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Isolation of Plasmid from Korean Copper-Resistant *Xanthomonas campestris* pv. *vesicatoria*

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한국에서 분리한 고추 더뎅이병균의 구리저항성 Plasmid

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ABSTRACT : In other country, copper-resistant strains of *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye have been isolated from where copper compounds were frequently applied, and the level of resistance was sufficient to reduce the efficiency of disease control with copper sprays. The copper resistance determinants of these strains are usually on the large conjugative plasmids; e.g. pXvCu, pXV10A, etc. The copper-resistant *X. c.* pv. *vesicatoria* strains HN94-2, -3, -4, -5, and -6 were isolated from Cheonan county, Korea by Lee *et al.* in 1994. The minimum inhibitory concentrations (MICs) for $CuSO_4$ of *X. c.* pv. *vesicatoria* HN94-2 and HN94-6 on nutrient agar medium were 1.6 and 1.5 mM, respectively. These strains were not resistant to zinc sulfate. Copper resistance of HN94-2 and HN94-6 was transferred to copper-sensitive strains MZ94-1R and KJ93-1R at the frequency of 4.3×10^{-3}~1.0×10^{-5} (transconjugant/donor). Copper resistance of HN94-6 was also transferred to *X. c.* pv. *campestris* MIS1R. Copper resistance determinants of HN94-2 and HN94-6 were suggested to be on the large conjugative plasmids, and designated pXVK9402 and pXVK 9406, respectively.

***Key words* : *Xanthomonas campestris* pv. *vesicatoria*, bacterial spot, copper resistance plasmid, conjugative plasmid.**

Pepper is one of the most important vegetable crops in Korea, and is damaged considerably by bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye (11, 15). The control of this disease has been achieved through the use of resistant plant varieties, agricultural practices, and more effectively by the application of agrochemicals containing copper (14).

However, strains of this bacterium resistant to copper-containing compounds have been reported in Florida (14), and other locations (1, 3, 16). Also, copper resistance strains occur in several pathovars of *Pseudomonas syringae* van Hall (2) and some nonpathogenic bacteria (8).

Copper resistance in *X. c.* pv. *vesicatoria* is associated with a large (approximately 200 kbp) self-transmissible plasmid, pXvCu (18). Other large plasmids in *X. c.* pv. *vesicatoria*, including pXV10A, share homology with the pXvCu-type plasmids, but pXV 10A does not show homology to IS476, which is present on the pXvCu-type plasmids (3).

An association between copper resistance and race 2 of the pepper strain of *X. c.* pv. *vesicatoria* was noted (14). It was reported that the loci for copper resistance and avirulence to pepper plants that have the *Bs1* gene for resistance to bacterial spot were linked and both loci were transferred with the large self-transmissible plasmid (18).

The copper-resistant *X. c.* pv. *vesicatoria* strains HN 94-2, -3, -4, -5, and -6 were isolated from Cheonan county, Korea by Lee *et al.* in 1994 (12). These strains were grown on sucrose peptone agar (SPA) supplemented with 200 μg/ml cupric sulfate. HN94-2 was determined as race 3 and HN94-3, -4, -5, and -6 were determined as race 1.

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The purpose of this research was to determine the presence of the plasmid in copper-resistant *X. c.* pv. *vesicatoria* strains isolated from Korea and the transfer of copper resistance with that plasmid to copper-sensitive strains by conjugation.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The copper-resistant *X. c.* pv. *vesicatoria* HN94-2 and HN94-6 were grown on SPA supplemented with 200 μg/ml cupric sulfate ($CuSO_4$) (12). Copper-sensitive strains were made resistant to 100 μg/ml rifampicin by culturing them in a series of increasing concentration of $CuSO_4$ in SPA medium.

Minimum inhibitory concentrations (MICs) determination. Bacteria were grown on nutrient agar, suspended in sterile water to about 5×10^8 cells per ml, and spotted in duplicate (10 μl per spot) onto nutrient agar containing different levels of cupric sulfate from 0 to 2.4 mM. The MIC of cupric sulfate was determined as the concentration that inhibited confluent growth of the culture after 3 days at 28°C. Strains of *X. c.* pv. *vesicatoria* were tested to MIC of zinc sulfate on nutrient agar media containing different levels of zinc sulfate from 0 to 1.2 mM.

