415 (pDAC103 and pDAC104) complemented PS61(pPT23D::Tn*5*-B) for production of β-galactosidase and copper resistance but did not complement PS61(pPT23D::Tn*5*-C) for either characteristic (Fig. 1B). PS61(pPT23D::Tn*5*-A) was only weakly complemented for β-galactosidase production, but not for copper resistance, by the 6.4-kb *Hin*dIII-*Bam*HI fragment in pDAC103 (Fig. 1B). The 6.4-kb *Hin*dIII-*Bam*HI fragment in the opposite orientation in pRK415 (pDAC104) did not complement PS61(pPT23D::Tn*5*-A) for β-galactosidase production or copper resistance (Fig. 1B). The 3.7-kb *Pst*I-*Pst*I fragment

TABLE 2. Requirement of *trans*-acting regulator for induction of the *cop* promoter

Strain	Introduced plasmid(s)	β-Galactosidase activity[a]	
		No $CuSO_4$	With $CuSO_4$
P. syringae			
PT23.2	pMP190	16.2	11.1
PT23.2	pCOP38	18.5	683.6
PT23.3	pMP190	8.7	2.8
PT23.3	pCOP38	6.6	6.0
PS61	pMP190	22.4	15.6
PS61	pCOP38	23.0	19.0
PS61	pCOP38, pPT23D	23.4	749.5
PS51	pMP190	18.8	10.7
PS51	pCOP38	20.4	23.5
PS51	pCOP38, pPT23D	39.6	1,274.0
PT12.2	pMP190	9.4	26.7
PT12.2	pCOP38	9.1	335.7
PT25.1	pMP190	23.3	27.2
PT25.1	pCOP38	34.7	959.8
PT16.1	pMP190	50.9	34.6
PT16.1	pCOP38	24.8	424.6
PT11.1	pMP190	12.5	11.8
PT11.1	pCOP38	40.9	625.6
PT17.3	pMP190	18.6	19.9
PT17.3	pCOP38	29.5	302.4
PT26.1	pMP190	30.8	34.1
PT26.1	pCOP38	34.9	687.4
PT27.1	pMP190	35.4	50.7
PT27.1	pCOP38	56.4	793.0
PT28.1	pMP190	33.7	53.5
PT28.1	pCOP38	20.9	30.2
PT29.1	pMP190	13.3	14.5
PT29.1	pCOP38	15.9	231.5
P. fluorescens			
078517	pMP190	21.0	50.1
078517	pCOP38	31.8	178.9
P. cichorii			
07881	pMP190	9.0	24.5
07881	pCOP38	21.3	1,084.2
Pseudomonas sp.			
07888	pMP190	22.8	30.5
07888	pCOP38	37.1	757.9
X. campestris			
108313	pMP190	29.0	18.8
108313	pCOP38	21.6	19.1
07882	pMP190	NT	NT
07882	pCOP38	24.8	18.1

[a] β-Galactosidase activity is expressed in Miller units (31). Cultures were grown for 12 h with 0.05 mM $CuSO_4$ for copper induction. The values shown are averages of two replicate cultures. Control cultures containing pMP190 represent the background levels of β-galactosidase present in these cultures. NT, not tested.

