Genetics

Cloning and Characterization of Genes Conferring Copper Resistance in Epiphytic Ice Nucleation-Active *Pseudomonas syringae* Strains

John S. Rogers, Ellen Clark, Gabriella Cirvilleri, and Steven E. Lindow

First author: ECOSTAT, Incorporated, P.O. Box 237, Highland City, FL 33846; second and fourth authors: Department of Plant Pathology, 147 Hilgard Hall, University of California at Berkeley 94720; and third author: Istituto di Patologia Vegetale, Universita Degli Studi di Catania, 95135 Catania, Italy.

Pseudomonas syringae pv. *tomato* and *Escherichia coli* containing the *cop* locus were provided by D. A. Cooksey.

We thank C. Pierce, B. Rotz, and L. Hancock for laboratory and greenhouse technical assistance and N. J. Panopoulos for helpful experimental advice.

Accepted for publication 26 April 1994.

ABSTRACT

Rogers, J. S., Clark, E., Cirvilleri, G., and Lindow, S. E. 1994. Cloning and characterization of genes conferring copper resistance in epiphytic ice nucleation-active *Pseudomonas syringae* strains. Phytopathology 84:891-897.

Many epiphytic *Pseudomonas syringae* strains obtained from asymptomatic host and nonhost plants are resistant to high concentrations of copper ions. A genomic DNA library of one such strain, AL513, was constructed in the broad host-range cosmid cloning vector pLAFR3. A cosmid designated pCOPR1.1 that conferred near wild-type levels of copper resistance when conjugated into the copper-sensitive (Cu^s) *P. syringae* strain AL487 was identified. A 6.5-kb *Pst*I fragment in pCOPR1.1 conferred this copper resistance. Insertional inactivation mutagenesis of this cosmid with the reporter transposon Tn*3*-Spice indicated that a region of approximately 5.5 kb, denoted *copJ*, is required for copper resistance. Transcriptional activity of *copJ*::Tn*3*-Spice gene fusions inserted into the *P. syringae* AL513 genome by marker exchange mutagenesis were induced within 30-45 min when as little as 0.6 μg of Cu^{2+} per milliliter was added to culture media. Zinc ions are toxic to this bacterial strain at concentrations of more than 10 μg/ml in culture media; however, zinc induced *copJ* at concentrations less than 1 μg/ml. Increases in the proportion of cells in the population that tolerated high doses of copper were correlated with increased transcriptional activity of *copJ*. In planta induction times of *copJ* were approximately sixfold longer than those observed in vitro.

Additional keywords: gene expression.

Metal-resistance genes have been identified in a variety of microorganisms from many diverse habitats (19,22,23). Metals toxic to bacteria and fungi bind to and nonspecifically disrupt multiple subcellular sites, including proteins essential for respiratory functions (5,45). In bacteria, metal-resistance genetic determinants are usually found on plasmids, many of them self-transmissible (13,16). Genetic loci in *Escherichia coli* conferring resistance to metals such as mercury (44) and copper (14,42) are relatively well characterized.

Among plant-pathogenic bacteria, both pseudomonads and xanthomonads have been identified that express resistance to copper (1,6,34). A 35-kb wild-type self-transmissible plasmid carrying copper-resistance genes was conserved among 12 copper-resistant (Cu^r) strains of the tomato pathogen *Pseudomonas syringae* pv. *tomato* (6,7). Subsequent analyses demonstrated that the *P. s. tomato* copper-resistance determinant contains four open reading frames (ORF) (35). This determinant, designated the *cop* locus, contains six genes, *copABCDRS*, that span about 6 kb (14). The *cop* locus hybridizes strongly to copper-resistant *P. s. tomato* strains, less strongly to chromosomal genes in Cu^s *P. s. tomato* pathovars (15,29), and to *Xanthomonas campestris* pv. *vesicatoria* strains from Florida, Oklahoma, and California under low-stringency conditions (51). Chromosomal genes from one Cu^s *P. s. tomato* strain resemble the *cop* operon in both structure and regulation (40). Structural similarities, including size and the presence of four ORFs, exist between copper-resistance genes from *P. s. tomato, X. campestris,* and the *pco* genes from *E. coli* (14,27). Resistant *P. s. tomato* cells appear to sequester copper outside the cytoplasm (11), but resistance mechanisms in *E. coli* and the xanthomonads, apparently involving efflux of copper from the cells, are not as well understood.

Diverse strains of the genus *Pseudomonas* also occur as epiphytes on many healthy plants and may incite freezing injury to frost-sensitive species (4,31). Andersen and Lindow (2,3) have reported the common occurrence of Cu^r ice nucleation-active *P. syringae* strains in northern California. The northern California strains survived higher concentrations of cupric ions (Cu^{2+}) (mean LC_{50} = 170 ppb) than did *P. s. tomato* (LC_{50} = 54 ppb) (3). Prior exposure of these northern strains to sublethal concentrations of copper increased the LC_{50} by more than 10-fold when compared with cells not receiving copper pretreatments. The growth and survival of several highly copper-resistant *P. syringae* strains has been reported (3). Interestingly, while colonies of *P. s. tomato* strains turn blue on copper-containing growth media (14), the northern California strains do not, suggesting different resistance mechanisms. Total genomic DNA from these highly resistant strains was hybridized to the 4.4-kb *cop* fragment from *P. s. tomato* to determine relative sequence homologies of the copper-resistance determinants. Several faint hybridization bands were detected at low stringencies, suggesting distantly related sequences (J. Wagner, *unpublished data*). *P. syringae* strain AL513, which exhibited the least homology to *cop*, was selected for more detailed genetic analysis. A principal objective of these studies was to construct fusions of an ice-nucleation reporter gene to copper-resistance determinants to provide information concerning the size and location of the resistance genes and to analyze their pattern of expression in culture and on plants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids and their relevant phenotypes and sources are listed in Table 1. Epiphytic *P. syringae* strains collected from asymptomatic almond and navel orange trees were stored in Luria broth (39) containing 15% glycerol at −80 C. Spontaneous rifampicin-resistant mutants were generated by methods described by O'Brien and Lindow (41). Approximately 10^9 cells of a bacterial suspension were plated on King's medium B (KB medium) (25) containing rifampicin at 100 μg/ml and incubated for 48 h at 30 C. Rifampicin-resistant mutants were isolated by selecting colonies with *P. syringae* characteristics (18,20), including ice nucleation activity, levan production (28), negative oxidase reaction (48), tobacco hypersensitivity (26), and negative arginine dihydrolase reaction (49). Prior to all experiments, *P. syringae* strains were recovered from storage and streaked onto KB medium containing appropriate antibiotics and incubated overnight at 30 C. *E. coli* strains were cultured on Luria agar. Strain and plasmid selections were carried out with the following antibiotic concentrations (micrograms per milliliter): rifampicin (Rf, Rif), 100; tetracycline (Tc), 12.5; nalidixic acid (Nal), 50; ampicillin (Amp), 50; streptomycin (Sm, Str), 20; and kanamycin (Km, Kan), 30.

