Chromosomal Gene Transfer by Conjugation in the Plant Pathogen *Xanthomonas axonopodis* pv. *vesicatoria*

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ABSTRACT

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Genes for copper resistance, located on the chromosome of strain XvP26 of *Xanthomonas axonopodis* pv. *vesicatoria*, were transferred by conjugation to a recipient strain of the bacterium. The chromosomal gene transfer was verified by analyses of the genomes of donor, recipient, and putative transconjugants for plasmid profiles, by polymorphism of DNA bands obtained by digesting total genomic DNA by a rare-cutting endonuclease and pulsed-field gel electrophoresis, and by Southern hybridization with a probe containing the copper genes. Transfer of kanamycin resistance to a recipient strain, associated with Tn*5* insertion into the chromosome of another strain of the bacterial spot pathogen, was also verified. The frequency of kanamycin resistance transfer to recipient was more than 75 times greater in pepper leaves than in vitro. The transfer of chromosomal sequences containing the hypersensitive reaction and pathogenicity (*hrp*) genes and pigmentation (*pig*) genes was linked with transfer of kanamycin resistance (Tn*5*). Horizontal transfer in planta of the chromosomal genes (i.e., *cop*, *pig*, *hrp*, and Tn*5* sequences) among strains of *X. axonopodis* pv. *vesicatoria* means that horizontal chromosomal gene transfer is possible in nature. This type of gene transfer may explain the presence of great diversity among strains of the bacterial spot pathogen in terms of DNA polymorphism and may also explain the apparent horizontal transfer of *hrp* sequences among pathovars of *Xanthomonas*.

Additional keywords: bacterial spot disease, *Capsicum annuum*, *Lycopersicon esculentum*.

Bacteria in the genus *Xanthomonas* cause diseases in over 124 monocotyledonous and 268 dicotyledonous plant species (15,17). The genus *Xanthomonas* comprises 20 DNA homology groups, which are considered to be genomic species (27). The xanthomonads that cause the bacterial spot disease of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) exhibit significant genetic diversity in restriction fragment length polymorphism, protein profiles, fatty acid profiles, and total DNA homology (24). Stall et al. (24) divided strains of the bacterial spot pathogen into two genetic groups, and Vauterin et al. (27) placed the groups into two separate species (i.e., *Xanthomonas axonopodis* pv. *vesicatoria* [A group] and *X. vesicatoria* [B group]).

Significant variation also occurs among the bacterial spot pathogens in plasmid composition, with plasmids varying in size from 3 to 300 kilobases (kb) among a population of 522 strains. Thirteen size classes of plasmids were differentiated, and 71 different plasmid profiles occurred among the strains (3). Most of the avirulence genes involved in host and cultivar specificities (19,23) and the genes for resistance to copper and streptomycin are located on plasmids (18,25). Moreover, plasmids that carry copper resistance genes are self-transmissible (25). Mobilization of plasmids among strains of *X. axonopodis* pv. *vesicatoria* has been demonstrated to occur in nature (3). Plasmids may also be important in gene transfer among strains of *Xanthomonas*. However, this type of gene transfer does not explain evidence obtained for the horizontal transfer of *hrp* gene sequences, which are located on the chromosome (2), among pathovars of *Xanthomonas* (16). Horizontal chromosomal gene transfer (20) among strains of the bacterium could account for such transfer. Horizontal transfer of chromosomal genes among strains of plant-pathogenic bacteria has been reported (5), but the significance of this transfer in causing genetic variation has not been studied.

