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Similarity between Copper Resistance Genes from *Pseudomonas syringae* pv. *actinidiae* and *P. syringae* pv. *tomato*

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ABSTRACT

Twenty-eight strains of *Pseudomonas syringae* pv. *actinidiae* isolated in 1984, 1987 and 1988 from kiwifruit orchards in Japan were tested for their resistance to copper sulfate. All strains isolated in 1984 were copper sensitive with a minimum inhibitory concentration (MIC) of cupric sulfate of 0.75 mM. However, some strains isolated in 1987 and 1988 were resistant, with the MIC ranging from 2.25 to 3.0 mM. All copper-resistant strains contained at least one of two plasmids, pPaCu1 (about 70.5 kb) or pPaCu2 (about 280 kb), or both. In a copper-resistant strain Pa429, the location of the copper-resistance gene(s) was examined by insertional inactivation with Tn*5*. The MIC of copper sulfate in the copper-sensitive mutant obtained by Tn*5* tagging decreased from 2.75 to 0.75 mM. The 14.5 kb *Bam*HI fragment, designated pPaCuB14, containing the same locus mutagenized with Tn*5* was cloned from pPaCu1. However, pPaCuB14 did not confer copper resistance in the transformant of copper-sensitive strain Pa21R, suggesting that this clone did not contain a full set of copper-resistance gene(s). Then a cosmid library of pPaCu1 was constructed and six cosmid clones hybridized with pPaCuB14 were selected. One of the six cosmids, designated pPaCuC1, conferred a near wild-type level of copper resistance in the transformant of the copper-sensitive strain. pPaCuC1 had a homologous region that hybridized with all of the PCR-amplified fragments of *copA*, *copB*, *copR*, and *copS* genes of *P. syringae* pv. *tomato*. DNA sequence analysis of the homologous region revealed the existence of four open reading frames (ORF A, B, R and S) oriented in the same direction. The predicted amino acid sequences of ORF A, B, R and S had 80, 70, 97 and 95% identity with CopA, B, R and S of *P. syringae* pv. *tomato*, respectively.

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Key words : copper resistance, *Pseudomonas syringae* pv. *actinidiae*, *P. syringae* pv. *tomato*.

INTRODUCTION

Bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* is an important disease of kiwifruit in Japan[29,32]. Previously, growers successfully controlled the disease with copper and streptomycin sprays. However, the efficacy of copper and of streptomycin has been reduced by the development of copper- and streptomycin-resistant bacterial strains, respectively. Resistance to copper has been demonstrated in the case of several phytopathogenic bacteria and some other bacteria, including *P. syringae* pv. *tomato*[4], *P. syringae* pv. *syringae*[31], *P. syringae*[2,9,26], *Xanthomonas campestris* pv. *vesicatoria*[1,6,20], *X. campestris* pv. *juglandis*[18], *Escherichia coli*[27,33], *Mycobacterium scrofulaceum*[12].

The genetic and molecular basis of the copper resistance of *P. syringae* pv. *tomato* has been well studied. The copper-resistance genes of *P. syringae* pv. *tomato* have been found to be located on a 35-kb plasmid (pPT23D), and a 4.5-kb *Pst*I fragment containing these genes has been cloned[5]. The *cop* operon contains four open reading frames designated *copA*, *copB*, *copC*, and *copD*[21]. Results of analyses of deletion mutants and mutants produced by site-specific mutagenesis suggest that *copA* and *copB* are essential for resistance. *copC* and *copD* are required for full resistance, but low-level resistance can be conferred in their absence. CopA and CopC are located in the periplasmic space, whereas CopB is on the outer membrane and CopD is on the inner membrane[8]. As the

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mechanism of copper resistance, it has been proposed that copper is excluded from the cytoplasm by three proteins (CopA, CopC, CopB) that trap copper in the periplasm and on the outer membrane[11,30]. Immediately downstream from *copD* are two genes, *copR* and *copS*[23]. These genes encode trans-acting factors that regulate the expression of the *cop* operon[23,24].