Recombinant DNA techniques. Restriction enzyme digestions, agarose gel electrophoresis, and Southern hybridizations were performed as described by Maniatis et al (33). Genomic DNA was isolated by a modification of the method of Ish-Horowicz and Burke (24). After extraction, DNA was purified by ethidium bromide-cesium chloride gradient centrifugation (33). Plasmid DNA was extracted by the method of Birnboim and Doly (9). Restriction endonucleases were obtained from either Amersham (Arlington Heights, IL) or Bethesda Research Laboratories (Gaithersberg, MD). Lysozyme and ribonuclease A was purchased from Sigma Chemical Co. (St. Louis). For Southern hybridizations, 5–10 μg of genomic DNA was digested with *Eco*RI, electrophoresed in 0.7% agarose, and transferred to nitrocellulose filters. DNA probes were radiolabeled with ^{32}P, using a random primer labeling kit (Multiprime; Amersham). Radioactive DNA-DNA hybridizations were carried out at relatively low stringencies as described by Maniatis et al (33). Filters were incubated for approximately 2 h at 65 C in a prehybridization solution consisting of 5× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 5× Denhardt's reagent (0.02% each of bovine serum albumin, polyvinyl pyrrolidine, and Ficoll), 50 mM sodium phosphate (pH 6.5), and denatured salmon sperm DNA at 50 μg/ml. Filters were incubated for at least 4 h at 50 C with hybridization solution (5× SSC, 1× Denhardt's reagent, 50 mM sodium phosphate [pH 6.5], denatured salmon sperm DNA at 100 μg/ml, and approximately 10 μCi of the labeled probe). After incubation with the probe, filters were washed twice for 5 min in 2× SSC and 0.1% sodium dodecyl sulfate (SDS), followed by a 30-min wash with 0.1× SSC containing 0.5% SDS, and a final wash of 0.5× SSC and 0.1% SDS at approximately 55 C for 2 h.

TABLE 1. Bacteria and plasmids used in molecular genetic studies and their relevant characteristics

Strain/plasmid	Characteristics[a]	Source
E. coli		
DH5α	del(*lac*ZYA-*arg*F)	33
HB101	Str^r	10
SF800	Nal^r, *pol*A	46
P. syringae		
AL487	Rif^r, Cu^s	2
AL513	Rif^r, Cu^r	2
Cit7del1	Rif^r, Ice^+, Cu^s	31
TLPdel1b	Rif^r, Ice^+, Cu^s	31
Km-ice	Rif^r, Kan^r, Cu^r, Ice^-	This study
MEX-11S	Rif^r, Kan^r, Cu^s, Ice^+	This study
MEX-13S	Rif^r, Kan^r, Cu^s, Ice^+	This study
Plasmids/transposons		
pLAFR3	*lac*, Tc^r	47
pRK2013	IncP, $TraRK2^+$, drep, RK2, repE1, Km^r	17
Tn*3*-Spice	Ice^+, Sm^r, Ap^r	30
pCOPR1	pLAFR3 derivative, Cu^r, Tc^r	This study
pCOPR1.1	pLAFR3 derivative, Cu^r, Tc^r	This study
pT11S	pLAFR3 derivative, Cu^r, Tc^r	This study
pT13S	pLAFR3 derivative, Cu^r, Tc^r	This study
pCOP2	pRK404 derivative, Cu^r, Tc^r	8

[a] Kan^r and Km^r = kanamycin resistance; Tc^r = tetracycline resistance; Rif^r = rifampicin resistance; Str^r and Sm^r = streptomycin resistance; Nal^r = nalidixic acid resistance; Amp^r and Ap^r = ampicillin resistance; Cu^s and Cu^r = copper sensitivity and resistance, respectively.

Construction of the genomic library. A library of total genomic DNA isolated from *P. syringae* strain AL513 was constructed in pLAFR3 (47). Genomic DNA was partially digested with *Sau*3A endonuclease and size fractionated by sucrose density centrifugation to approximately 17–20 kb (J. Wagner, *unpublished data*). The size-fractionated genomic DNA was dephosphorylated with calf intestinal alkaline phosphatase and ligated with pLAFR3 arms (47). Recombinant cosmids were packaged with commercially available reagents (Amersham) and transduced into *E. coli* DH5α. Colonies containing pLAFR3 with inserted AL513 DNA were identified as white colonies on Luria agar containing tetracycline and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma).

Screening transconjugants for resistance to copper. Individual and mass triparental matings between *E. coli* DH5α cosmid-containing strains, *E. coli* HB101 (pRK2013), and the Cu^s *P. syringae* strain AL487 were carried out for 24 h at 30 C on nutrient yeast extract glycerol agar (NYGA). The ratio of donor/helper/recipient was 1:1:2. Mating mixtures were incubated overnight at 30 C and plated on NYGA or KB medium containing Rf and Tc to select transconjugants. Bacteria were screened for resistance to copper ions on casitone-yeast extract glycerol (CYE) agar containing indicated concentrations of copper. Metal ions are complexed in growth media in varying amounts (43). CYE, however, is a complex medium providing all required resources for normal growth that retains a limited copper ion-binding capacity (37,38). Copper was added to the CYE medium as $CuSO_4$ (Sigma) and adjusted to pH 7.0.

Bacteria were screened by one of two methods: 1) a qualitative screening in which resistance was scored as growth or no growth on media containing a given $CuSO_4$ concentration; 2) a quantitative screening in which colony development on media containing various amounts of copper was enumerated. In the qualitative method, bacteria were streaked on CYE medium containing added Cu^{2+} at 20–50 μg/ml, incubated for 48 h at 30 C, and examined for evidence of growth. In the quantitative method, bacteria were enumerated after spreading 10-fold serial dilutions of cells onto CYE agar containing added cupric ion, from 0 to 60 μg/ml.

