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Short communication

Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. *actinidiae*

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Abstract

Copper-containing bactericides have been used to control bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae*. However, the efficacy of copper has been reduced by the occurrence of copper-resistant strains. Analysis of the DNA sequence of a cluster region containing the copper-resistance genes from *P. syringae* pv. *actinidiae* suggested the presence of three possible different systems for copper resistance: copper-trapping, copper-efflux and copper-transport systems. Transposon insertional inactivation analysis indicated that the copper-trapping system was essential for copper resistance.

Bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* is an important disease of kiwifruit in Japan (Serizawa et al., 1989; Takikawa et al., 1989). Growers have successfully controlled the disease with copper and streptomycin. However, the efficacies of copper and streptomycin have been reduced by the occurrence of copper- and streptomycin-resistant bacterial strains. Resistance to copper has been demonstrated in the case of several phytopathogenic bacteria and some other bacteria, including *P. syringae* pv. *tomato* (Bender and Cooksey, 1986), *P. syringae* pv. *syringae* (Sundin and Bender, 1993), *P. syringae* pv. *actinidiae* (Nakajima et al., 2002), *P. syringae* (Andersen et al., 1991; Cooksey, 1990; Rogers et al., 1994), *Xanthomonas campestris* pv. *vesicatoria* (Adaskaveg and Hine, 1985; Bender et al., 1990; Marco and Stall, 1983), *X. campestris* pv. *juglandis* (Lee et al., 1994), *Escherichia coli* (Rouch et al., 1985; Tetaz and Luke, 1983), *Mycobacterium scrofulaceum* (Erardi et al., 1987), *Alcaligenes eutrophus* (Collard et al., 1994), *Enterococcus hirae* (Odermatt et al., 1993), *Helicobacter pylori* (Ge et al., 1995; Melchers et al., 1996) and *Helicobacter felis* (Bayle et al., 1998). Previous work on *P. syringae* pv. *actinidiae* demonstrated the involvement of a 70.5 kb plasmid (pPaCu1) in copper resistance (Nakajima et al., 2002). A cosmid library of pPaCu1 was constructed and one cosmid clone (pPaCuC1) that conferred near wild-type levels of copper resistance upon transformation of a copper-sensitive strain was selected for further analysis. Copper resistance determinants isolated from pPaCuC1 had a cluster region homologous to *copA*, *copB*, *copR* and *copS* genes of *P. syringae* pv. *tomato*. These open reading frames (ORFs) were designated ORF A, B, R and S, respectively. The region between *copB* and *copR* in *P. syringae* pv. *tomato* is reported to include *copC* and *copD* and is 1.4 kb in size (Mellano and Cooksey, 1988), whereas the corresponding region in the case of *P. syringae* pv. *actinidiae* was found to be about 11 kb in size (Nakajima et al., 2002). To further understand the basis of copper-resistance mechanisms in *P. syringae* pv. *actinidiae*, complete DNA sequence of the 11 kb region was determined. In the present

study, we suggest the possible copper-resistance mechanism of *P. syringae* pv. *actinidiae* consists of three different systems on the basis of the sequence data and the transposon insertional inactivation analysis.

Subclones of pPaCuC1, a plasmid encoding the copper-resistance genes of *P. syringae* pv. *actinidiae*, were produced by digestion of pPaCuC1 with an appropriate restriction enzyme and ligation of the resulting fragments with pBluescript II KS+ (Stratagene, La Jolla, CA, USA) or pGEM-5Zf(+) (Promega Corp., Madison, WI, USA). Deletion derivatives of the plasmids were made using Exonuclease III. Sequences were determined by the cycle sequencing method (Thermosequenase Fluorescent-Labeled Primer Cycle Sequencing Kit; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and analyzed using GENETYX software (Software Development, Tokyo, Japan). The nucleotide sequence data have been deposited in the DDBJ database under the accession numbers AB044351 and AB044355.