Homology with these *cop* genes was detected in an analysis of chromosomal DNA from copper-resistant strains of *P. cichorii* and *P. fluorescens*. Homology was also detected with chromosomal DNA from copper-sensitive strains of *P. cichorii*, *P. fluorescens* and *P. syringae* pv. *tomato*, indicating that *cop* homologs may be indigenous to certain *Pseudomonas* species and have functions other than copper resistance[10,19,36].

In the present study, we describe the cloning, DNA sequences of copper-resistance genes from *P. syringae* pv. *actinidiae*, and their relation to copper-resistance genes of *P. syringae* pv. *tomato*.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions Bacterial strains and plasmids used in this study and their relevant phenotypes and sources are listed in Table 1. *P. syringae* pv. *actinidiae* strains were isolated from leaf spots, blighted blossoms and canker lesions on leaders and shoots of infected kiwifruit. They were identified on the basis of laboratory and pathogenicity tests[32]. *P. syringae* pv. *actinidiae* strains were grown at 25°C on potato dextrose agar (PDA; Difco, pH 6.8) or in LB broth. *E. coli* was cultured at 37°C on LB agar or in LB broth. Appropriate antibiotics were added if necessary for maintaining the selection markers. Concentrations of antibiotics were as follows: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 12.5 μg/ml; and rifampicin, 50 μg/ml.

MIC determination All strains of *P. syringae* pv. *actinidiae* were tested for their sensitivity or resistance to copper sulfate. The strains to be screened were grown for 48 hr on PDA medium at 25°C. The bacteria were then suspended in sterile water at about 1×10^8 cells per ml and spotted onto PDA (pH 6.8) plates containing copper sulfate at concentrations ranging from 0 to 3.0 mM. The MIC (minimum inhibitory concentration) was expressed as the concentration of $CuSO_4$ at which inhibition of growth was visibly evident after 48 hr of incubation at 25°C[13].

Transposon mutagenesis Bacterial conjugation between *E. coli* 2492 (pJB4JI) (donor strain) and *P. syringae* pv. *actinidiae* Pa429R (recipient strain) was performed on an LB plate by mixing a loopful of cells from 2-day-old plates, approximately 10^{10} cells, in a 1 : 1, donor : recipient ratio. After incubation at 25°C for 24 hr, cells were suspended in sterile distilled water, serially diluted, and plated onto LB plates containing appropriate antibiotics to select for Tn*5*-containing transconjugants[7]. After incubation at 25°C for 2 days, kanamycin-resistant derivatives of *P. syringae* pv. *actinidiae* were transferred to kanamycin-containing PDA plates. Copper-sensitive mutants were selected on PDA plates containing 1.2 mM cupric sulfate. The position of the transposon insertion was mapped by single and double digestions with various restriction endonucleases[15].

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
Pseudomonas syringae		
pv. *actinidiae* Pa429R	Rif^r mutant of Pa429, Cu^r, Sm^r, containing pPaCu1	This study
Pa21R	Rif^r	This study
Escherichia coli		
DH5α		
2492	Km^r, Gm^r, containing pJB4JI	
Plasmids		
pPaCu1	Cu^r, Sm^r, about 70.5 kb	This study
pPaCuB14	14.5 kb *Bam*HI fragment of pPaCu1, cloned in pLAFR5, Tc^r	This study
pJB4JI	Km^r, Gm^r, containing Tn*5*	
pPaCuC1	Copper-resistance gene(s) of pPaCu1, cloned in pLAFR5, Tc^r	This study
pPT23D	Copper-resistance plasmid from *P. syringae* pv. tomato PT23	Cooksey, D.A.[9]
pLAFR5	Tc^r	Keen, N.T. *et al.*[17]
pGEM-5Zf(+)	Ap^r	Promega Corp.
pBluescript KS(+)	Ap^r	Stratagene Cloning Systems

a) Ap^r, Cu^r, Gm^r, Km^r, Rif^r, Sm^r, Tc^r indicate resistance to ampicillin, copper sulfate, gentamycin, kanamycin, rifampicin, streptomycin and tetracycline, respectively.

