JOURNAL OF BACTERIOLOGY, June 1988, p. 2879–2883
0021-9193/88/02879-05$02.00/0

Vol. 170, No. 6

Nucleotide Sequence and Organization of Copper Resistance Genes from *Pseudomonas syringae* pv. *tomato*

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The nucleotide sequence of a 4.5-kilobase copper resistance determinant from *Pseudomonas syringae* pv. *tomato* revealed four open reading frames (ORFs) in the same orientation. Deletion and site-specific mutational analyses indicated that the first two ORFs were essential for copper resistance; the last two ORFs were required for full resistance, but low-level resistance could be conferred in their absence. Five highly conserved, direct 24-base repeats were found near the beginning of the second ORF, and a similar, but less conserved, repeated region was found in the middle of the first ORF.

Copper compounds have been widely used in agricultural systems to control undesirable microorganisms, and in recent years, copper-tolerant microbes have been isolated from such environments (19). Plasmid-encoded resistance to copper has been demonstrated for several bacterial species, including *Escherichia coli* (18), *Mycobacterium scrofulaceum* (8), and the plant pathogens *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* (1, 17). Previous work on *P. syringae* pv. *tomato* demonstrated the involvement of a 35-kilobase (kb) plasmid (pPT23D) in copper resistance (1, 2). This plasmid was highly conserved among copper-resistant strains of *P. syringae* pv. *tomato* isolated in southern California (5). Copper resistance determinants were isolated on a 4.5-kb *Pst*I-*Pst*I fragment cloned from pPT23D. Tn*5* insertions indicated that at least 3.9 kb of this fragment was involved in copper resistance (2). To further understand the basis of copper resistance in the *P. syringae* pv. *tomato* system, the DNA sequence of the 4.5-kb fragment was determined. This paper presents the structure, organization, and relative contribution to copper resistance of four genes contained within this fragment.

DNA sequence analysis. The 4.5-kb *Pst*I-*Pst*I fragment was subcloned from pCOP2 (2) into pUC119 (20) in both orientations, and overlapping deletions differing by approximately 100 base pairs (bp) were made by the method of Henikoff (11) using exonuclease III and S1 nuclease. Single-stranded template DNA was prepared by coinfecting with the helper bacteriophage M13K07 (20). Both strands were sequenced by the Sanger dideoxy method (14) using ^{35}S-labeled dATP (3). Sequence data were analyzed by the computer programs of BIONET (Intelligenetics, Palo Alto, Calif.).

Four open reading frames (ORFs) of 1,827, 984, 378, and 930 bp occurred in the same orientation and were preceded by sequences resembling those of *E. coli* consensus ribosome-binding sites (15). These ORFs were designated A, B, C, and D, respectively (Fig. 1). Several additional, widely dispersed ORFs with possible ribosome-binding sites occurred in the opposite orientation with sizes of 234, 243, 291, 510, 225, 342, and 417 bp. However, Tn*5* insertions affecting copper resistance (2) and the deletions and frameshift mutations described later in this paper were not consistent with the involvement of these ORFs in copper resistance.

The sequence had an overall G+C content of 60%, similar to that of genomic DNA of *P. syringae* (13). Codon usage in all the ORFs showed that there was a strong preference (74.5%) for codons containing G or C at position 3. All four of the predicted protein products contained unusually high levels of methionine (7.1, 4.9, 5.6, and 4.9% in ORFs A, B, C, and D, respectively). The hydropathic nature of the four predicted proteins, determined by the algorithm of Kyte and Doolittle (12), indicated that ORFs A, B, and C lacked any significant hydrophobic regions with the exception of fairly large hydrophobic stretches at the beginning of each ORF (data not shown). The nature of these hydrophobic stretches is similar to that expected for signal peptides (16, 21, 22), each possessing a charged amino acid at the N terminus, a hydrophobic core region, and a polar C-terminal region. The predicted product from ORF D had several potential membrane-spanning hydrophobic regions and also possessed a leader region resembling that of a signal peptide.

Five direct repeats of 24 bases each were present near the beginning of ORF B (Fig. 1). This region would encode a highly conserved polypeptide of eight amino acids (Asp-His-Ser-Gln/Lys-Met-Gln-Gly-Met) directly repeated five times (Fig. 2). A similar methionine-rich repetitive region was found in ORF A (Fig. 1); it was not as highly conserved as the region in ORF B, nor were the repeats tandemly linked throughout the entire region. However, the general structure Asp-His-X-X-Met-X-X-Met was conserved between several of the ORF A repeats and those in ORF B (Fig. 2). The function of the repeated sequences is not yet known, but a computer search of protein data bases with the ORF A protein indicated similarities between a region of about 50 amino acids just preceding the repeated sequences and a region of the azurin proteins from *Pseudomonas aeruginosa*, *Pseudomonas dentrificans*, *Bordetella bronchiseptica*, and *Alcaligenes denitrificans*. In addition, a region of 18 amino acids with similarity to plastocyanin from *Chlorella fusca* was detected 10 amino acids upstream from the region with similarity to azurin. The plastocyanin and azurin blue copper proteins bind copper between the sulfur atoms of a cysteine and a methionine and between nitrogen atoms in the imidazole groups of two histidine residues (4). Although there are no cysteines near the repeated region of ORF A and only one in the entire protein, the region is rich in methionine and