Transposon mutagenesis and construction of reporter gene fusions. Reporter transposon mutagenesis was carried out according to the procedure of Lindgren et al (30). The target plasmid, pCOPR1.1, containing the *P. syringae* AL513 Cu^r locus was transformed into *E. coli* HB101 containing pTn*3*-Spice and pSShe. Transformants of the transposon-donor strain were mated with *E. coli* HB101 (pRK2013) and the recipient, *E. coli* SF800. Selection for *E. coli* SF800 (pCOPR1.1::Tn*3*-Spice) transconjugants was made on Luria agar containing Nal, Tc, Sm, and Amp. The location and orientation of Tn*3*-Spice insertions within the *P. syringae* AL513 target DNA was determined by restriction analysis. Plasmids pT11S and pT13S, containing Tn*3*-Spice insertions 11 and 13, respectively, were used to measure transcriptional activity in *trans* and to introduce the reporter transposon into the genome of *P. syringae* by marker-exchange mutagenesis.

Construction of Ice^- derivatives of *P. syringae* AL513. Since ice nucleation activity was used to report on the transcriptional activity of copper-resistance genes, an Ice^- derivative of *P. syringae* strain AL513 was constructed. Plasmid pLK2 was used in the construction of the Ice^- mutant of *P. syringae* AL513. The pLAFR3 library of *P. syringae* AL513 in *E. coli* DH5α was screened for ice nucleation at −5 C by a replica-freezing technique (32). A cosmid expressing ice nucleation activity in *E. coli* DH5α was designated pGC1. The ice nucleation gene, designated *iceG*, was subcloned from pGC1 to produce plasmid pGC11 by partial digestion of pGC1 with *Sau*3A and ligation into the *Bam*HI site of pUC118. An approximately 1-kb *Sal*I fragment was then deleted from pGC11, and the APH fragment, conferring Km resistance, was excised from pUC4K with *Sal*I and inserted into the *Sal*I site to produce pKR11, which no longer conferred ice nucleation in *E. coli*. pLK2 was subsequently constructed by cloning the APH-containing *iceG* gene from pKR11 into the *Eco*RI/*Hin*dIII site of pLAFR3.

An Ice$^-$ *P. syringae* AL513 recombinant strain containing an APH insertion in *iceG* was constructed via marker-exchange mutagenesis with plasmid pLK2. Ice$^+$ merodiploid transconjugants were selected on KB medium containing Rf, Tc, and Km. Merodiploids were then grown for 12 h in KB broth containing Rf. This process was repeated 12 times. Serial dilutions of the final culture were plated onto KB agar supplemented with Km, and marker-exchange mutants were identified as Tc^s colonies by replica plating onto KB medium containing Tc. One such recombinant, designated *P. syringae* Km-ice, was devoid of ice nucleation activity.

Four derivative bacterial strains were subsequently constructed for use in determining transcriptional activity of the Cu^r locus: 1) *P. syringae* Km-ice containing plasmid pT11S was designated PLS-11S; 2) *P. syringae* Km-ice containing plasmid pT13S was designated PLS-13S; 3) *P. syringae* Km-ice containing Tn*3*-Spice fusion 11 marker-exchanged into its genome was designated MEX-11S; and, 4) *P. syringae* Km-ice containing Tn*3*-Spice fusion 13 marker-exchanged into its genome was designated MEX-13S.

Ice nucleation assays. Expression of copper-resistance genes was assayed by measuring the ice nucleation activity of *P. syringae* Km-ice strains containing the reporter transposon Tn*3*-Spice after growth in KB broth for 24 h at 24 C. Ice nucleation activity was assayed at −5 or −9 C by a droplet-freezing assay (30) and quantified as described by Vali (50).

Statistical analyses. All data were analyzed by the Statview 4.0 statistical software package (Abacus Concepts, Berkeley, CA). Measurements of ice nucleation activity were log-transformed prior to analysis. Standard errors of the means or 95% confidence intervals were calculated from the three replicate estimates of ice nucleation activity in each experiment. Mean ice nucleation activities were compared by Duncan's new multiple range test. All experiments were repeated at least three times.

RESULTS

Identification of copper-resistance determinants. No *E. coli* DH5α transfectants containing cosmids from the *P. syringae* AL513 genomic library were found to be Cu^r. To determine if copper-resistance genes from *P. syringae* AL513 might be expressed in a Cu^s *P. syringae* strain, mass matings were carried out to mobilize the pLAFR3 cosmid library from *E. coli* DH5α into the Cu^s *P. syringae* strain AL487. Of 2,020 transconjugants screened, 19 Cu^r *P. syringae* AL487 transconjugants were identified. All except one of the 19 cosmid-containing *P. syringae* AL487 transconjugants grew on CYE medium containing more than 45 μg of added Cu^{2+} per milliliter, whereas the wild-type strain AL487 did not grow well on CYE medium with more than about 20 μg of added Cu^{2+} per milliliter.

To better establish the size of the DNA determinants conferring copper resistance to AL513, copper-resistance cosmids were digested with *Eco*RI/*Hin*dIII or *Pst*I, and the size and similarity of each insert were determined. A visual comparison of the restriction patterns permitted the categorization of each insert into one of five groups. One *Pst*I and at least two *Eco*RI/*Hin*dIII restriction fragments of equal size were common to all groups. A representative cosmid having the fewest total number of *Eco*RI/*Hin*dIII fragments was chosen for further study and designated pCOPR1.

pCOPR1 was introduced by conjugation from *E. coli* DH5α into the Cu^s *P. syringae* strain AL487, and its resistance to copper concentrations ranging from 0 to 70 μg/ml was quantitatively assessed. While the wild-type *P. syringae* strain AL487 did not survive when more than 10–15 μg of Cu^{2+} was added per milliliter to CYE medium, *P. syringae* AL487 (pCOPR1) showed inducible resistance to concentrations of copper approaching those tolerated by the *P. syringae* AL513 wild-type DNA source strain (Fig. 1). Variations in the composition of CYE medium may explain why these 10–15 μg/ml inhibitory concentrations of copper were about 5 μg/ml less than the 20 μg/ml observed in the prior screening experiments. This copper resistance-range evaluation was repeated at least five times over several months with similar results.