The purpose of this research was to investigate if chromosomal gene transfer by conjugation occurs among strains of *X. axonopodis* pv. *vesicatoria* in a natural niche of the bacterium. This work was begun after discovery of a strain of *X. axonopodis* pv. *vesicatoria* that had copper resistance genes located on the chromosome. It was speculated that the copper genes, which are usually plasmid-borne in this bacterium, may have integrated into the chromosome and thus might mobilize the chromosome between strains. Chromosomal movement between strains of *X. axonopodis* pv. *vesicatoria* by conjugation in nature could be an important source of genetic variation, potentiating changes of ecological and evolutionary significance for the plant pathogen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study and their sources are listed in Table 1. Strain XvP26, originally isolated from pepper in Taiwan, was used as one donor, and the determinants for copper resistance were located on the chromosome. Strain 85-13, isolated from pepper in Florida, was used as another donor, and transposon Tn*5* was inserted into its chromosome to provide a strain with kanamycin resistance. Streptomycin-resistant mutants of both donors were obtained by selection of colonies on plates containing 200 µg of streptomycin sulfate per ml. Strain 82-8, originally isolated from pepper in Florida, was used as a recipient throughout these experiments, and colonies resistant to nalidixic acid and rifamycin were selected successively on media containing those antibiotics. Strain 82-8 was found to be a good recipient in previous conjugation studies (25).

All strains of *X. axonopodis* pv. *vesicatoria* were grown on nutrient agar (NA) made from nutrient broth (Becton Dickinson, Cockeysville, MD) containing 1.5% agar. Nutrient broth cultures

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(NB) were grown for 24 h on a rotary shaker (150 rpm) at 28°C. Matings between strains were performed on nutrient yeast glycerol agar (NYGA) (8). All strains were stored in sterile tap water at room temperature and in 30% glycerol at –70°C. Antimicrobial agents were used to maintain selection for resistance markers at the following final concentrations: rifamycin, 80 μg ml^{-1}; nalidixic acid, 50 μg ml^{-1}; kanamycin sulfate, 50 μg ml^{-1}; streptomycin, 50 μg ml^{-1}, and copper sulfate, 200 μg ml^{-1}.

Plant material and plant inoculation. Pepper plants (6 weeks old) of cv. Early Calwonder (ECW) were maintained in a growth chamber at 28 to 30°C during inoculation and incubation. The mesophyll spaces of leaves of pepper plants were infiltrated with bacterial suspensions with the aid of a 1-ml plastic syringe and 27-gauge needle. The concentrations of bacteria in suspensions in sterile tap water were approximately 10^8 CFU ml^{-1}, as determined by measuring the optical density (absorbance at 600 nm [A_{600}] = 0.3) in a spectrophotometer (Spectronic 20; Bausch and Lomb, Inc., Rochester, NY).

Conjugation for chromosomal gene transfer. Both donor and recipient strains were grown overnight at 28°C on NA containing appropriate antimicrobial agents. Bacteria were collected from solid media, suspended in 1 ml of sterile tap water, washed two times by centrifugation, and resuspended in sterile tap water. Equal amounts of donor (10^9 CFU ml^{-1}) and recipient (10^9 CFU ml^{-1}) cells were mixed thoroughly by pipetting, plated on NYGA, and incubated for 0, 8, 16, 24, 48, and 72 h. At the designated times, bacteria were scraped from the NYGA plates, adjusted to approximately 10^9 CFU ml^{-1}, and 400 μl were transferred to each of two plates of the appropriate selective media. The number of donor cells in each suspension was determined by 10-fold serial dilution and plating proper dilutions onto media containing copper or kanamycin, depending on the donor.

As a comparison, the same bacterial mixtures were infiltrated into the mesophyll spaces of pepper leaves and subsequently incubated for 0, 8, 16, 24, 32, 40, and 48 h at 28°C. After an appropriate time interval, 3 cm^2 of inoculated leaf tissue was sequentially triturated in 1 ml of sterile tap water, pelleted at 16,000 × *g* in an Eppendorf microfuge for 2 min, washed in 1 ml of sterile tap water, and centrifuged at 16,000 × *g* for 2 min. The pellets were resuspended in 800 μl of sterile tap-water and, depending upon donor strain, 400 μl of the suspension was spread onto each of two plates of media containing copper, rifamycin, nalidixic acid, and chlorothalonil (80 μg ml^{-1}), or kanamycin, rifamycin, nalidixic acid, and chlorothalonil (80 μg ml^{-1}). Chlorothalonil was added to prevent the growth of fungi present in leaf tissue. Plates were kept at 28°C for 3 to 4 days. Colonies of transconjugants and donors were counted, and transfer frequencies were expressed as transconjugants per donor cell. The experiment was repeated several times, and results from a representative experiment are reported.