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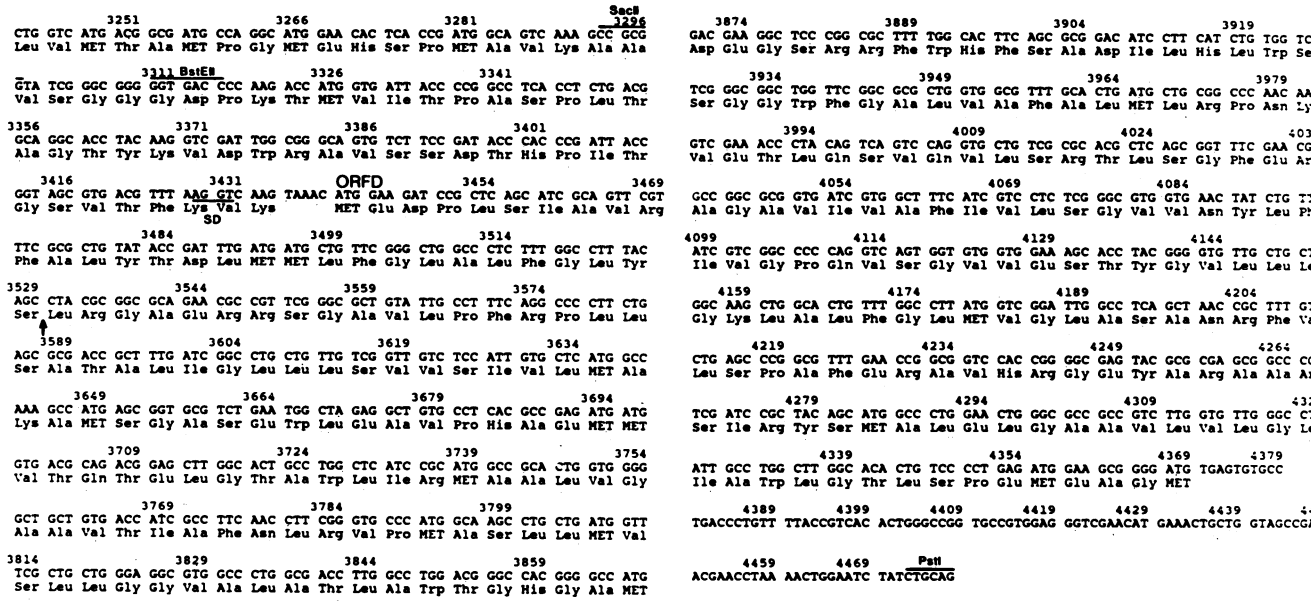
FIG. 1. Nucleotide sequence and predicted translation products of the copper resistance gene cluster from *P. syringae* pv. *tomato* PT23. Nucleotides are numbered 1 to 4478. Pertinent restriction sites are indicated as overstrikes. Potential Shine-Dalgarno (SD) ribosome-binding sites (15) are underlined. Repeating segments are underlined with arrows. Potential signal sequence cleavage sites are designated by vertical arrows.



histidine and might contain repeated copper-binding domains that could play a role in copper resistance.

Deletion analysis and site-specific mutations. Various exonuclease III deletion derivatives generated for sequencing and specific restriction fragments from the copper resistance gene cluster were subcloned from pUC119 into the broad-host-range vector pRK415 (7; N. T. Keen, personal communication) or pDSK519 (N. T. Keen, personal communication). Most of the deletion derivatives were cloned in both orientations with respect to the *lac* promoter in these vectors. These subclones were mobilized into *P. syringae* pv. *syringae* PS61 by using the helper plasmid pRK2013 (9) as described previously (2), and they were screened for their level of resistance to copper sulfate. Removal of a 60-bp *Pst*I-*Hin*dIII fragment from the 5′ end of the 4.5-kb fragment up to the *Hin*dIII site (pCOP6A and pCOP6B) had no significant effect on the level of copper resistance as compared with that of the intact fragment (Fig. 3). Deletion of 86 bp (pCOP7B) or 124 bp (pCOP8B) from the 5′ end reduced the level of copper resistance, but resistance was elevated when these derivatives were cloned with the ORFs oriented behind the *lac* promoter (pCOP7A and pCOP8A). This result suggested that sequences essential for expression of copper resistance were present well upstream from the ORF A start codon, although *E. coli* or *Pseudomonas* spp. consensus promoter regions (6, 10) were not observed in this region. Deletions from the 5′ end into ORFs A, B, C, and D generally inactivated resistance to copper, regardless of orientation to the *lac* promoter. Deletions from the 3′ end into ORFs D and C generally reduced resistance to intermediate levels. Further deletions from the 3′ end into ORFs B and A inactivated resistance.