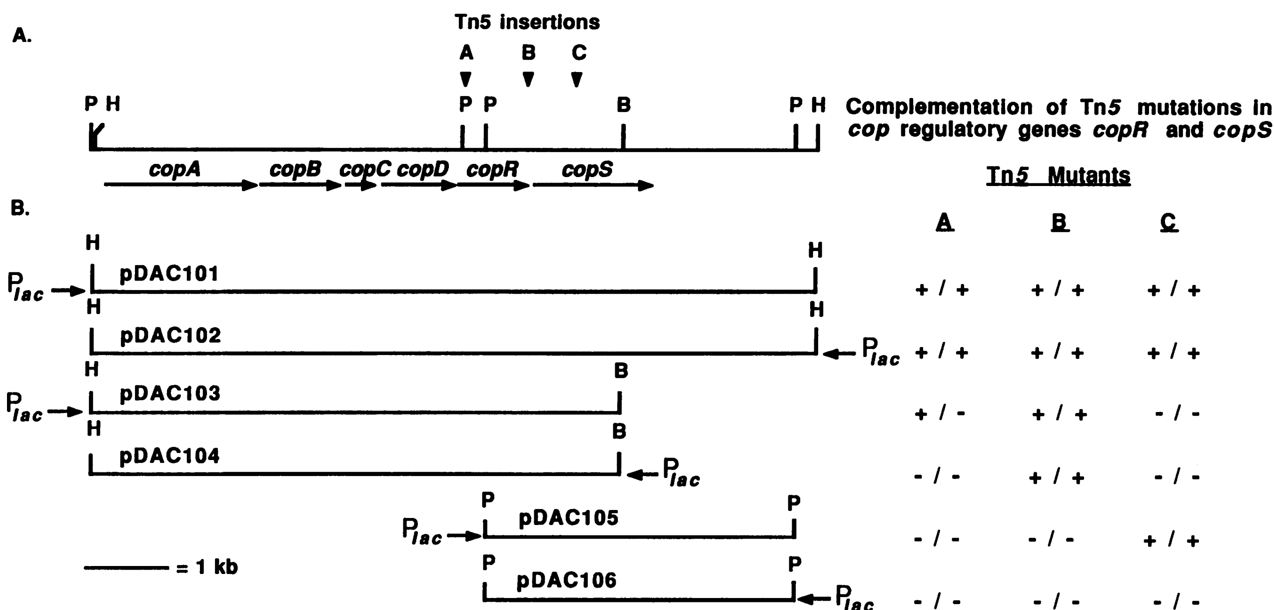

FIG. 1. Localization and complementation of Tn*5* mutations in regulatory genes adjacent to the *cop* operon. (A) Restriction endonuclease map of the 8.85-kb fragment containing the copper resistance operon (*copA*, *copB*, *copC*, and *copD*) and regulatory genes (*copR* and *copS*) required for activation of *cop* operon expression. Tn*5* insertions causing disruption of regulatory genes *copR* and *copS* are mapped as A, B, and C, which correspond to PS61(pPT23D::Tn*5*-A), PS61(pPT23D::Tn*5*-B), and PS61(pPT23D::Tn*5*-C), respectively. (B) Data showing the abilities of different clones from the 8.85-kb *Pst*I-*Hin*dIII fragment shown in panel A to complement Tn*5* regulatory mutants (A, B, and C) for (i) copper-inducible β-galactosidase production (+ or −) from the *cop* promoter in pCOP38 (shown at the left of the slash in each column) and (ii) copper resistance (+ or −) from the *cop* operon (shown at the right of the slash in each column). Plasmid clones and mutated plasmids are listed in Table 1. Abbreviations: P, *Pst*I, H, *Hin*dIII, B, *Bam*HI.

complemented PS61(pPT23D::Tn*5*-C) for β-galactosidase production and copper resistance only when expressed from the *lac* promoter in pRK415 (pDAC105). pDAC106 did not complement the Tn*5* mutation in PS61(pPT23D::Tn*5*-C) (Fig. 1B).

Organization and expression of *cop* regulatory genes. The pattern of complementation observed suggested that two genes are required for induction of the *cop* promoter. The direction of transcription for these genes was the same as that of the *cop* operon. These genes appeared to be organized as an operon because the second gene, contained within the 3.7-kb *Pst*I-*Pst*I fragment, was expressed only if it was driven from the *lac* promoter in pDAC105 (Fig. 1). The first gene was designated *copR*, and the second was designated *copS*. Reporter vector pDAC138 was constructed with pMP190 containing a DNA fragment that encodes the 3′ end of *copD* and the 5′ end of the first regulatory gene (*copR*) ending at the *Pst*I site 54 nucleotides into *copR* (Fig. 2). Expression studies using pDAC138 showed that an active promoter region 5′ to *copR* was constitutively expressed (Table 3). This promoter region did not function when cloned into pMP190 in the opposite orientation to make pDAC139 (Table 3). pDAC138 produced the same levels of β-galactosidase in PS61 whether or not pPT23D was present in the strain and whether or not these strains were grown in the presence or absence of 0.05 mM $CuSO_4$.