General DNA manipulations In the case of mini-scale preparations of *E. coli* plasmid DNA, the plasmids were isolated by the boiling method, and in the case of large-scale preparations, cleared lysates were prepared and the plasmids were isolated by density gradient centrifugation in cesium chloride-ethidium bromide solution[3,28]. Plasmid DNA was isolated from *P. syringae* pv. *actinidiae* by the method of Kado and Liu[16] and further purified on cesium chloride-ethidium bromide gradients when necessary. Digestion of DNA with restriction enzymes and subsequent agarose gel electrophoresis were performed by standard procedures[28]. Fragments for subcloning were isolated using a Geneclean kit (BIO 101 Inc., La Jolla, CA, USA) after separation in agarose. Southern blotting was performed as described by Sambrook *et al.*[28]. Fragments used as probes in Southern hybridization were separated by agarose gel electrophoresis, the appropriate pieces of the gel were excised, and the DNA was extracted with a Geneclean kit. The fragments were then labeled with digoxigenin-11-dUTP using a nonradioactive DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). The sequences of the primers used for amplification of copper-resistance genes of *P. syringae* pv. *tomato* are listed in Table 2.

Construction of the pLAFR5 cosmid library Total plasmid DNA was isolated from Pa429 by the method of Kado and Liu[16] with slight modifications, purified on a cesium chloride gradient, and partially digested with *Sau*3AI to produce various fragments, the majority of which were in the 20-30 kb range. These fragments were dephosphorylated by treatment with calf intestinal alkaline phosphatase and then ligated with pLAFR5[17]. Recombinant cosmids were packaged *in vitro* and introduced into DH5α cells by transduction.

Electroporation and isolation of copper-resistant clones *P. syringae* pv. *actinidiae* competent cells were prepared and transformed with cosmids by electroporation[35]. Transformants were purified and then plated onto PDA plates containing 1.2 mM cupric sulfate to test for copper resistance.

DNA sequencing 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-Labelled Primer Cycle Sequencing Kit; Amersham Pharmacia Biotech), and analyzed using GENETYX software (Software Development, Tokyo , Japan).

Table 2. Primer sequences used for amplification of copper-resistance genes of *P. syringae* pv. *tomato*

Genes	Primer sequences[a]
copA	5′-ATGGAATCAAGAACTTCTCGACGT
	5′-CTCCTCTACCCGAACTTCGCGGAAC
copB	5′-CCGCGGACTGTTTTGAATAGACTCCAC (CCGCGG: *Sac*II)
	5′-AACCACATGCGCACGCCCAGGACTAA
copC	5′-CGCATGTTGTTGAACCGCACAAGT
	5′-ACTAGTCTTGACCTTAAACGTCACGCT (ACTAGT: *Spe*I)
copD	5′-AACATGGAAGATCCGCTCAGCATC
	5′-ACTAGTTCCATCTCAGGGGACAGTGT (ACTAGT: *Spe*I)
copR	5′-AGGGTCGAACATGAAACTGCTG
	5′-ACTAGTAGCATGTAACCCATTCCCCGG (ACTAGT: *Spe*I)
copS	5′-ACTAGTGCGCTTGACCCTTCTGTTTGT (ACTAGT: *Spe*I)
	5′-GCAGTGACAACCGGTTTCATGT

a) Added cleavage sites for the restriction endonucleases are underlined.