Mutations in pigmentation (*pig*) and pathogenicity (*hrp*) genes of the recipient strain. Cells of the recipient strain 82-8 were grown in 4 ml of nutrient broth to the mid-log phase growth stage (5×10^8 CFU ml^{-1}). To pellet the cells, 1 ml of the culture was centrifuged for 2 min at 16,000 × *g*, and the cells were resuspended in an equal volume of a Tris-maleic acid buffer at pH 6.0 (4). A final concentration of 100 μg ml^{-1} of a freshly prepared solution of nitrosoguanidine (NTG) was added to the suspension of the recipient strain and incubated at 28 to 30°C for 30 min. The cells were then pelleted, resuspended in sterile tap water, and 10-fold dilutions were plated onto NA. White colonies were selected and one (82-8P), which was complemented back to a yellow color with a cosmid that contained cloned pigmentation genes from *X. axonopodis* pv. *vesicatoria*, was used as the *pig*$^-$ recipient strain in chromosomal gene transfer experiments. Complementation was accomplished by transferring plasmid EC744 from *Escherichia coli* DH5α to cells of the white strain by triparental matings using pRK2073 as a helper plasmid (9,12,22).

To discount revertants of the pigmentation (*pig*) mutation, strain 82-8P was grown overnight to 10^9 CFU ml^{-1}. The suspension was added at a final concentration of 10^3 CFU ml^{-1} to 30 test tubes containing 3 ml of NB, grown for 48 h to 10^9 CFU ml^{-1}, and dilutions from the suspension in each tube were plated on NA. White and yellow colonies were counted at appropriate dilutions to determine reversion frequency.

To obtain a Hrp$^-$ recipient strain, approximately 300 individual colonies of 82-8 selected after the NTG treatment described above were inoculated into the mesophyll of pepper leaves at a concentration of 10^8 CFU ml^{-1}. Plants were kept at 28 to 30°C for 3 days for disease development. Colonies selected for no disease development were tested for mutations in the *hrp* cluster by complementation experiments. The plasmid EC993 containing the *hrp* gene cluster was transferred by triparental conjugation from *E. coli* DH5α to several nonpathogenic strains. Transconjugants from triparental matings were inoculated into pepper leaves to test for complementation to pathogenicity. One strain (82-8H) with the *hrp*$^-$ phenotype that could be complemented with the cloned *hrp* gene cluster was selected as the *hrp*$^-$ recipient for the conjugation tests described later.

To discount the possibility for pathogenic revertants of the *hrp* mutation, strain 82-8H was grown overnight to 10^9 CFU ml^{-1}, added at a final concentration of 10^3 CFU ml^{-1} to 50 test tubes containing 3 ml of NB, grown for 48 h to 10^9, and then diluted to 10^7 and 10^8 CFU ml^{-1} for inoculation into pepper leaves. The inoculated plants were incubated in the greenhouse for 3 weeks and then checked for lesion development.