The 6.4-kb *Hin*dIII-*Bam*HI fragment in pDAC103 and pDAC104 (Fig. 1) complemented the Tn*5*-induced mutation in PS61(pPT23D::Tn*5*-B) but not the mutation in PS61(pPT23D::Tn*5*-A). This suggested that the Tn*5* insertion in PS61(pPT23D::Tn*5*-B) provided promoter activity for transcribing the second gene, whereas the polar effect caused by the Tn*5* insertion in PS61(pPT23D::Tn*5*-A) completely disrupted expression of both genes (4; Fig. 1).

Sequence of the activator genes and similarity to two-component regulatory systems. The 312-bp *Pst*I-*Pst*I fragment immediately 3′ to the *cop* operon (described above) was cloned and sequenced. Additionally, overlapping DNA fragments from the 3.7-kb *Pst*I-*Pst*I fragment were also subcloned and sequenced (Fig. 1A and 2). A putative ribosome-binding site and translational start codon immediately 3′ to *copD* was identified in the previously published sequence of the 4.5-kb *Pst*I-*Pst*I fragment containing the *cop* operon (27). An open reading frame (*copR*) that encodes 227 amino acids followed this start site and continued through the 312-bp *Pst*I-*Pst*I fragment into the adjacent 3.7-kb *Pst*I-*Pst*I fragment (Fig. 1 and 2). A second open reading frame (*copS*) that encodes 487 amino acids and overlapped the termination codon of *copR* was identified (Fig. 2).

The predicted amino acid sequence from *copR* shared strong similarities with other known regulatory proteins belonging to two-component regulatory systems (Fig. 3). The conserved phosphorylation site (aspartic acid) observed in the other two-component activator proteins (Fig. 3) was also present in CopR at residue 51. The amino acid sequence predicted from *copS* showed similarities to three conserved regions of sensor proteins from two-component systems, including the histidine kinase autophosphorylation site at H-245 (Fig. 4). The relative positions of two hydrophobic regions in CopS were also similar to those of sensor proteins PhoB and EnvZ (44; Fig. 5).

DISCUSSION

Two-component sensory transduction is a common mechanism for bacteria to alter gene expression in response to environmental stimuli (9, 30). The current study suggests that *P. syringae* employs this strategy for regulation of

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     CopD→
   1 AGCATGGCCC TGGAACTGGG CGCCGCCGTC TTGGTGTTGG GCCTGATTGC CTGGCTTGGC ACACTGTCCC CTGAGATGGA AGCGGGGATG TGAGTGTGCC
     S M A L   E L G   A A V   L V L G   L I A   W L G   T L S P   E M E   A G M   *                    310
                                    RBS         CopR→                                             PstI
 101 TGACCCTGTT TTACCGTCAC ACTGGGCCGG TGCCGTGGAG GGTCGAACAT GAAACTGCTG GTAGCCGAAG ACGAACCTAA AACTGGAATC TATCTGCAGC
                                                         M K L L   V A E D   E P K   T G I   Y L Q Q   18