Identification and characterization of the *copJ* locus. Previous Southern blot hybridizations at relatively low stringencies established that some sequence homology existed between DNA from AL513 and the *cop* sequence from pCOP2. Further hybridizations of pCOPR1 and *cop* were carried out to characterize the location and size of the *P. syringae* AL513 copper-resistance determinant on pCOPR1. As expected, blots obtained with low-stringency posthybridization washes revealed a faint band indicating homology between pCOPR1 and the 4.4-kb *Pst*I *cop* probe from pCOP2. This homology was restricted to an 8.0-kb *Bgl*II fragment and an approximately 6.5-kb *Pst*I fragment of pCOPR1. The 6.5-kb *Pst*I fragment was subcloned from pCOPR1 and ligated into pLAFR3 to produce pCOPR1.1. *P. syringae* AL487 containing pCOPR1.1 exhibited resistance to a range of cupric ions comparable to that of strain AL513 (up to 45–50 μg of Cu^{2+} per milliliter was added to CYE medium). The copper-resistance determinant in this locus was designated *copJ*. A restriction map of pCOPR1.1 was generated for *Pst*I, *Eco*RI, *Hin*dIII, and *Sal*I (Fig. 2). No cleavage sites internal to the 6.5-kb *Pst*I fragment were identified for *Bgl*II, *Kpn*I, or *Sst*I.

Insertional inactivation of *copJ*. The location, orientation, and effect on expression of copper resistance of 11 insertions of the reporter transposon Tn*3*-Spice within the 6.5-kb *Pst*I *copJ* fragment are shown in Figure 2. Insertions of Tn*3*-Spice within approximately 5.5 kb of the 6.5-kb *copJ Pst*I fragment completely inactivated expression of Cu^r in *P. syringae*. Insertions from near the *Hin*dIII proximal end to about 1 kb from the *lac* proximal end of the insert completely inactivated copper resistance. The

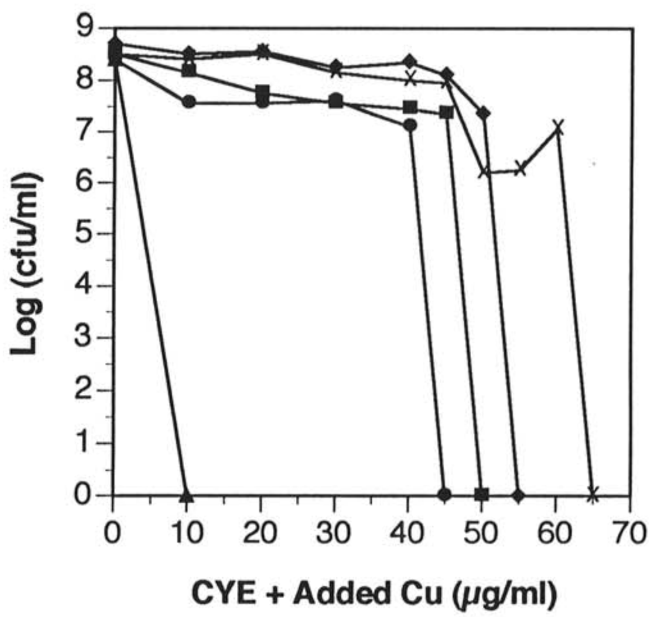

Fig. 1. Survival of *Pseudomonas syringae* AL487, *P. syringae* AL487 (pCOPR1), and wild-type *P. syringae* strain AL513 on casitone-yeast extract glycerol (CYE) agar containing concentrations of added Cu^{2+} ranging from 0 to 70 μg/ml. Copper concentrations are expressed as the concentration of divalent ion (Cu^{2+}) added to CYE medium in micrograms per milliliter. Induced cultures first were grown on CYE to which Cu^{2+} was added at 5 μg/ml prior to application to the screening media. Cells of uninduced and induced populations of *P. syringae* AL513, the *P. syringae* AL487 wild-type strain, and *P. syringae* AL487 (pCOPR1) were grown on CYE media containing concentrations of added Cu^{2+} ranging from 0 to 60 μg/ml. ▲ = AL487 wild-type; ● = AL487 (pCOPR1) uninduced; ■ = AL487 (pCOPR1) induced; ◆ = AL513 uninduced; × = AL513 induced.

direction of transcription of all Tn*3*-Spice insertions into *copJ* is opposite in direction from the *lac* promoter. The *copJ*::Tn*3*-Spice fusion plasmids containing insertions 11 and 13 and designated pT11S and pT13S, respectively (Fig. 2), were selected for subsequent gene-expression experiments. Only very low levels of ice nucleation activity (<10^{-6} ice nuclei per cell) were observed in *E. coli* DH5α (pT11S), regardless of whether Cu^{2+} was added to the culture medium.

Induction of *copJ* by copper ions in vitro. To evaluate the effects of copper ions on the transcription of *copJ* and the relationship of *copJ* activity to copper resistance, ice nucleation activity expressed by genomically located *copJ*::Tn*3*-Spice marker exchange mutants was measured in cells exposed to different concentrations of added cupric ions in vitro. Added Cu^{2+} at concentrations from 0.10 to 1.00 μg/ml increased ice nucleation

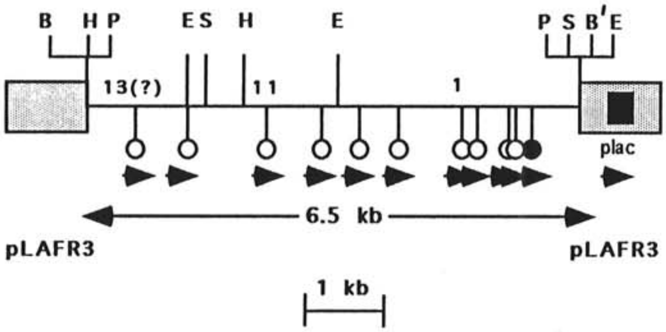

Fig. 2. Tn*3*-Spice insertions into the *copJ* locus of *Pseudomonas syringae* AL513. Insertions represented by open circles resulted in complete inactivation of the copper-resistance phenotype. The insertion represented by the single closed circle located at the plac proximal end did not abolish cell growth on casitone-yeast extract glycerol agar containing 20 μg of Cu^{2+} added per milliliter. The arrows indicate the direction of transcription of *inaZ* in each insertion. Plac refers to the *lac* promoter located in pLAFR3. Restriction sites: H = *Hin*dIII; P = *Pst*I; E = *Eco*RI; S = *Sal*I; B' = *Bam*HI. No sites for *Bgl*II, *Kpn*I, or *Sst*I were observed internal to the *copJ* locus.