RESULTS

Copper resistance and plasmids

Twenty-eight strains of *P. syringae* pv. *actinidiae* isolated in 1984, 1987, and 1988 in kiwifruit orchards in Japan were tested for resistance to copper sulfate. All strains isolated in 1984 had copper sensitivity with an MIC of 0.75 mM. However, some strains isolated in 1987 and 1988 from orchards in which copper bactericides had been routinely applied were copper resistant with an MIC in the range of 1.75-3.0 mM. All 28 strains were screened for plasmids and were found to have an indigenous, cryptic plasmid of approximately 40 kb. In addition to that, all copper-resistant strains contained one of two plasmids, pPaCu1 (about 70.5 kb) and pPaCu2 (about 280 kb), or both (Table 3), and also exhibited resistance to streptomycin. A restriction map of pPaCu1 is shown in Fig. 1.

Cloning the copper-resistance gene(s)

The region of pPaCu1 that encodes the copper-resistance gene(s) was located by Tn*5* mutagenesis. Only one copper-sensitive mutant was obtained. The location of the Tn*5* insertion was determined by single or double digestions with *Bam*HI, *Eco*RI and *Hin*dIII. Restriction enzyme mapping showed that the insertion site was within a 14.5-kb *Bam*HI fragment (Fig. 2).

The 14.5-kb *Bam*HI-*Bam*HI fragment (pPaCuB14) from pPaCu1 cloned in pLAFR5 was able to complement the Tn*5* mutation in a copper-sensitive mutant. However, pPaCuB14 did not confer copper resistance in a transformant of copper-sensitive strain Pa21R. Six overlapping clones that hybridized with pPaCuB14 were isolated from the cosmid library. Restriction maps of these clones, designated pPaCuC1 to pPaCuC6, are shown in Fig. 2. When these six cosmids were introduced subsequently into the copper-sensitive strain Pa21R, five of the cosmids, but not pPaCuC4, conferred copper resistance (Fig. 2). These cosmids allowed the transformed bacteria to grow on PDA containing more than 1.5 mM copper sulfate, whereas growth of the wild-type strain was inhibited in the presence of 0.75 mM copper sulfate.

Homology to copper-resistance genes of *P. syringae* pv. *tomato*

When PCR-amplified probes specific for the six genes of the *cop* operon from pPT23D of *P. syringae* pv. *tomato* were used to detect the homologous regions in six cosmid clones, the probes specific for *copA*, *B*, *R* and *S* were found to hybridize with these cosmid clones. The approximate regions that hybridized with these probes were mapped (Fig. 2). These regions were confirmed by sequence analysis. Regions homologous to *copC* and *D* were not detected. 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[21], whereas the corresponding region in the case of *P. syringae* pv. *actinidiae* was found to be about 11 kb in size.

The regions that hybridized with *copA*, *B*, *R* and *S* were cloned and sequenced. The nucleotide sequence data have been deposited in the DDBJ database under the accession numbers AB033419 and AB033420. Two successive ORFs with lengths of 1878 and 918 bp occur in the region hybridized with *copA* and *B*. These ORFs were designated ORF A and ORF B, respectively (Fig. 3). No consensus *E. coli* promoter sequences[14] were detected in the region upstream of these ORFs. The sequence of a *cop* box (A/C-A-G-C-T-T-A-C-A/G-G-A-A-A-T-G-T-A-A-T-C/T), previously identified as a CopR binding site in *P. syringae* pv. *tomato*[24], was found in the region upstream of ORF A. Comparison of the deduced amino acid sequence of the ORF A product and CopA from *P. syringae* pv. *tomato* revealed 80% identity and 84% similarity. CopA contains four tandem repeats of the octapeptide (D-H-X-X-M-X-X-M)[21], whereas the ORF A product has three tandem repeats of the same octapeptide. The ORF

Table 3. Minimum inhibitory concentration (MIC) of copper sulfate for strains of *P. syringae* pv. *actinidiae*