 201 AAGGCCTGCG GGAGGCCGGC TTCAACGTGG ACCGCGTGGT CACAGGCACA GATGCCGTTG ACCAGGCGCT CAATGAAGCC TATGACCTGT TGATTCTCGA
       G L R   E A G   F N V D   R V V   T G T   D A V D   Q A L   N E A   Y D L L   I L D               51
 301 CGTGATGATG CCAGGCCTTG ATGGCTGGGA AGTCATCAGA CGCCTTCGCA CCGCTGGCCA GCCCGTACCG GTGCTGTTTC TGACCGCACG GGACGGGGTA
       V M M   P G L D   G W E   V I R   R L R T   A G Q   P V P   V L F L   T A R   D G V               84
 401 GACGACCGGG TCAAGGGCCT GGAGCTGGGC GCCGATGATT ATCTGGTCAA GCCGTTTGCC CTTTCAGAAC TGCTGGCACG GGTGCGAACG CTGTTACGGC
     D D R V   K G L   E L G   A D D Y   L V K   P F A   L S E L   L A R   V R T   L L R R            118
                PstI
 501 GCGGCAGCAG CCTGCAGGTT CAAACGAGCC TGCAAATCGG AGACCTTCAG GTGGATTTGC TCAAGCGTCG GGCGACTCGG GGCGGCAAGC GAATCGAGCT
       G S S   L Q V   Q T S L   Q I G   D L Q   V D L L   K R R   A T R   G G K R   I E L              151
 601 TACGGCCAAA GAGTTTGCAC TGCTGGAACT GCTGATGCGC CGGCAGGGTG AAGTGCTGTC CAAATCGCTG ATCGCCTCCC AGGTCTGGGA CATGAATTTC
       T A K   E F A L   L E L   L M R   R Q G E   V L S   K S L   I A S Q   V W D   M N F              184
 701 GACAGCGACA CAAATGTTAT CGAGGTGGCG ATACGACGGT TGAGGGCCAA GATTGACGAT GACTTCGAGG TAAAACTGCT GCATACCTGC CGGGGAATGG
     D S D T   N V I   E V A   I R R L   R A K   I D D   D F E V   K L L   H T C   R G M G            218
                RBS             CopS→
 801 GTTACATGCT AGAGGCGCAG GACGAAGGAT GAAGCCTGGC TCACTCACCC TGCGCTTGAG CCTTCTGTTT GTGGTCGCGG TCGCCGCCGT CCTAATCATT
       Y M L   E A Q   D E G *                                                                            227
                                M K P G   S L T L   R L S   L L F   V V A V   A A V   L I I            24
 901 GTAGGGGTTG CCTTCAACGA GCTGAGCCGT CACCACTTTC GCGCCCTGGA TGCGCAGGCG CTGGGAGAAA AGCTGGAAGC GATTACCCAG ATTGCCAAGG
     V G V A   F N E   L S R   H H F R   A L D   A Q A   L G E K   L E A   I T Q   I A K E             58
1001 AATCAGGCGC AAACCCGGAA CTGCTCAAGG CGCGCTGGCA CACCCTCCTG GGGGCACATC CCGATCTCAG TGCTGTATTT CTGAAGACCG ATGGAACGCC
       S G A   N P E   L L K A   R W H   T L L   G A H P   D L S   A V F   L K T D   G T P              91
1101 TTTTTTTGCA GAACCTCCCC AATCAGCGGT GCCGTCGCTC GCGCAGGCCA CCCAGCGCGA TGGCGTCTGG GAGTGGGAAA AAGAAGGCCG CATGTTCAGG
       F F A   E P P Q   S A V   P S L   A Q A T   Q R D   G V W   E W E K   E G R   M F R             124
     ApaI
1201 GCCCTGACCG CCTCGGTATC GTTGCCCACT GCCAGCCCGC CGCTGACCGC GTGGCTTGTC CTGGACGTCA CCACGCATAT GCACTTCTTT GCCATGCTTG
     A L T A   S V S   L P T   A S P P   L T A   W L V   L D V T   T H M   H F F   A M L E            158
1301 AACGCTGGTT CTGGGGCGTC CTATTGGCTA GTACCGTGTT AAGCGCGGCC CTGGGGTGGC TGGTCGCCAA GAACGGGCTA CGCCCGGTAG CCAGGGTGAC
       R W F   W G V   L L A S   T V L   S A A   L G W L   V A K   N G L   R P V A   R V T            191
                          SacII