Conjugation experiments. Matings between copper-resistant *X. c.* pv. *vesicatoria* strains and copper-sensitive recipients were done as described by Bender *et al.* (3) with some modifications. Donor or recipient strains were grown in 5 ml of nutrient broth overnight at 28°C. One hundred microliters of each culture was inoculated in 5 ml of nutrient broth with appropriate chemicals or antibiotics. Donor and recipient strains were grown for 4 hours and 10 hours at 28°C, respectively. The culture was washed twice with 0.85% saline solution and centrifuged at 12,000 rpm for 30 sec. The pellets were resuspended with 50 μl of 0.85% saline solution. Mixed donor and recipient suspension was plated on membrane filter (25 mm in diameter, 0.45 μm in pore-size) on mating medium (nutrient agar with 1% water agar overlay) and incubated for 20 hours at 28°C. Bacterial cultures were then removed by vortexing the filters in 5 ml of nutrient broth and 0.1 ml of the culture was plated on selective media to enumerate donors and putative transconjugants. Colonies that grew on nutrient agar supplemented with 200 μg/ml of cupric sulfate and 100 μg/ml of rifampicin were considered as putative transconjugants. Controls consisting of donor and recipient cells alone were treated similarly to determine the frequency of spontaneous resistant mutants in the population. One to five transconjugant bacterial colonies

Table 1. Bacterial strains used in this study

Bacterial strains and plasmids	Relevant characteristics[a]	Source
Xanthomonas campestris pv. *vesicatoria*		
HN94-2	Cu^r, race 3	Lee, S.D.
HN94-6	Cu^r, race 1	Lee, S. D.
07882	Cu^r	Cooksey, D. A.
E3	Cu^r, race 2	Stall, R. E.
EJB1	Cu^s, race 1	Yoon, Y. C.
MZ94-1	Cu^s	This work
MZ94-1R	Rif^r derivative of MZ94-1	This work
KJ93-1	Cu^s, race 1	Lee, S. D.
KJ93-1R	Rif^r derivative of KJ93-1	This work
CE93-1	Cu^s, race 1	Lee, S. D.
CE93-1R	Rif^r derivative of CE93-1	This work
MZC2	Cu^r, transconjugant between HN94-2 and MZ94-1R	This work
KJC6	Cu^r, transconjugant between HN94-2 and MZ94-1R	This work
MIS1C6		This work
Xanthomonas campestris pv. *campestris*		
MIS1	Cu^s	Myung, I. S.
MIS1R	Rif^r derivative of MIS1	This work
Alcaligenes eutrophus (pJP4)	81.9 kb plasmid	Ka, J. O.
Agrobacterium tumefaciens NT1 (pTi15955)	200 kb plasmid	Farrand, S. K.

[a] Cu^r=copper resistance; Cu^s=copper sensitive; Rif^r=Rifampicin resistance.

were randomly selected per mating for plasmid isolation.

Some matings were also performed in pepper leaves (19). Approximately 10^8 donor and 5×10^8 recipient cells in 0.01 M phosphate-buffered saline (PBS, pH 7.2) were mixed and drawn into a sterile 10 ml plastic syringe without the needle. Approximately 0.2 ml of the cell suspension was infiltrated into the underside of the individual leaves of pepper (*Capsicum annum* cultivar Kusung). The plants were incubated in growth chamber for 48 hours at 28°C. After which, the infiltrated areas were dipped in 95% ethanol for 30 sec, and washed with sterile water. The infiltrated areas were blended in 2 ml of 0.01 M PBS (pH 7.2) and the 0.1 ml aliquots were plated onto selective media as membrane filter mating. Controls consisted of donor and recipient cells infiltrated alone to determine the frequency of spontaneous mutation.

Plasmid isolation. Plasmids of bacterial strains were isolated by QIAGEN Maxi kits (Chatsworth, CA) according to the manufacture's instruction. Bacterial strains grown in 100 ml of nutrient broth overnight at 28°C were harvested by centrifugation, and washed twice with 0.85% saline. Bacterial pellets were resuspended in 10 ml of 50 mM Tris/HCl, 100 mM EDTA, pH 8.0 with RNase 100 μg/ml, and added with 10 ml of 200 mM NaOH and 1% SDS. After incubation for 5 min at room temperature, 10 ml of 3.0 M prechilled potassium acetate (pH 5.5) was added for neutralization, and incubated on ice for 30 min. After centrifugation at 4°C for 2×15 min at $20,000\times g$, the supernatant was transferred to equilibrated QIAGEN column. The column was washed with washing buffer, and plasmid DNA was eluted with elution buffer. DNA was precipitated with 0.7 volumes of isopropanol, washed with 70% ethanol, and dried in the air. Finally, precipitated DNA was resolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