A	1295	Asp	His	Gly	Ser	Met	Asp	Gly	Met
	1395	Asp	His	Ser	Lys	Met	Ser	Thr	Met
	1427	Asp	His	Gly	Ala	Met	Ser	Gly	Met
	1451	Asp	His	Gly	Ala	Met	Gly	Gly	Met
B	2113	Asp	His	Ser	Gln	Met	Gln	Gly	Met
	2137	Asp	His	Ser	Lys	Met	Gln	Gly	Met
	2161	Asp	His	Ser	Gln	Met	Gln	Gly	Met
	2185	Asp	His	Ser	Lys	Met	Gln	Gly	Met
	2209	Asp	His	Ser	Gln	Met	Gln	Gly	Met

FIG. 2. Predicted amino acid sequences of homologous repeating units of ORFs A and B. The numbers at the left indicate the positions (in base pairs) of the first codon of each repeating unit. Conserved amino acids are boxed.

Frameshift mutations were generated in ORFs A and B by digesting the full-length clone with either *Kpn*I (pCOP27), *Apa*I (pCOP31), or *Eag*I (pCOP28), followed by deletion of the 3′ overhangs or filling in of the 5′ overhangs by using DNA polymerase I (Klenow fragment) and subsequent ligation. A second frameshift and deletion in ORF B (pCOP29) was generated by removal of the *Bsm*I-*Hpa*I fragment (pCOP29). A deletion and frameshift in ORF C (pCOP30) was generated by removal of the 94-bp *Bst*EII-*Bst*EII fragment. Each frameshift construct was subcloned into pRK415 or pDSK519 with the ORFs either behind the *lac* promoter (A) or in the opposite orientation (B) and conjugated into *P. syringae* pv. *syringae* PS61 to screen for expression of copper resistance. Frameshift mutations in ORF A or ORF B completely inactivated resistance regardless of orientation (Fig. 3). Resistance was also inactivated in the ORF C deletion derivative when cloned with the ORFs in the opposite orientation to that of the *lac* promoter (pCOP30B), but an intermediate resistance was observed when this derivative was cloned with the ORFs oriented behind the *lac* promoter (pCOP30A). Together with earlier observations of the inducibility of copper resistance in this system (D. A. Cooksey, Phytopathology **76**:1076, 1986), these data suggest a possible role for ORF C in the positive regulation of copper resistance, especially since similarities were detected in computer searches of protein data bases between a region near the beginning of the ORF C protein and several DNA-binding proteins.

These data support the involvement of ORFs A, B, C, and D in copper resistance. Deletions and site-specific frameshift mutations in ORFs A and B suggest that these two ORFs are