1401 CCAGACCGCC GCCTCGATGT CCGCGGGCTC GCTAAAGGAA CGCATACCCT TGGAGCCAGT GCCGGATGAG TTGCGCGCGC TCATCACCGC GTTCAATTCC
       Q T A   A S M S   A G S   L K E   R I P L   E P V   P D E   L R A L   I T A   F N S            224
1501 ATGCTCGGTC GCCTGGATGA TTCCTTCATG CGCCTATCGA ATTTCTCCGC TGACATCGCG CATGAGCTTC GCACCCCGAT CAGCAACCTG CGCACCCACA
     M L G R   L D D   S F M   R L S N   F S A   D I A   H E L R   T P I   S N L   R T H T            258
                                                             SacI           SacI
1601 CCGAGGTGAT CCTGGCCAAA AAGCGTGCGC CCGAGGTGTA CGAGGAGAAT TTGAGCTCCA ACCTGGAAGA GCTCAACCGT CTTTCGGGAA TCATCGACGG
       E V I   L A K   K R A P   E V Y   E E N   L S S N   L E E   L N R   L S G I   I D G            291
     SphI
1701 CATGCTGTTC CTGGCCAAGT CCGACAACGG GCTAATTGTG CCTGAGGCTG TTGAACTGGA TCTGAGAACA GTCATCAGCA AGCTGTTCGG CTATTACGAA
       M L F   L A K S   D N G   L I V   P E A V   E L D   L R T   V I S K   L F G   Y Y E            324
1801 TTTCTGGCCG AGGACAAGGG GATTCAGCTA CAAGCATCAG GCAACGCCTC GATCTTCGCG GACAGCGTGA TGATCGACCG TGTCGTGTCG AACCTGCTGT
     F L A E   D K G   I Q L   Q A S G   N A S   I F A   D S V M   I D R   V V S   N L L S            358
1901 CCAATGCCCT GCGCTATACG TCGTCCGGAG AGACGATCAA GGTCAGCATC CACGATCACG GAGGAAGGGT AGAGCTTCGA TTGGAAAATC CTGGCCCTGA
       N A L   R Y T   S S G E   T I K   V S I   H D H G   G R V   E L R   L E N P   G P E            391
                                                       BamHI
2001 AATAGTACCG CAGCACCTAG ACCGCATCTT TGACCGGTTC TACCGGGTGG ATCCGGCGCG GCGCGAAGGC CGGGAATGCG GGGCTGGGGC TAGCGATTGC
       I V P   Q H L D   R I F   D R F   Y R V D   P A R   R E G   R E C G   A G A   S D C            424
2101 CCGGTCCTTG ATGCAAGCGC ATGGCGGCAC TATCTCGTGT ACATCCCACG AGGGCCGAAC GACCTTCATC CTCACCTTCA TGCGATCGCC TGCCCCACGA
     P V L D   A S A   W R H   Y L V Y   I P R   G P N   D L H P   H L H   A I A   C P T N            458
2201 ACCTGACATG CCGGCCTGAC AGCCTGGGCA CCGCTAAGCC AGGCCATACC AGGCTTGGCG AACATGAAAC CGGTTGTCAC TGCGCCGGGT AGTCAGCCAC
       L T C   R P D   S L G T   A K P   G H T   R L G E   H E T   G C H   C A G *                    487
                                                                                          SphI
2301 CACCTCCTGC TCGCCGGTCT TTTTCAGTCC GATGACCTGA TAGGCGCTTT TATGCCCTGT TTCCATGCCC GGTGAGCCCA TTGCATGC
```

FIG. 2. Nucleotide sequence and predicted translation products of regulatory genes *copR* and *copS*. The end of *copD* of the previously published copper resistance operon sequence (27) is shown before the beginning of *copR*. Potential ribosome-binding sites (RBS) are underlined. Pertinent restriction endonuclease recognition sites used for subcloning and sequencing are underlined and labeled above the sequence. A potential transcriptional termination sequence is also underlined. The asterisks indicate translational stop codons.