TABLE 1. List of bacterial strains and plasmids used

Strains and plasmids	Relevant characteristics	Source/source of reference[a]
Bacteria		
Xanthomonas axonopodis pv. *vesicatoria*		
XvP26	Pepper race 3, Cop^r and Str^r	R. E. Stall
82-8	Pepper race 1, Rif^r and Nal^r	R. E. Stall
85-13	Pepper race 2, Str^r and Kan^r (Tn*5*)	R. E. Stall
75-3	Large plasmid (284 kb)	R. E. Stall
E3	Large plasmid (213 kb)	R. E. Stall
82-8H	Hrp$^-$ mutant of strain 82-8	This study
82-8P	Pig$^-$ mutant of strain 82-8	This study
Pantoea stewartii subsp. *stewartii* SW2	13 plasmids in different sizes	D. L. Coplin
Escherichia coli DH5α	F-*recA* 80d*lac*ZM15	Bethesda Research Laboratories
Plasmids		
pLAFR3	Tet^r rlx$^+$ RK2 replicon	Staskawicz et al. (26)
pEC744	pLAFR3 containing *pig*	R. E. Stall
pEC993	pLAFR3 containing *hrp*	R. E. Stall
pXv26Cu	pLAFR3 containing Cop^r genes	Basim and Stall (1)
pRZ102	Contains Tn*5* transposon	Rothstein et al. (21)
pRK2073	ColEI replicon, Tra$^+$ Mob$^+$ Sp^r	Figurski and Helinski (12)

[a] Bethesda Research Laboratories, Gaithersburg, MD; R. E. Stall, University of Florida, Gainesville; and D. L. Coplin, Ohio State University, Columbus.

Plasmid profiles. Bacterial strains were grown for 20 to 30 h at 28°C with vigorous shaking in tubes containing 3 ml of NB and proper antibiotics. Cells were harvested by centrifugation, cell density was adjusted to A_{600} = 0.3 in sterile deionized water, and plasmid DNA was extracted as described previously (3). The plasmid DNA was then subjected to electrophoresis until a bromophenol blue front had moved 10 cm from the well. The electrical potential was 5 V cm^{-1} for the submerged gel (Model H5; BRL, Grand Island, NY). Samples were electrophoresed through 0.5% agarose gels (SeaKem, DNA grade; FMC BioProducts, Rockland, ME) in Tris-acetate-EDTA buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH 8.0), stained with ethidium bromide (0.5 μg ml^{-1}) for 30 min, and photographed using transmitted UV light. In each gel, a plasmid preparation of *Pantoea stewartii* subsp. *stewartii* strain SW2 (7) was included as a molecular weight marker.

The presence of a megaplasmid in the donor, recipient, or putative transconjugants was determined by pulsed-field gel electrophoresis (PFGE) technique. Cells of donor, recipient, and transconjugants and other strains of *X. axonopodis* pv. *vesicatoria*, which were known to have large plasmids (Table 1), were incorporated into agarose blocks and then lysed as described previously (10). The total undigested genomic DNAs from those strains were subjected to PFGE as described below.

Preparation of genomic DNA in agarose blocks and restriction enzyme digestion. Bacterial genomic DNA in agarose blocks was prepared as described by Hacioglu et al. (14). Agarose blocks containing lysed bacterial cells were sectioned into four to five pieces, about 3- to 4-mm sections, across the width of the block with a sterile glass coverslip. The small agarose pieces were rinsed twice in 1 ml of Tris-EDTA buffer for 1 h. The buffer was changed, and blocks were transferred into Eppendorf tubes containing 100 ml of 1× *Spe*I restriction enzyme buffer. After 20 min, the buffer was replaced with fresh enzyme buffer, and 18 units of *Spe*I (Promega Corp., Madison, WI) was added. The DNA was digested overnight at 37°C.