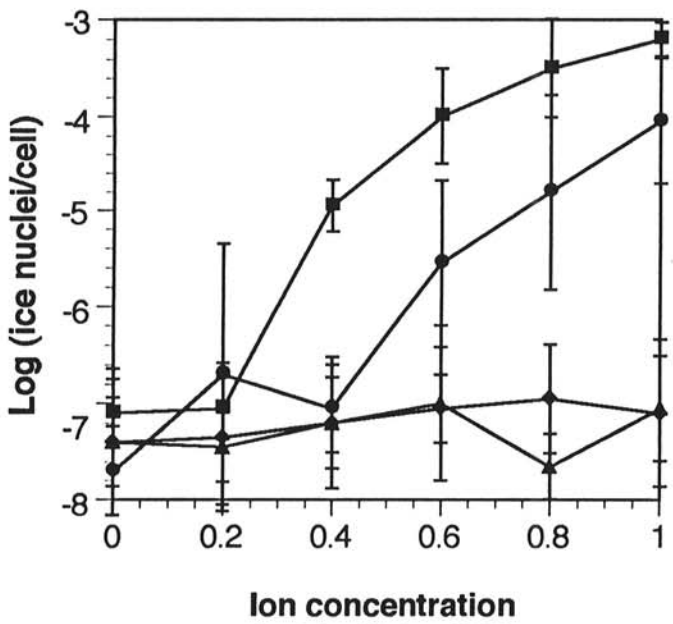

Fig. 3. Effect of different metal ions on expression of *copJ*. *Pseudomonas syringae* MEX-11S was grown overnight for 12 h at 25 C in casitone-yeast extract glycerol agar in the presence of various concentrations of copper (■), zinc (●), magnesium (▲) or no metals (◆) shown on the abscissa. After 12 h, aliquots were removed from each culture and assayed for ice nucleation activity as described in the text. The vertical bars indicate 95% confidence intervals for the estimation of the mean.

activity by several orders of magnitude. MEX-11S produced 10^{-7} or less ice nuclei per cell when 0.01 μg or less of Cu^{2+} was added per milliliter to culture media but produced up to 10^{-3} ice nuclei per cell when from 0.10 to 1.00 μg of Cu^{2+} was added per milliliter (Fig. 3). In contrast, Mn^{2+}, Mg^{2+}, Ca^{2+}, and K^+ did not increase the numbers of ice nuclei produced. Addition of Zn^{2+}, however, greatly increased transcriptional activity of *copJ* (Fig. 3). This Zn^{2+}-mediated induction of copper resistance was unexpected because *P. syringae* AL513 does not grow in the presence of more than about 10 μg of Zn^{2+} per milliliter. Although up to 1 μg of Zn^{2+} was added per milliliter, actual available ionic Zn^{2+} was probably less due to the metal ion complexing capacity of CYE medium (37).

Increases in ice nucleation activity, indicating increased transcriptional activity of *copJ*, occurred within about 30 min of addition of Cu^{2+} ions (Fig. 3). Maximum expression of *copJ* occurred after approximately 4 h. Increases in the number of cells that grew on CYE medium containing added Cu^{2+} at 60 μg/ml were correlated with increased transcriptional activity of *copJ* (Fig. 4).

Induction of *copJ* by copper ions in planta. The time required for full induction of *copJ* was significantly longer in planta than in vitro (Figs. 4 and 5). In contrast to the results obtained in vitro, induction of *copJ* was not observed within 1 h after the application of copper to bean plants. Little or no increase in ice nucleation activity on copper-sprayed plants occurred before approximately 4 h after application of a foliar (copper hydroxide, Kocide 101, Griffin Corp., Valdosta, GA) spray. Maximum induction of *copJ* in planta was usually not observed until 24 h or more after applying Cu^{2+} to the surfaces of bean leaves (Fig. 5).

Expression of plasmid-borne *copJ*::Tn*3*-Spice gene fusions. Cells containing cosmid-borne *copJ*::Tn*3*-Spice gene fusions that were not exposed to Cu^{2+} expressed high ice nucleation activity. Cells of *P. syringae* strain Km-ice (pT11S) produced about 10^{-2} ice nuclei per cell regardless of whether 1 μg of Cu^{2+} was added per milliliter to culture media. Similarly, cells of Km-ice (pT13S) produced about 10^{-3} ice nuclei per cell regardless of whether Cu^{2+} was added to the culture medium. The number of ice nuclei produced by cells containing plasmid-borne fusions in the absence of Cu^{2+} was very similar to that produced by cells when the

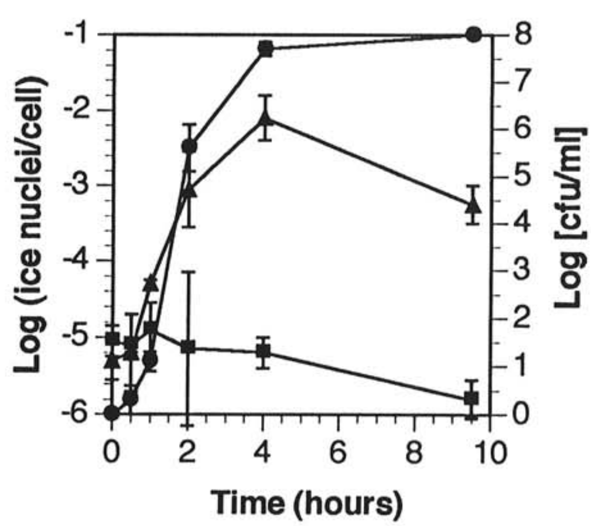

Fig. 4. Induction of copper resistance and transcription of *copJ* in *Pseudomonas syringae* MEX-11S over time in vitro. *P. syringae* MEX-11S was grown overnight in casitone-yeast extract glycerol (CYE) agar and a 1:50 dilution of each strain was then made into fresh CYE and allowed to grow approximately 2 h at 25 C. $CuSO_4$ (Cu^{2+} at 1 μg/ml) was then added to the medium. Cultures with (▲) and without (■) the added Cu^{2+} were allowed to grow an additional 3 h at 25 C and ice nucleation activity was assayed at various times as shown on the absissa. The number of cells surviving on CYE containing 60 μg of Cu^{2+} added per milliliter after the assay times indicated also was measured (●). The vertical bars indicate 95% confidence intervals for the estimation of the mean.

same fusions where introduced into the genome by marker exchange and exposed to copper. Thus, MEX-11S and MEX-13S produced about 10^{-2} and 10^{-3} ice nuclei per cell, respectively, when grown in Cu^{2+}-amended media but only about 10^{-6} and 10^{-7} ice nuclei per cell, respectively, when grown in media without added copper.