copper resistance gene expression. The amino acid sequence predicted from *copR* shares strong similarity to activator proteins from two-component regulatory systems, including *phoB* (24), *ompR* (10), the putative regulatory gene for copper metabolism in *Streptomyces lividans*, *cutR* (46), and the copper resistance gene activator from *E. coli*, *pcoR* (6; Fig. 3). CopR also contains the conserved aspartic acid residue thought to be the phosphorylation site important for regulatory activation (6). The amino acid sequence predicted from the *copS* gene showed homology with sensor proteins of other two-component systems in three important regions (Fig. 4), including the histidine kinase autophosphorylation site (44). The similarity between CopS and other sensor proteins was low outside of these three regions. Identification of *pcoS* in *E. coli*, corresponding to *copS*, has been previously reported but no sequence data have been published for comparison (40). CopS also contains two highly hydrophobic β-sheet regions, like EnvZ and PhoR (25, 44), suggesting that it is also a transmembrane protein. The overall structure of CopS resembles that of the common

TABLE 3. Expression of β-galactosidase activity from the *copRS* promoter region in reporter vector pMP190

Plasmid(s)[b] introduced into strain PS61	β-Galactosidase activity[a]	
	No $CuSO_4$	With $CuSO_4$
pDAC138	284	282
pDAC138,pPT23D	278	298
pDAC139	27	26
pDAC139,pPT23D	23	17

[a] β-Galactosidase activity is expressed in Miller units (31). Cultures were grown for 6 h with 0.1 mM $CuSO_4$ for copper induction.

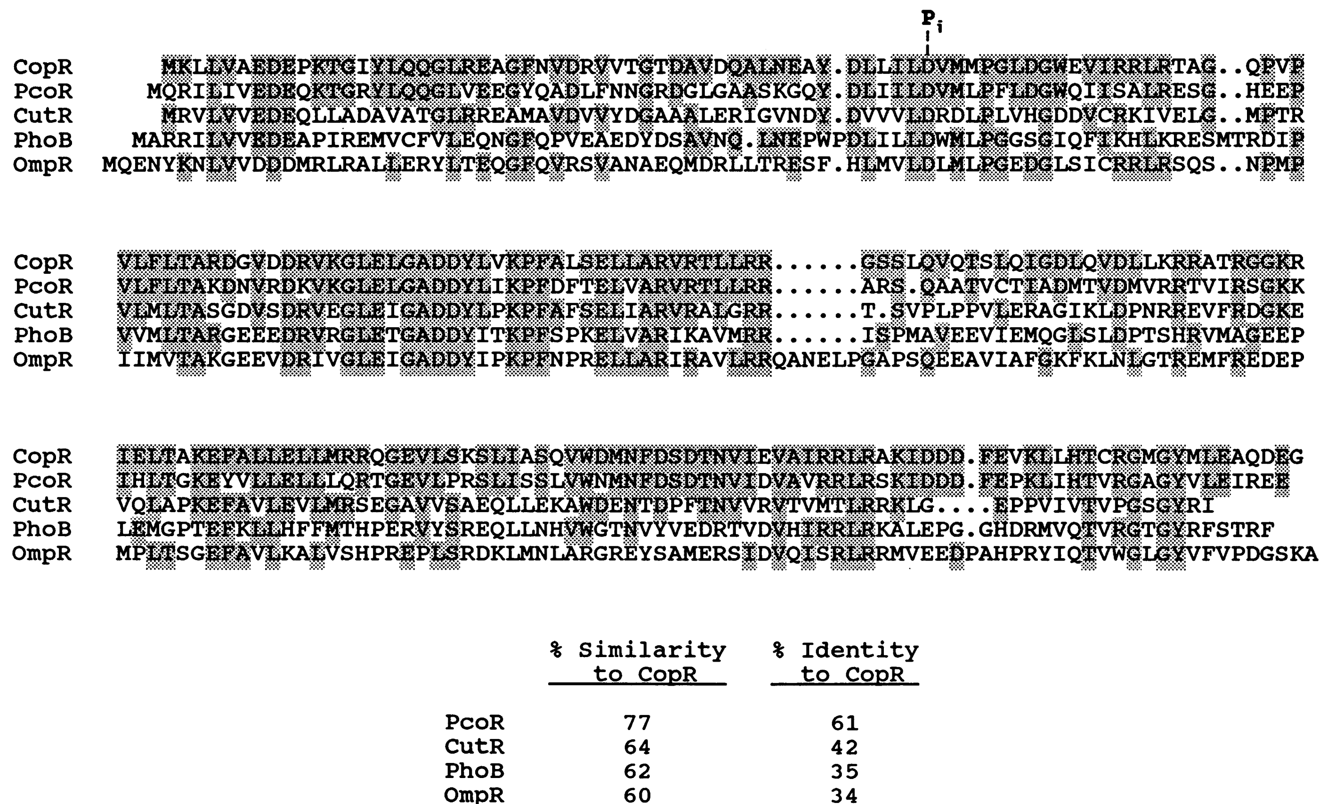
[b] pDAC138 contains the *copRS* promoter region in pMP190 in the correct orientation to drive expression of *lacZ*. pDAC139 contains the *copRS* promoter region in the opposite orientation (Table 1).