Strain	Cu^r plasmids	MIC (mM)	Year isolated
Pa1	not detected	0.75	1984
Pa11	not detected	0.75	1984
Pa21	not detected	0.75	1984
Pa31	not detected	0.75	1984
Pa52	not detected	0.75	1984
Pa423	pPaCu2	2.25	1987
Pa429	pPaCu1	2.75	1987
Pa430R	pPaCu1	3.0	1987
Pa430S	pPaCu1	3.0	1987
Pa431	pPaCu2	1.75	1987
Pa436R	not detected	0.75	1984
Pa436S	not detected	0.75	1984
Pa438	not detected	0.75	1984
Pa440R	not detected	0.75	1984
Pa440S	not detected	0.75	1984
Pa445	not detected	0.75	1984
Pa447R	not detected	0.75	1984
Pa447S	not detected	0.75	1984
Pa450	not detected	0.75	1984
Pa453	pPaCu1	3.0	1987
Pa454	pPaCu2	2.25	1987
Pa454W	pPaCu2	2.25	1987
Pa457	not detected	0.75	1987
Pa459	pPaCu1 and pPaCu2	3.0	1988
Pa460	not detected	0.75	1987
Pa462	not detected	0.75	1987
Pa712	pPaCu2	2.25	1987
Pa722a	not detected	0.75	1987

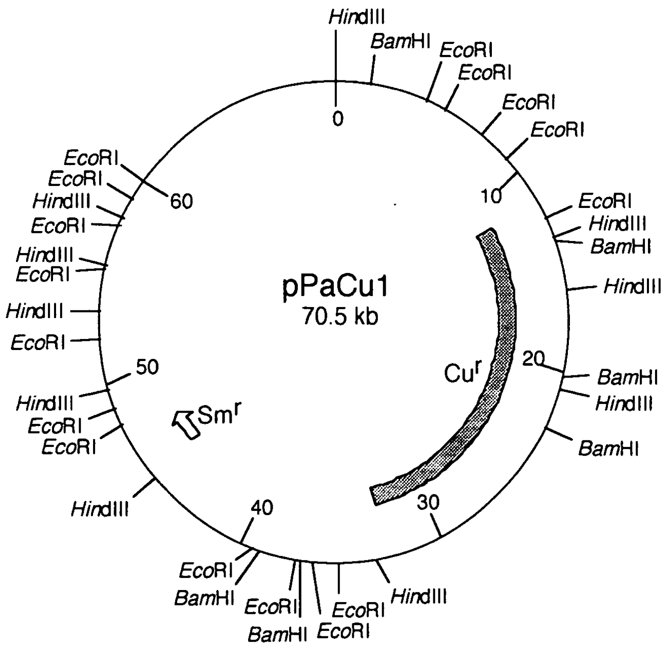

Fig. 1. Restriction enzyme map of plasmid pPaCu1 from *P. syringae* pv. *actinidiae* Pa429. The location of copper-resistance genes defined in this study is indicated with a thick line, streptomycin-resistance genes with an arrow.

A product also contains one copy of multicopper oxidase signature 1 (G-X-[FYW]-X-[LIVMFYW]-X-[CST]-X{8}-G-[LM]-X{3}-[LIVMFYW]) and one copy of multicopper oxidase signature 2 (H-C-H-X{3}-H-X{3}-[AG]-[LM]). Putative copper ligands for type 1, type 2 and type 3 identified in ascorbate oxidase[22] are conserved in the ORF A product. The amino terminus of the ORF A product has a putative secretory signal peptide sequence[34]. Comparison of the deduced amino acid sequence of the ORF B product and CopB from *P. syringae* pv. *tomato* revealed 73% identity and 77% similarity. The ORF B product contains two tandem repeats of the octapeptide (D-H-X-X-M-X-X-M), which is repeated tandemly five times in CopB[21].

Two successive ORFs with lengths of 687 and 1464 bp occur in the region that hybridized with *copR* and *S*. These ORFs were designated ORF R and ORF S, respectively. Comparison of the deduced amino acid sequence of the ORF R product and CopR and that of the ORF S product and CopS revealed 97% identity (98% similarity) and 95% identity (98% similarity), respectively.