DISCUSSION

Both *copJ* and *cop* increase the survival of bacteria in culture and on plants in the presence of copper dosages that would be lethal to bacteria not containing either of the two genetic determinants. Higher levels of copper resistance are conferred by the *copJ* locus of the epiphytic ice-nucleating bacterial strain *P. syringae* AL513 than are conferred by the *cop* operon to the pathogen *P. s. tomato*, however. Colonies of *Pseudomonas* strains carrying *cop* turn blue on copper-containing media, suggesting that they sequester copper (11). Evidence indicates that this copper sequestration in *P. s. tomato* is mediated by periplasmic and outer membrane proteins (11). In contrast, Cu^r *E. coli, X. c. vesicatoria,* and *P. syringae* AL513 do not become blue when grown on media containing high copper concentrations. Thus, the possibility exists that *copJ*-containing strains employ an efflux mechanism of copper resistance similar to that believed to occur in *E. coli* and *X. campestris* (14,51). Although restriction maps are different, weak homology determined from low-stringency Southern blot analyses suggest that at least some structural similarities exist between *copJ* and *cop*. Based on similar hybridization results obtained by Voloudakis (51), it appears as if *copJ* may be about as related to *cop* as are the *X. c. vesicatoria* copper-resistance genes. The copper-resistance determinants from *E. coli*, the xanthomonads, and both *copJ* and *cop* apparently are related and are likely to be similar to *copABCDRS* in overall structure, irrespective of the mechanism of copper tolerance (14).

E. coli DH5α apparently does not phenotypically express *copJ*. The *copJ* genes in *E. coli* DH5α were transcribed only at very low levels as determined by measurements of ice nucleation activity of cells containing *copJ* in gene fusions. In contrast, copper-sensitive *P. syringae* strains containing the cloned *copJ* locus express copper resistance approaching that of the Cu^r wild-type DNA source strain. Similarly, copper-resistance genes from *P. s. tomato* and *X. c. vesicatoria* were not expressed in *E. coli* but were expressed in other Cu^s *P. syringae* and *X. campestris* strains (8,51). Like *cop*, there may be additional genetic elements required for transcription of *copJ* that are not present in *E. coli* but that are present in other *P. syringae* strains. Cooksey et al (15) suggested that expression of plasmid-encoded copper resistance in pseudomonads may be facilitated by a chromosomal regulatory determinant found in some, but not all, *P. syringae* species, including both sensitive and resistant strains. Such chromosomal determinants may be associated with other more generalized "housekeeping" processes in the host species, because the genes conferring copper resistance in *P. syringae* may be descendants of genes with functions other than that of copper resistance (13).

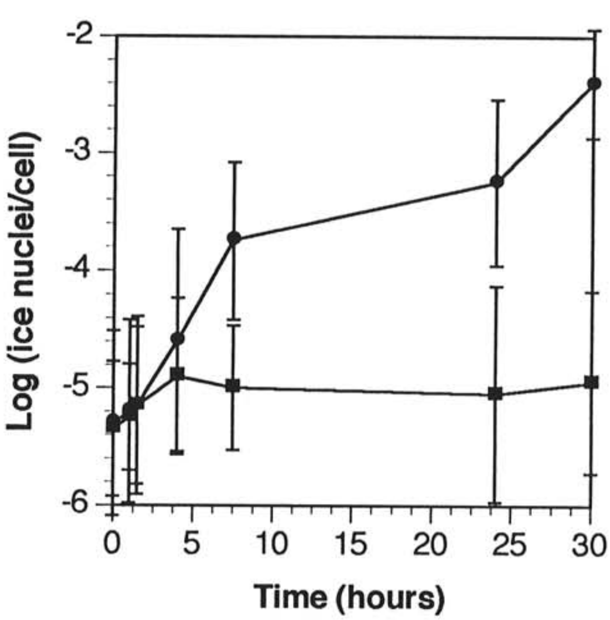

Fig. 5. Induction of *copJ* as a function of time on plants treated with $Cu(OH)_2$. *Pseudomonas syringae* MEX-11S was inoculated onto *Phaseolus vulgaris* L. 'Bush Blue Lake' and allowed to grow for 24 h under high-humidity conditions. Plants were then sprayed to runoff with 0.5 g of Kocide 101 per liter (77% cupric hydroxide) (●) or with water alone (■). Leaves were excised at different time intervals, and bacterial populations were enumerated by dilution plating and assayed for ice nucleation activity. Vertical bars represent one standard error of the mean.

The complete abolition of copper resistance by insertional mutations over the length of *copJ* differs somewhat from that observed for the *cop* operon (35). Of four open reading frames (ORFs) in *cop*, the two nearest the promoter provided partial resistance, whereas the presence of all four ORFs was required for full resistance. Mutations in or near the regulatory region of *cop* result in complete elimination of the copper-resistance phenotype. Polar effects on *cop* transcription may account for these observations because mutations downstream from the regulatory region of *cop* in ORFs 3 and 4 still enable transcription of ORFs 1 and 2, resulting in at least partial expression of copper resistance (35). In contrast, it is apparent that genes spanning nearly 5.5 kb of *copJ* are required for even partial expression of copper resistance in *P. syringae* AL513; thus, strong polar effects on *copJ* expression were observed. Similarly, at least 5.2 kb was required for full expression of copper resistance in *X. c. juglandis* (27), and 6.0 kb was required for resistance in *X. c. vesicatoria* (51).

Other heavy metal resistance genes (such as those conferring mercury resistance) (21) are induced and confer higher levels of resistance when exposed to sublethal doses of the heavy metal. Increases in copper resistance in *P. s. tomato* occurred subsequent to induction of transcription of the *cop* operon on the addition of sublethal doses of copper ions to the growth medium (36). Induction of copper resistance was also observed in *X. campestris* (51). Epiphytic *P. syringae* strains from northern California, including strain AL513, also exhibited high levels of copper resistance only if previously exposed to sublethal doses of this metal (2,3). This increase in copper resistance is apparently due to the large increase in transcription of *copJ* on the exposure of *P. syringae* AL513 to sublethal doses of Cu^{2+} in vitro and in planta (Figs. 3 and 5).