input-transmitter modular arrangement of sensor proteins, such as EnvZ and PhoR (33).

In a hypothetical model, the putative transmembrane protein CopS may sense high levels of free Cu^{2+} ions in the periplasm and phosphorylate the CopR protein present in the cytoplasm. Phosphorylation of CopR would convert it from an inactive to an active state to induce expression of the *cop* operon.

The results from this study show that *copR* and *copS* are both transcribed from the same promoter located 5′ to *copR*. However, we have not ascertained whether or not they are also expressed from the *cop* promoter. This is a possibility because we could not identify a *rho*-independent termination sequence 3′ to the *copD* open reading frame. In comparison, *phoB* and *phoR* are also organized as an operon under the control of a promoter 5′ to *phoB* that is, in this case, induced under conditions of phosphate starvation (26). The *copRS* promoter appears to be expressed constitutively (Table 3). In the *phoB-phoR* system, it is suggested that phosphorylation of Asp-53 of *phoB* changes its conformation so that it facilitates binding of RNA polymerase to the promoters of the phosphate regulon which lack a −35 promoter consensus sequence (22, 23). The *cop* operon promoter also lacks a good consensus sequence for RNA polymerase binding (27). It is possible that an activated CopR may facilitate binding of RNA polymerase to the *cop* promoter. We are currently investigating the interaction of DNA-binding proteins with the *cop* promoter.

Induction of the *cop* promoter is specifically induced by copper, since no induction was observed in cultures grown in the presence of subinhibitory levels of other cations, including Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Ru^{2+}, Pd^{2+}, Cd^{2+}, Sn^{2+}, Hg^{2+}, and Pb^{2+} (12). These results suggest that metal specificity is determined by the CopS transducer protein. If so, CopS must specifically recognize or transduce a signal in response only to copper. This would require specific binding or placement of copper in the periplasmic (or membrane) domain, but possibly in the cytoplasmic domain of CopS as well. We did not observe proportional spacing of potential copper ligands in CopS for the type I copper-binding domains previously published (32), nor did we



	% Similarity to CopR	% Identity to CopR
PcoR	77	61
CutR	64	42
PhoB	62	35
OmpR	60	34

FIG. 3. Alignment of CopR with regulatory proteins from two-component signal transduction pathways. PcoR is responsible for activation of copper resistance genes in *E. coli* (21), CutR is suggested to regulate uptake or efflux of copper in *S. lividans* (46), PhoB regulates phosphate uptake and assimilation in *E. coli* (22–24), and OmpR is a regulatory protein in *E. coli* that modulates gene expression in response to changes in osmolarity (10). The putative phosphorylation site (aspartic acid residue) is indicated (P_i). Conserved residues are shaded.