Agarose gel electrophoresis. Ten microliters of the DNA-dye mixture (8 : 2) was placed in a 1×6 mm well in 0.7% agarose for horizontal electrophoresis. The agarose bed was 3 mm thick. Electrophoresis was accomplished at 65 V in a 0.5× Tris borate buffer (0.045 M Tris borate, 0.001 M EDTA, pH 8.0). The gels were stained with ethidium bromide at 0.5 μg/ml, viewed, and photographed with short wave length ultraviolet illumination.

RESULTS

MICs determination. The levels of copper resistance in two strains of *X. c.* pv. *vesicatoria* isolated in Korea were high enough compared with those of previously described copper-resistant strains; *X. c.* pv. *vesicatoria* 07882 and E3. MICs of cupric sulfate for strains HN94-2 and HN94-6 were 1.6 and 1.5 mM, respectively (Table 2). MICs for cupric sulfate of copper-sensitive strains were 0.2~0.5 mM. MICs of zinc sulfate for HN94-2, and HN94-6 were 0.5, and 0.3 mM, respectively (Table 2). Copper-resistant strains were not resistant to zinc sulfate. MICs of zinc sulfate of all tested stains, regardless of copper sensitivity, were between 0.3 to 0.6 (Table 2). Therefore, the copper resistance of Korean isolates was not linked to zinc resistance.

Plasmid involvement in copper resistance. Stall and coworkers previously demonstrated that copper-resistant (Cu^r) strains of *X. c.* pv. *vesicatoria* isolated from diseased pepper plants in Florida have contained Cu^r genes on a conjugative plasmid designated pXvCu (18). Therefore, an experiment was conducted to determine whether the strains of *X. c.* pv. *vesicatoria* isolated in Korea contained Cu^r genes on a self-transmissible plasmid. The plasmid profiles of E3, HN94-2, and HN94-6 were similar; each strain contained a large plasmid which comigrated with the 200 kb plasmid present in *Agrobacterium tumefaciens* NT1 (Fig. 1). Copper-sensitive strains MZ94-1R and KJ93-1R contained smaller plasmids, and *X. c.* pv. *campestris* MIS1R had

Table 2. Minimum inhibitory concentrations (MICs)[a] of $CuSO_4$ and $ZnSO_4$ for *Xanthomonas campestris* pv. *vesicatoria*

Bacterial strain	Copper sensitivity[b]	MIC (mM) on nutrient agar	
		$CuSO_4$	$ZnSO_4$
X. c. pv. *vesicatoria*			
HN94-2	R	1.6[c]	0.5
HN94-6	R	1.5	0.3
07882	R	1.8	0.6
E3	R	1.5	0.6
EJB1	S	0.5	0.4
MZ94-1	S	0.4	0.6
KJ93-1	S	0.3	0.5
CE93-1	S	0.2	0.3

[a] The lowest concentration that inhibited confluent growth of the culture after 3 days.
[b] R : Resistant, S : Sensitive.
[c] Each value is the mean of three replications.