As little as 1 μg of Cu^{2+} added per milliliter to CYE medium was sufficient to induce the *copJ* genes of *P. syringae* AL513 (Fig. 3). Menkissoglu and Lindow (37) demonstrated that CYE medium exhibits a considerable ability to complex copper in a soluble but nonionic form. It is believed that only free copper ions act as inducers of *copJ*, and that other forms of copper, such as complexed but soluble forms, play little or no role in induction. Based on copper ion-availability curves in CYE medium obtained by Menkissoglu and Lindow (37), the addition of 1 μg of Cu^{2+} per milliliter to CYE medium results in less than 7×10^{-7} μg/ml that remains in an ionic state in the medium. The *copJ* regulatory system is apparently highly responsive to free divalent copper ions, because it is induced in media containing only nanomolar concentrations of copper ions. Mellano and Cooksey (36) observed that no metals except copper induced expression of the *cop* operon from *P. s. tomato*. They concluded also that the *cop* system conferred resistance only to copper. *CopJ*, in contrast, is induced by similarly low concentrations of both Cu^{2+} and Zn^{2+} but not by other metal ions (Fig. 3) even though it does not confer tolerance of zinc ions. Because the *P. s. tomato cop* operon is not inducible with zinc, *cop* and *copJ* may differ significantly in their regulation. Thus, even very small amounts of copper such as those encountered on a plant surface may be sufficient to increase the resistance of *P. syringae* AL513 to the high levels of copper that may subsequently be encountered in its leaf-surface habitat on bactericide application.

Relative to the in vitro induction of operons such as *lac*, which occurs within 5 min of the addition of isopropyl-D-thiogalactoside (IPTG) in *E. coli*, induction of copper resistance is slow but apparently occurs within about one generation time. This induction time is comparable to that observed for the induction of the *cop* genes in *P. s. tomato* by Mellano and Cooksey (36). The relatively long time required for induction of *copJ* by Cu^{2+} may reflect the time required for Cu^{2+} ions to be actively transported into cells. Because induction times occur within one generation time, at least some fraction of the cell population appears to survive initial challenges by copper.

Broth cultures provide an excellent environment for the study of basic biochemical processes in bacterial populations; however, bacterial cells on leaves are likely to be in varied physiological states and apparently respond to copper ions quite differently than a physiologically uniform laboratory culture. Plant compounds apparently do not induce the *copJ* genes, because the apparent transcription rate of *copJ* was very low unless $Cu(OH)_2$ was applied to the leaf (Fig. 5). Several factors may affect the time required for induction of *copJ* on plant surfaces, including the varied physiological states of the cells and the availability of free divalent copper ions on the plant surface. Many bacterial cells on plants exposed to the open environment may not be physiologically active and are, thus, incapable of induction of copper resistance. The availability of copper ions on the plant surface may also differ markedly from the availability of ions in uniform culture. Menkissoglu and Lindow (38) determined that the free copper ion concentrations on plants treated with $Cu(OH)_2$ at 0.5 g/L was equivalent to that when 6 μg of Cu^{2+} is added per milliliter to CYE medium, sufficient to induce *copJ* in vitro. Therefore, all bacterial cells located on a leaf surface may not be exposed immediately to ionic copper applied through a foliar spray. The ability of at least some cells of copper-resistant populations to induce to higher levels of copper resistance when exposed to low concentrations of copper ions on leaves increases the chances that those populations will survive compared to those strains lacking that ability.

The *cop* genes from *P. s. tomato* are under the control of a two-component, probably positive, regulatory system (40). Evidence also suggests that there is a chromosomally located, *trans*-acting regulatory element involved in *cop* expression (14). Under conditions of low copper-ion concentrations, however, there is an apparent low constitutive level of expression of *copJ* measured in genomic *copJ*::Tn*3*-Spice fusions. In contrast, the high-level constitutive expression of some plasmid-borne *copJ*::Tn*3*-Spice gene fusions suggests that a copper-responsive, *cis*-acting regulatory element may be involved that prevents transcription of *copJ* in the absence of Cu^{2+}. If *copJ* is *cis*-regulated by a locus immediately upstream from the copper-resistance operon, and if the regulatory region was not present or interrupted in the plasmid-borne *copJ*::Tn*3*-Spice fusions, then a high level of constitutive expression with little or no induction by Cu^{2+} would be expected. The similarity in transcription rates of the fused *copJ* region in the presence and absence of copper indicate that *copJ* is under constitutive expression at near maximum levels in pCOPR1.1. The regulatory region of *copJ* was likely truncated during the subcloning of the 6.5-kb *Pst*I fragment into pCOPR1.1 from the larger pCOPR1.

Although Southern hybridization studies indicate that *copJ* and *cop* share some structural similarities, it does appear as if there are important functional and possibly regulatory distinctions between the two copper-resistance determinants. Sequestration is associated with the *cop* sequence from *P. s. tomato* as a resistance mechanism. However, the lack of development of blue colonies in AL513 cultures on exposure to copper suggests that *copJ* confers a different, possibly efflux, mechanism for copper resistance. Copper-resistance loci in *E. coli* and *X. campestris* also confer high levels of copper resistance similar to *copJ*, and all confer higher tolerance than does the *cop* operon in *P. s. tomato*. It is believed an efflux mechanism is responsible for resistance in these strains, but it is not known if efflux always confers resistance to greater concentrations of copper than sequestration. Size, structural, and physiological similarities provide strong circumstantial evidence that *copJ* may be an operon structurally similar to resistance determinants found in *P. s. tomato, X. campestris,* and *E. coli*. Additional studies will show how specific levels and mechanisms of copper resistance conferred by *copJ* compare to those in the *E. coli* and *X. campestris* systems. Further analyses of the *copJ* region will be needed to reveal the degree of structural similarity to the copper-resistance operons from *E. coli, P. s. tomato,* and the xanthomonads and why this locus confers such high levels of copper resistance compared to those observed in other *P. syringae* strains.