	Pi * I		II *		* III		* *
CopS	241 ADIAHELRTPIS	346	SVMIDRVVSNLLSNA	385	LENPGPEIVP	[20]	RECGAGASDC
PhoR	209 ANVSHELRTPLT	315	EDQLRSAISNLVYNA	354	VEDNGPGIAP	[21]	GGSGLGLAIV
EnvZ	239 AGVSHDLRTPLT	334	PLSIKRAVANMVVNA	371	VEDDGPGIAP	[19]	SGTGLGLAIV

FIG. 4. Alignment of three conserved regions of CopS and sensor proteins of two-component regulatory systems. PhoR is a sensor corresponding to activator PhoB in *E. coli* (25, 26), and EnvZ is a sensor in *E. coli* that corresponds to activator OmpR (10). The numbers indicate the amino acid residue at the start of each conserved region or the number of amino acids between conserved sequences (in brackets). The putative autophosphorylation residue (histidine) is indicated (P_i). Conserved residues are shaded.

observe similarities to putative copper-binding sequences previously reported for *cop* structural genes *copA* and *copB* (27). However, the deduced amino acid sequence from *copS* contains regions of high methionine, histidine, and cysteine content that may serve as ligands for copper binding. The cysteine residues are all located on the carboxyl end of CopS, suggesting that they reside in the cytoplasmic domain. There are a number of histidine and methionine residues on either side of the second hydrophobic region of CopS that may facilitate copper binding.

Induction of the *cop* promoter in pCOP38 was observed for *P. syringae* pv. tomato strains containing pPT23D and was also observed for some strains of *P. syringae* pv. tomato and other *Pseudomonas* species with no copper resistance plasmids. Therefore, in addition to the *cop* structural homologs previously reported in the chromosomes of several *Pseudomonas* species (15), regulatory homologs of *copR* and *copS* probably also exist in the chromosomes of some pseudomonads. However, not all strains of *P. syringae* pv. tomato contain functional *copR-copS* chromosomal homologs that activated the *cop* promoter introduced in pCOP38. Similarly, no functional *copR-copS* homologs were present in two strains of *P. syringae* pv. syringae or in either a copper-sensitive strain of *X. campestris* or a copper-resistant strain carrying a plasmid with homology to *cop* (15). All strains of *P. syringae* that we have examined do contain genes that hybridize with *cop*, at least under conditions of low stringency, and the *cop*-related copper resistance genes of *X. campestris* are copper inducible (15). These bacteria may contain genes similar to *copR-copS* but with differences in promoter specificity.

Genetic data suggest that the *E. coli* chromosomal genes, designated *cutR* and *cutS*, may be homologous to plasmid-encoded *pcoR* and *pcoS*, since Tn*5* mutations in *pcoR* can be complemented by *cutR* (6, 40). The *cutR* and *cutS* genes have been proposed to regulate several *cut* genes involved in copper uptake and management in *E. coli* (34, 35, 41). Together with arguments that support a general requirement for copper transport and management systems to avoid damage caused by free copper ions (34, 35), the finding of related copper-responsive chromosomal regulatory genes in *Pseudomonas* spp. and *E. coli* suggests that copper transport systems are common features of many bacterial species.

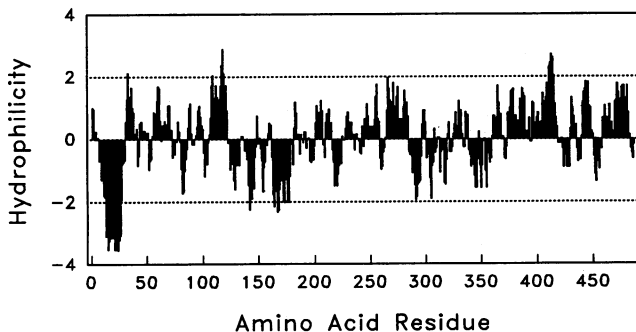


FIG. 5. Hydrophilicity profile of the predicted amino acid sequence of CopS. The algorithm of Kyte and Doolittle (20) was used, with a window setting of seven. Negative values indicate regions with predicted hydrophobic secondary structure. Hydrophobic regions of 20 amino acids or greater are present from amino acid residues 5 to 30 and 162 to 181.

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