DISCUSSION

All copper-resistant strains of *P. syringae* pv. *actinidiae* contained at least one of two plasmids, pPaCu1 and pPaCu2, or both. This result suggested that these plasmids contain the copper-resistance gene(s). The copper-

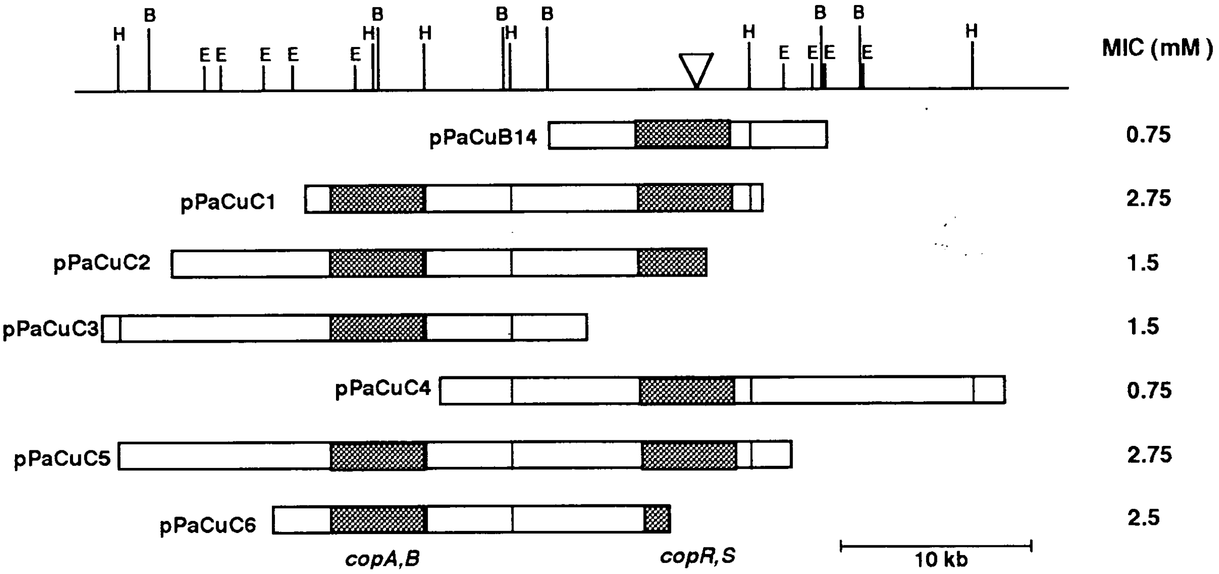

Fig. 2. Restriction endonuclease map of the region containing the copper-resistance genes of pPaCu1 and localization of overlapping cosmid clones. The site of Tn*5* insertion is represented by a triangle. pPaCuB14 contains a 14.5-kb *Bam*HI fragment complemented Tn*5* mutation. pPaCuC1 to pPaCuC6 hybridized with pPaCuB14 were isolated from the cosmid library of pPaCu1. The column on the right indicates MIC of $CuSO_4$ in the transformed Pa21R strains with these clones. The shaded areas are where *cop* probes hybridized. B, E and H depict the cleavage sites for the restriction endonucleases *Bam*HI, *Eco*RI and *Hin*dIII, respectively.