From this sequence data, six ORFs, 384, 936, 1320, 3147, 2385 and 198 bp in size were detected, preceded by the sequences identical to those of *E. coli* consensus ribosome-binding sites. These ORFs were designated ORF C, D, E, F, G and H, respectively (Figure 1). No consensus *E. coli* promoter sequences (Hawley and McClure, 1983) were detected in the region upstream of these ORFs. Comparison of the deduced amino acid sequences of the ORF C product and CopC from *P. syringae* pv. *tomato* revealed 69% identity and 78% similarity. The ORF D product and *P. syringae* pv. *tomato* CopD showed 58% identity and 71% similarity. Therefore, ORF C and ORF D are assumed to be genes functionally similar to *copC* and *copD*, respectively. A fifth putative ORF, ORF E, is located 2.4 kb downstream of the ORF D termination codon. The deduced amino acid sequences of the ORF E product and CnrB, a protein responsible for nickel and cobalt resistances in *A. eutrophus* (Liesegang et al., 1993), shared 27% identity and 39% similarity. However, no highly conserved regions were found in these two proteins. The ORF F product and CzcA, a cation efflux system protein from *A. eutrophus* (Nies et al., 1989), showed 34% identity and 55% similarity in terms of amino acid sequence. *Alcaligenes eutrophus* strain CH34 has two different plasmid-encoded cation efflux systems. In this bacterium, resistance to cobalt, zinc and cadmium are determined by the *czc* operon and resistance to cobalt and nickel are determined by the *cnr* operon (Liesegang et al., 1993; Nies et al., 1989). It has been suggested that these two operons have evolved from a common ancestor operon because of the extensive homology observed when comparing the *czc* and *cnr* structural genes and similar types of exported cations (Liesegang et al., 1993). There are three structural genes (*czcA*, *czcB* and *czcC*) in the *czc* system. CzcA, CzcB and CzcC are considered to form a membrane-bound cation efflux protein complex CzcABC (Nies, 1992). The CzcABC complex is a cation–proton antiporter (Nies, 1995). Therefore, it is possible that the ORF E and F products might act together in cupric ion efflux. The deduced amino acid sequences of the ORF G product and a potential copper-transporting ATPase from

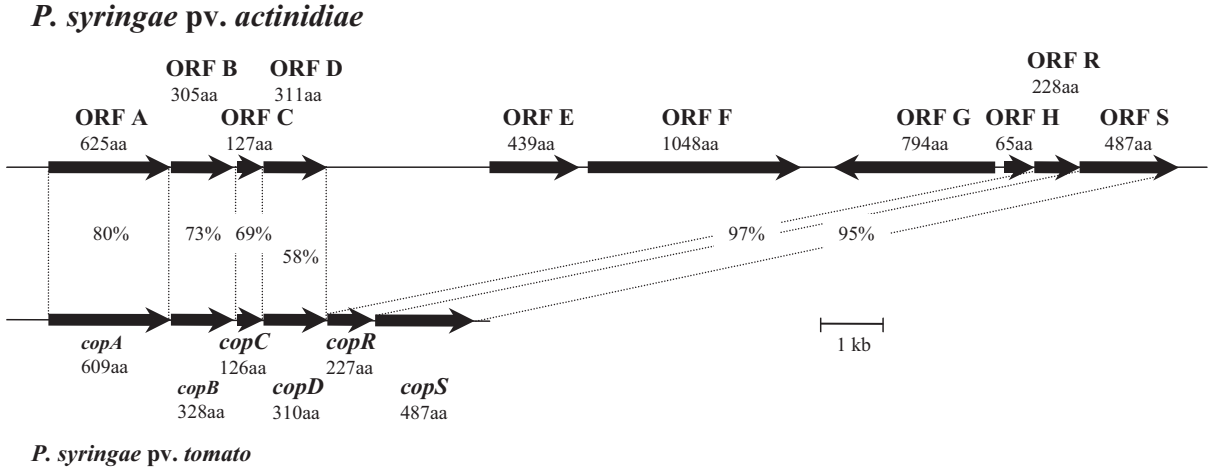

Figure 1. Schematic structure of copper-resistance genes of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *tomato*. Amino acid identities between the corresponding genes are indicated in percentages.