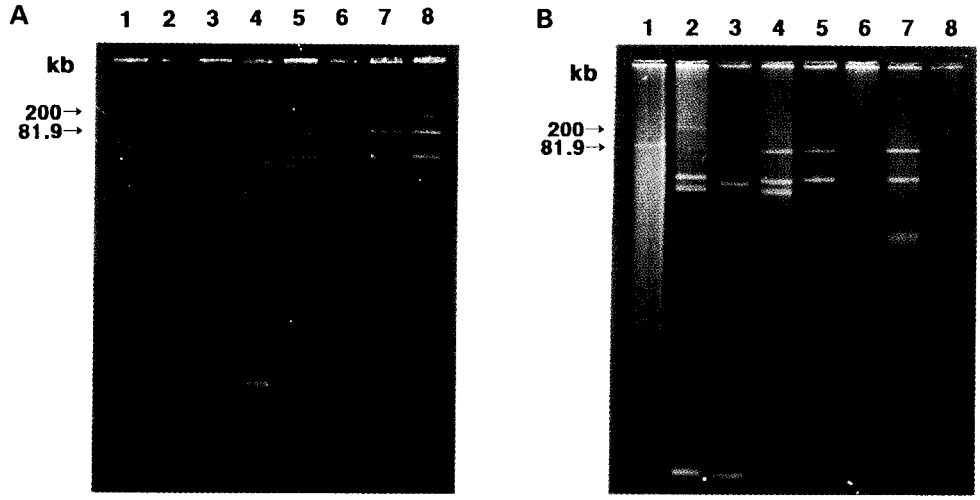

Fig. 1. Transfer of copper resistance plasmid from HN94-2 and HN94-6 to MZ94-1R and MIS1R of *Xanthomonas campestris*. pv. *vesicatoria*. Plasmids are isolated from; A. lane 1 : *Alcaligenes eutrophus* (pJP4), 2 : *Agrobacterium tumefaciens* (pTi15955), 3 : *X. c.* pv. *vesicatoria* E3 (the largest plasmid is about 200 kb), 4 : *X. c.* pv. *vesicatoria* 07882, 5 : *X. c* pv. *vesicatoria* HN94-2, 6 : *X. c.* pv. *vesicatoria* MZ94-1R, 7 and 8 : *X. c.* pv. *vesicatoria* MZC2, transconjugants between MZ94-1R and HN94-2. B. lane 1 : *Alcaligenes eutrophus* (pJP4), 2 : *X. c.* pv. *vesicatoria* E3 (the largest plasmid is about 200 kb), 3 : *X. c.* pv. *vesicatoria* 07882, 4 : *X. c.* pv. *vesicatoria* HN94-6, 5 : *X. c.* pv. *vesicatoria* KJ93-1R, 6 : *X. c.* pv. *campestris* MIS1R, 7 : *X. c.* pv. *vesicatoria* KJC6, transconjugant between KJ93-1R and HN94-6, 8 : *X. c.* pv. *vesicatoria* MIS1C6, transconjugant between MIS1R and HN94-6.

Table 3. Frequency of the conjugative transfer of copper resistance (Cu^r) *Xanthomonas campestris* pv. *vesicatoria* strains

Donor strain	Frequency[a] of Cu^r in recipient strains		
	X. c. pv. *vesicatoria* MZ94-1	*X. c.* pv. *vesicatoria* KJ93-1	*X. c.* pv. *campestris* MIS1
X. c. pv. *vesicatoria* HN94-2	4.0×10^{-4}	3.9×10^{-5}	–[b]
X. c. pv. *vesicatoria* HN94-6	1.0×10^{-5}	4.3×10^{-3}	4.1×10^{-5}

[a] Frequency is expressed as no. of transconjugants per donor cell.
[b] Frequency was lower than that of spontaneous mutation (1.0×10^{-10}).

no plasmid.

To test the self-transmissibility of those large plasmids, conjugation was performed. Copper-resistant strains HN94-2 and HN94-6 were used as putative donors, and copper-sensitive strains MZ94-1R, KJ93-1R and MIS1R were used as putative recipients. Copper resistance was transferred to copper-sensitive strains (Table 3). Copper resistance of HN94-2 was transferred to copper-sensitive strains MZ94-1R and KJ93-1R at the frequency of 4.0×10^{-4} and 3.9×10^{-5} (transconjugant/donor), respectively. Copper resistance of HN94-6 was transferred to copper-sensitive strains MZ 94-1R and KJ93-1R at the frequency of 1.0×10^{-5} and 4.3×10^{-3}, respectively, and also transferred to *X. c.* pv. *campestris* MIS1R at the frequency of 4.1×10^{-5}. Spontaneous mutants arose rarely in controls of donor and recipient cells alone, and the observed rates of spontaneous mutation to copper or rifampicin resistance were always less than 10^{-10} per cell.

Plasmids were reisolated from putative transconjugants (Fig. 1). The largest plasmid in the transconjugant MZC2 comigrated with the largest plasmid of HN94-2. Also the largest plasmid in the transconjugants KJC6 and MIS1C6 comigrated with the largest plasmid of HN94-6. Thus, the largest plasmid in HN94-2 and HN94-6 appears to be the self-transmissible copper resistance plasmid, and designated pXVK 9402 and pXVK9406, respectively.