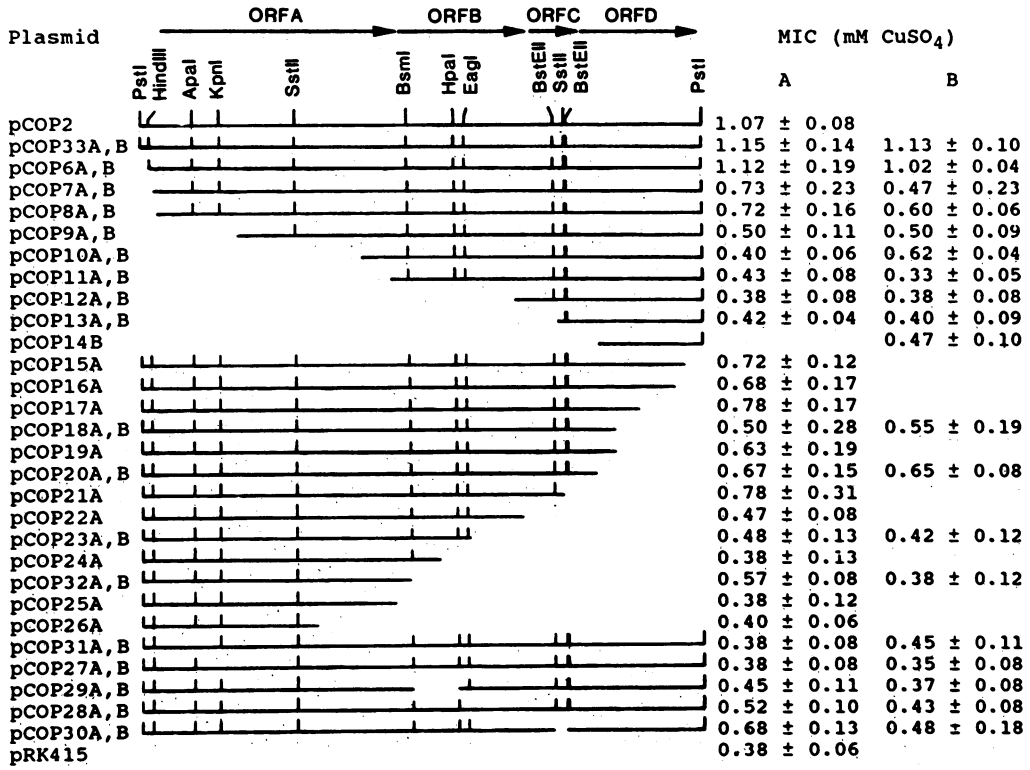


FIG. 3. MIC analysis of 5′ deletions, 3′ deletions, and frameshift mutations of a copper resistance gene cluster from *P. syringae* pv. *tomato* PT23. Derivatives were cloned in the broad-host-range vector pRK415 or pDSK519, with the ORFs oriented either behind the *lac* promoter (A) or in the opposite orientation (B). Horizontal lines to the right of each plasmid designation represent subcloned derivatives of the 4.5-kb *PstI-PstI* fragment. Vertical lines indicate important restriction sites. Missing restriction sites and gaps indicate positions of site-specific mutations and deleted DNA. Each MIC (tested at 0.1 mM $CuSO_4$ increments) was the mean ± the standard deviation of two transconjugants replicated three times each. pRK415 was the negative control and represents the level of copper tolerance of the recipient *P. syringae* pv. *syringae* PS61 used to monitor expression of all mutant derivatives.

essential for resistance. Deletions and site-specific mutations in ORFs C and D suggest that they are required for full resistance, but low-level resistance can be conferred in their absence.

We thank J. Cha and H. E. Stone for technical assistance and N. T. Keen, B. C. Hyman, S. J. Tamaki, and D. Y. Kobayashi for helpful discussions and review of the manuscript.

This work was supported in part by a grant from the University of California Toxic Substances Research and Teaching Program and from University of California Statewide Critical Applied Research funds.

LITERATURE CITED

1. **Bender, C. L., and D. A. Cooksey.** 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. J. Bacteriol. **165**:534–541.
2. **Bender, C. L., and D. A. Cooksey.** 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. **169**:470–474.
3. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
4. **Coleman, P. M., H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw, and M. P. Venkatappa.** 1978. X-ray crystal structure analysis of plastocyanin at 2.7Å resolution. Nature (London) **272**:319–324.
5. **Cooksey, D. A.** 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. *tomato*. Appl. Environ. Microbiol. **53**:454–456.
6. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Alginate biosynthesis: a model system for gene regulation and function in *Pseudomonas*. Bio/Technology **5**:469–477.
7. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X. Liang, D. Guiney, and D. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and monitoring gene expression. Plasmid **13**:149–153.
8. **Erardi, F. X., M. L. Failla, and J. O. Falkinham III.** 1987. Plasmid-encoded copper resistance and precipitation by *Mycobacterium scrofulaceum*. Appl. Environ. Microbiol. **53**:1951–1954.
9. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **79**:1648–1652.
10. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *E. coli* promoter sequences. Nucleic Acids Res. **15**:2343–2361.
11. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**: 351–359.
12. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
13. **Palleroni, N. J.** 1984. Genus I. *Pseudomonas* Migula 1894, 237^{AL}, p. 141–199. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
14. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci.

USA **74**:5463–5467.
15. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.
16. **Sjöström, M., S. Wold, A. Wieslander, and L. Rilfors.** 1987. Signal peptide amino acid sequences in *Escherichia coli* contain information related to final protein localization. A multivariate data analysis. EMBO J. **3**:823–831.
17. **Stall, R. E., D. C. Loschke, and J. B. Jones.** 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **76**:240–243.
18. **Tetaz, T. J., and R. K. J. Luke.** 1983. Plasmid-controlled resistance to copper in *Escherichia coli*. J. Bacteriol. **154**:1263–1268.
19. **Trevors, J. T.** 1987. Copper resistance in bacteria. Microbiol. Sci. **4**:29–31.
20. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
21. **von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133**:17–21.
22. **von Heijne, G.** 1985. Signal sequences: the limits of variation. J. Mol. Biol. **184**:99–105.