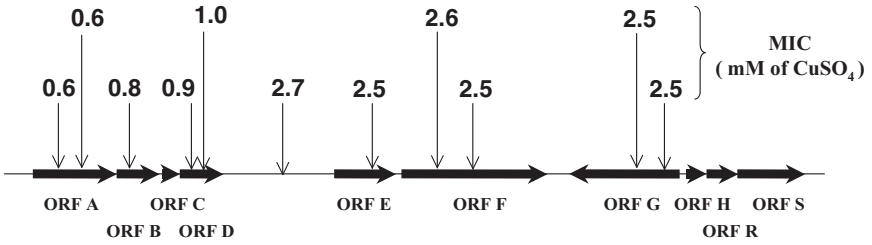

Figure 2. Effect of transposon insertions on the level of copper resistance encoded by plasmid pPaCuC1 within *P. syringae* pv. *actinidiae* Pa21. Transposon insertion locations are indicated by arrows. The numbers above arrows represent the minimal inhibitory concentrations (MICs) of copper sulfate. Pa21 is a copper-sensitive strain (MIC of copper sulfate; 0.7 mM). Pa21 containing pPaCuC1 is copper resistant (MIC of 2.7 mM).

Bacillus subtilis (EMBL accession number O32220) shared 38% identity and 53% similarity. The ORF G product contained one copy of the phosphorylation site characteristic of E1-E2 ATPases (D-K-T-G-T-[LIVM]-[TI]) (Prosite ID: ATPASE_E1_E2 (PS00154)) and two copies of a heavy-metal-associated domain ([LIVN]-X{2}-[LIVMFA]-X-C-X-[STAGCDNH]-C- X{3}-[LIVFG]-X{3}-[LIV]-X{9, 11}-[IVA]-X-[LVFYS]) (Prosite ID: HMA (PS01047)). The deduced amino acid sequence of the ORF H product and CopZ, a regulatory protein controlling expression of copper-ATPases of *E. hirae* (Odermatt et al., 1995), shared 35% identity and 51% similarity. The ORF H product also contains the heavy-metal-associated domain described above. Consequently, the putative ORF G product might be a P-type ATPase which is regulated by the product of ORF H and associated with copper-efflux.

In order to investigate the roles of these genes in copper resistance, each gene was disrupted using GPS-Mutagenesis System (New England Biolabs, Inc. Beverly, MA, USA) and the resulting disruptants were analyzed for their copper resistances. The position of insertion of the transposon was confirmed by sequencing. Transposon insertion derivatives of cosmid pPaCuC1, which carries the copper-resistance genes of *P. syringae* pv. *actinidiae*, were subsequently introduced into the copper-sensitive strain Pa21 by electroporation. Sensitivity of the transconjugants to copper sulfate was assayed on PDA plates supplemented with copper sulfate at concentrations of 0–3.0 mM at 0.1 mM intervals. This experiment was repeated three times with same results. All derivatives with disrupted ORFs reduced the level of copper resistance to a greater or lesser extent as compared with that of the intact cosmid (MIC; 2.7 mM $CuSO_4$) (Figure 2). However, the disruptants of ORF A, B and D showed remarkable decreases in copper resistance among them, which indicated that the copper-trapping system was essential for the resistance. The other disruptants exhibited only slight decreases. All of the disruptants did not express any increased sensitivities to cobalt, cadmium, nickel and zinc (data not shown).

These results suggest that the mechanism of copper resistance in *P. syringae* pv. *actinidiae* consists of three different systems, that is, copper-trapping by copper-binding proteins, copper-efflux mediated by a cation efflux protein, and copper-transport mediated by a copper-transporting ATPase. The interactions among these three systems remain to be further investigated.

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