LITERATURE CITED

1. Adaskaveg, J. E., and Hine, R. B. 1985. Copper tolerance and zinc sensitivity of Mexican strains of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. Plant Dis. 69:993-996.
2. Andersen, G. L., and Lindow, S. E. 1986. Occurrence and control of copper tolerant strains of *Pseudomonas syringae* on almond and citrus in California. (Abstr.) Phytopathology 76:1118.
3. Andersen, G. L., Menkissoglu, O., and Lindow, S. E. 1991. Occurrence and properties of copper-tolerant strains of *Pseudomonas syringae* isolated from fruit trees in California. Phytopathology 81:648-656.
4. Arny, D. C., Lindow, S. E., and Upper, C. D. 1976. Frost sensitivity of *Zea mays* increased by application of *Pseudomonas syringae*. Nature (Lond.) 62:282-284.
5. Avakyan, Z. A. 1971. Comparative toxicity of free ions and complexes of copper and amino acids to *Candida utilis*. Microbiology 40:363-368.
6. Bender, C. L., and Cooksey, D. A. 1985. Plasmid-mediated copper resistance in *Pseudomonas syringae* pv. *tomato*. (Abstr.) Phytopathology 75:1325.
7. Bender, C. L., and Cooksey, D. A. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: Conjugative transfer and role in copper resistance. J. Bacteriol. 165:534-541.
8. Bender, C. L., and Cooksey, D. A. 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. 169:470-474.
9. Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.
10. Boyer, H. W., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459-472.
11. Cha, J.-S., and Cooksey, D. A. 1991. Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. Proc. Natl. Acad. Sci. USA 88:8915-8919.
12. Cooksey, D. A. 1990. Genetics of bactericide resistance in plant pathogenic bacteria. Annu. Rev. Phytopathol. 28:201-219.
13. Cooksey, D. A. 1990. Plasmid-determined copper resistance in *Pseudomonas syringae* from impatiens. Appl. Environ. Microbiol. 56:13-16.
14. Cooksey, D. A. 1993. Copper uptake and resistance in bacteria. Mol. Microbiol. 7:1-5.
15. Cooksey, D. A., Azad, H. R., Cha, J.-S., and Lim, C.-K. 1990. Copper resistance gene homologs in pathogenic and saprophytic bacterial genes from tomato. Appl. Environ. Microbiol. 56:431-435.
16. Davies, J., and Smith, D. I. 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. 32:460-518.
17. Ditta, D. W., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium melilota*. Proc. Natl. Acad. Sci. USA 27:7347-7351.
18. Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliott, R. A., and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Annu. Rev. Phytopathol. 59:153-168.
19. Erardi, F. X., Failla, M. L., and Falkinham, J. O., III. 1987. Plasmid-encoded copper resistance and precipitation by *Mycobacterium scrofulaceum*. Appl. Environ. Microbiol. 53:1951-1954.
20. Fahy, P. C., and Lloyd, A. B. 1983. *Pseudomonas*. The fluorescent pseudomonads. Pages 141-188 in Plant Bacterial Diseases: A Diagnostic Guide. P. C. Fahy and G. J. Persley, eds. Academic Press, Sidney, Australia.
21. Foster, T. J., and Ginnity, F. 1985. Some mercurial resistance plasmids from different incompatibility groups specify *merR* regulatory functions that both repress and induce the *mer* operon of plasmid R100.

J. Bacteriol. 162:773-776.
22. Gadd, G. M., and Griffiths, A. J. 1978. Microorganisms and heavy metal toxicity. Microb. Ecol. 4:303-317.
23. Hendrick, C. A., Haskins, W. P., and Vidaver, A. K. 1984. Conjugative plasmid in *Corynebacterium flaccumfaciens* subsp. *oortii* that confers resistance tó arsenite, arsenate, and antimony (III). Appl. Environ. Microbiol. 48:56-60.
24. Ish-Horowicz, D., and Burke, J. F. 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. 9:2989-2998.
25. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.
26. Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature (Lond.) 199:299-300.
27. Lee, Y. A., Hendson, M., and Schroth, M. N. 1992. Cloning and characterization of copper-resistance genes from *Xanthomonas campestris* pv. *juglandis*. (Abstr.) Phytopathology 82:1125.
28. Lelliott, R. A., Billing, E., and Hayward, A. C. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.
29. Lim, C. K., and Cooksey, D. A. 1993. Characterization of chromosomal homologs of the plasmid-borne copper resistance operon of *Pseudomonas syringae*. J. Bacteriol. 175:4492-4498.
30. Lindgren, P. B., Frederick, R., Govindarajan, A., Panopoulos, N., Staskawicz, B., and Lindow, S. E. 1989. An ice nucleation reporter gene system: Identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. *phaseolicola*. EMBO J. 8:1291-1301.
31. Lindow, S. E. 1985. Ecology of *Pseudomonas syringae* relevant to the field use of Ice$^-$ deletion mutants constructed *in vitro* for plant frost control. Pages 23-25 in: Engineering Organisms in the Environment: Scientific Issues. American Society for Microbiology, Washington, D. C.
32. Lindow, S. E., Arny, D. C., and Upper, C. D. 1978. Distribution of ice nucleation active bacteria on plants in nature. Appl. Environ. Microbiol. 36:831-838.
33. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
34. Marco, G. M., and Stall, R. E. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Dis. 67:779-781.
35. Mellano, M. A., and Cooksey, D. A. 1988. Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. 170:2879-2883.
36. Mellano, M. A., and Cooksey, D. A. 1988. Induction of the copper resistance operon from *Pseudomonas syringae*. J. Bacteriol. 170:4399-4401.
37. Menkissoglu, O., and Lindow, S. E. 1991. Relationship of free ionic copper and toxicity to bacteria in solutions of organic compounds. Phytopathology 81:1258-1263.
38. Menkissoglu, O., and Lindow, S. E. 1991. Chemical forms of copper on leaves in relation to the bactericidal activity of cupric hydroxide deposits on plants. Phytopathology 81:1263-1270.
39. Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
40. Mills, S. D., Jasalavich, C. A., and Cooksey, D. A. 1993. A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. J. Bacteriol. 175:1656-1664.
41. O'Brien, R. D., and Lindow, S. E. 1987. Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. Phytopathology 79:619-627.
42. O'Halloran, T. V. 1993. Transition metals in control of gene expression. Science 261:715-725.
43. Ramamoorthy, S., and Kushner, D. J. 1975. Binding of mercury and other heavy metal ions by microbial growth media. Microb. Ecol. 2:162-176.
44. Robinson, J. B., and Tuovinen, O. H. 1984. Mechanisms of microbial resistance and detoxification of mercury and organomercurial compounds: Physiological, biochemical, and genetic analyses. Microbiol. Rev. 48:95-124.
45. Silver, S., and Misra, T. K. 1988. Plasmid-mediated heavy metal resistances. Annu. Rev. Microbiol. 42:717-743.
46. Stachel, S. E., An, A., Flores, C., and Nester, E. 1985. A Tn*3*/-*lacZ* transposon for the random generation of β-galactosidase gene fusions: Application to the analysis of gene expression in Agrobacterium. EMBO J. 4:891-898.
47. Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.
48. Steele, F. J. 1961. The oxidase reaction as a taxonomic tool. J. Gen. Microbiol. 25:297-301.
49. Thornley, M. J. 1960. The differentiation of *Pseudomonas* from other gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. 23:37-52.
50. Vali, G. 1971. Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. J. Atmos. Sci. 28:456-459.
51. Voloudakis, A. E., Bender, C. L., and Cooksey, D. A. 1993. Similarity between copper resistance genes from *Xanthomonas campestris* and *Pseudomonas syringae*. Appl. Environ. Microbiol. 59:1627-1634.