Conjugation on pepper leaves. To test the possibility of the transfer of the copper resistance plasmid in the field, bacterial matings were performed on pepper leaves. Copper resistance of HN94-2 was transferred to copper-sensitive strains MZ94-1R on pepper leaves at the frequency of 5.2×10^{-5} (transconjugant/donor). The observed frequency of the transfer was approximately 10 times lower than that of the filter mating involving the two strains. Spontaneous mutants arose rarely in controls of donor and recipient cells infiltrated alone, and the rates of spontaneous mutation were less than 10^{-10} per cell.

DISCUSSION

Copper sprays are used widely for control of the bacterial spot of pepper and tomato caused by *X. c.* pv. *vesicatoria*. Copper resistance in this pathogen has been reported first in Florida (14), and also observed in Mexico (1), California (7), Oklahoma (3), North Carolina, Barbados, and Italy (16).

Most of these strains, except California strain 07882, contain large plasmids with homology to pXvCu which was identified first in copper-resistant strains from Florida (7, 18). The pXvCu-type of plasmid is usually self transmissible, and its role in copper resistance can therefore be assessed by conjugation experiments between copper-resistant and -sensitive strains (3, 18). These plasmids are polymorphic; varying considerably in restriction enzyme digestion profiles even between different isolates from the same site (3). The plasmids also vary in size but are usually 200 kb or larger. One of such plasmids, pXV10A, was transmissible to several pathovars of *X. campestris* but not to other plant pathogenic species (7).

The copper-resistant *X. c.* pv. *vesicatoria* strains were first isolated from Cheonan county in Korea (12). The copper resistance of Korean strains HN94-2 and HN94-6 were able to grow in 3 to 15 times more copper containing media than those of copper-sensitive strains, and similar to those of 07882 and E3. Colors of their colonies and their surroundings were not changed when they were grown on copper-supplemented media (data not shown). Therefore, the mechanisms for copper resistance of these strains were concerned with copper efflux rather than copper sequestrations in the cell wall. These Korean strains were not resistant to zinc sulfate and agrochemicals containing zinc which were recommended for the control of these copper-resistant strains. However, it was reported that a strain initially insensitive to copper and sensitive to zinc developed to tolerate to zinc after repeated exposure (1).

Copper-resistant Korean strains seem to contain copper resistance genes on a self-transmissible plasmid since the copper resistance was cotransferred with a large plasmid of about 200 kb into copper-sensitive strains. This plasmid has copper-resistant determinants and it was designated as a pXVK. Copper resistance of these strains was transferred to copper-sensitive strains by conjugation very easily. The spread of copper resistance within field populations of *X. c.* pv. *vesicatoria* is likely enhanced by conjugation. The demonstration of conjugation on pepper leaves indicates that the transfer of the 200 kb plasmid associated with copper resistance is able to happen in the field. This is the first report in Korea that the self-transmissible plasmids carry copper resistance. More detail and careful investigations for copper resistance in Korea should be done.

요 약

세계적으로 구리약제에 대해서 저항성을 나타내는 균주들이 발견되었으며, 이들은 구리약제의 방제효과를 감소시켰다. 국내에서도, 구리 저항성 균주 *Xanthomonas campestris*. pv. *vesicatoria* HN94-2, -3, -4, -5, -6 들이 천안지역의 고추재배지에서 처음으로 분리되었으며, 이들 균주들의 nutrient agar에서 황산구리($CuSO_4$)에 대한 최소억제농도(minimum inhibitory concentration, MIC)는 1.4~1.6 mM이었다. 이들은 모두 황산아연($ZnSO_4$)에 대해서는 감수성을 보여, 구리저항성 균주에 대한 방제약제로서 아연 함유 약제를 사용할 수 있을 것이다. 분리된 균주중 HN94-2와 HN94-6을 이용하여 접합(conjugation)을 통한 구리저항성의 전파를 실험한 결과, 이들 두 균주 모두 구리감수성 균주에게 4.3×10^{-3}에서 1.0×10^{-5}(transconjugant/donor)의 정도로 구리 저항성이 전이되었다. 이들 HN94-2와 HN94-6 균주의 구리저항성 유전자들은 약 200 kb 정도의 커다란 플라스미드(plasmid)에 존재하며, 이들은 각각 pXVK9402와 pXVK9406이라 명명되었다.

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