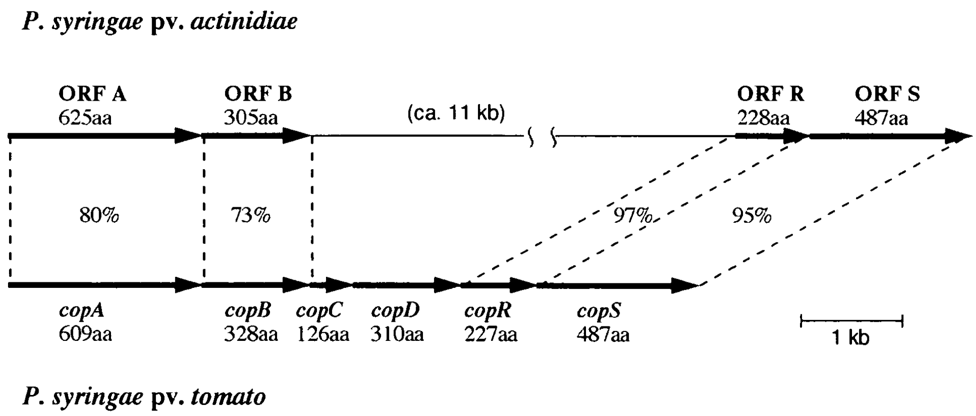

Fig. 3. Schematic structure of copper-resistance genes of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *tomato*. Amino acid identities between the corresponding genes are indicated as percentages.

resistance gene(s) of strain Pa429 were found to be located in the plasmid pPaCu1 by insertional inactivation with Tn*5*. We previously reported that the streptomycin-resistance genes in pPaCu1 are homologous to those in the non-conjugative IncQ plasmid RSF1010[25]. Therefore, it was revealed that pPaCu1 contains both streptomycin- and copper-resistance genes. Presumably, the continued practice of repeated applications of copper and streptomycin in kiwifruit orchards may result in a population that is uniformly resistant to both bactericides. Copper resistance and pPaCu1 were transferred in filter matings to copper-sensitive recipient strains of *P. syringae* pv. *actinidiae* (data not shown). Plasmid mobilization could have played an important role in the spread of copper-resistant strains of *P. syringae* pv. *actinidiae*.

A cosmid designated pPaCuC1 conferring near wild-type levels of copper resistance in the transformed copper-sensitive strain Pa21R was obtained. The size of the copper-resistance gene region (about 20 kb) in pPaCuC1 is much larger than that of *P. syringae* pv. *tomato*, *X. campestris* pv. *juglandis* or *E. coli*. Regions of pPaCuC1 hybridized with *copA* and *B* and the copper-responsive regulatory genes *copR* and *copS* of *P. syringae* pv. *tomato*. DNA sequence analysis of the region hybridized with *copA*, *B*, *R* and *S* revealed that the copper-resistance genes of *P. syringae* pv. *actinidiae* contain at least four ORFs (ORF A, B, R and S).

In *P. syringae* pv. *tomato*, tandem repeats of a highly conserved octapeptide have been proposed to comprise a copper-binding domain responsible for exclusion of copper from the cytoplasm[11]. These repeats were also found in ORF A and ORF B of *P. syringae* pv. *actinidiae*, indicating that they may function in the same manner to trap cupric ions. The N terminus of the ORF A product has characteristics similar to those of a signal peptide sequence, suggesting that the protein might be located in the cell membrane or the periplasm[34]. CopB also has a signal peptide, and this protein is tightly associated with the outer membrane[8]. ORF B has a cleavable N-terminal signal peptide sequence, consistent with the view that it is either a periplasmic protein or an outer membrane protein but not an inner membrane protein. CopC and CopD are reported not to be essential for copper resistance but are required for maximum resistance, and it has been proposed that they serve to maintain uptake of small amounts of copper essential for cell growth when expression of the copper-sequestering CopA and CopB proteins is fully induced[11]. In *P. syringae* pv. *actinidiae*, genes functionally similar to *copC* and *copD* may exist in the region downstream of ORF B, although the both homologous genes were not detected.

Expression of the copper-resistance genes in *P. syringae* pv. *actinidiae* appears to be also under the control of a two-component regulatory system[23]. Although pPaCuC2, pPaCuC3 and pPaCuC6 did not contain the complete ORF R and ORF S, these cosmids conferred copper resistance. It seems that functional chromosomal homologs to ORF R, S activated the promoter. Aside from the lack of homologous genes with *copC* and *copD*, the mechanism of the copper resistance in *P. syringae* pv. *actinidiae* may be similar to that in *P. syringae* pv. *tomato*, suggesting that the *cop* homologs may be indigenous to certain *Pseudomonas* species.

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