PFGE. Fragments of digested DNA were separated electrophoretically in 0.9% SeaKem-GTG agarose (FMC BioProducts) in 0.5× Tris-borate-EDTA buffer (TBE; 44.5 mM Tris-HCl, 44.5 mM boric acid, and 1 mM EDTA, pH 8.0) by using a Bio-Rad DRII Contour-Clamped Homogeneous Electric Field (CHEF) apparatus (Bio-Rad Laboratories, Richmond, CA) (6). The blocks containing the digested DNA were washed with 500 μl of wash solution (0.5 mM EDTA, pH 9.5, and 1% *N*-lauroyl sarcosine in sterile deionized water) at 55°C for 2 h and were rinsed once at room temperature in 500 μl of wash solution for 15 min. The agarose blocks were then placed into 1-cm-wide wells in an agarose gel (14 × 12.5 cm) and sealed with 2% Seakem-GTG agarose at 55°C. The gel was placed in a Bio-Rad DR II CHEF unit containing 1.6 liters of 0.5× TBE buffer that was cooled to 14°C throughout the run. Electrophoresis was accomplished using pulse times ramped from 5 to 45 s for 22 h at 200 V and from 1 to 13 s for 12 h at 200 V for separation of the size range 225 to 2,200 kb and 0.13 to 194 kb, respectively. Gels were stained with 0.5× TBE buffer containing ethidium bromide (0.5 μg ml^{-1}) for 30 min, destained with 0.5× TBE buffer for 30 min, and photographed over a UV light source with type 55 Polaroid film (Polaroid Corp., Cambridge, MA). After electrophoresis, sizes of DNA fragments were determined by measuring the distances the bands migrated compared with *Saccharomyces cerevisiae* chromosome markers (Bio-Rad Laboratories) and low-range PFGE markers (New England Biolabs, Inc., Beverly, MA).

Southern hybridization analysis. Southern hybridization was done as described previously (16), with slight modifications. DNA separated by PFGE was exposed to UV light for 2 min. DNA depurination was performed in 0.25 N HCl twice for 20 min on a shaker at room temperature to enhance the transfer of large DNA fragments. DNA of the plasmid pXv26Cu and the *Hin*dIII fragment of pRZ102 were labeled using the Genius Non-radioactive Chemiluminescent DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The membranes were washed twice for 5 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) at room temperature and then twice for 15 min in 0.5× SSC plus 0.1% SDS at 65°C after hybridization. Posthybridization washes and chemiluminescent development were carried out according to the instructions of the manufacturer. For reuse, these membranes were washed in distilled water for 1 min and then the probe was removed by soaking them twice in 0.4 N NaOH plus 0.1% (wt/vol) SDS for 20 min at 37°C on a shaker. Finally, the membranes were rinsed in 2× SSC for 2 min and then dried at room temperature until use.

RESULTS

Selection of putative transconjugants in vitro. When strain XvP26 Cop^rStr^r was mated in vitro with strain 82-8 Rif^rNal^r for 24 h, putative transconjugants developed on NA medium containing copper, nalidixic acid, and rifamycin. The frequency was low (approximately 1 × 10^{-9} transconjugants per donor cell). However, no growth

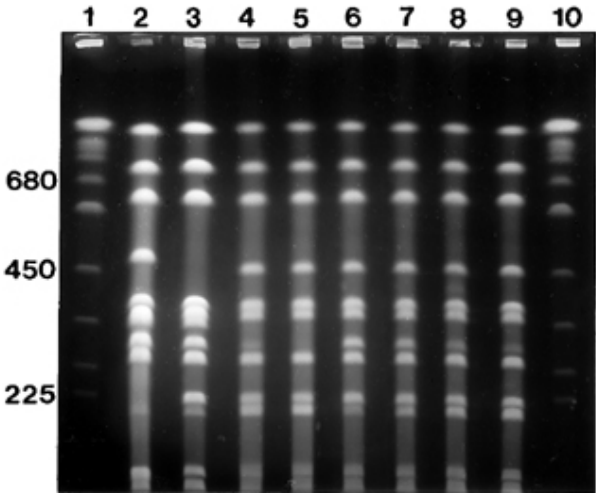


Fig. 1. Restriction profiles after pulsed-field gel electrophoresis of *Spe*I digests of genomic DNA of donor XVP26 (Cop^rStr^r), recipient 82-8 (Rif^rNal^r), and transconjugants ($Cop^rRif^rNal^r$). Lanes 1 and 10, DNA size standards from S*accharomyces cerevisiae*; lane 2, donor; lane 3, recipient; and lanes 4 to 9, transconjugants. The ramped pulse times were 5 to 45 s for 22 h at 14°C and 200 V. The gel was 1.0% Seakem-GTG agarose in 0.5× Tris-borate-EDTA buffer. Molecular sizes of marker bands are indicated in kilobases.

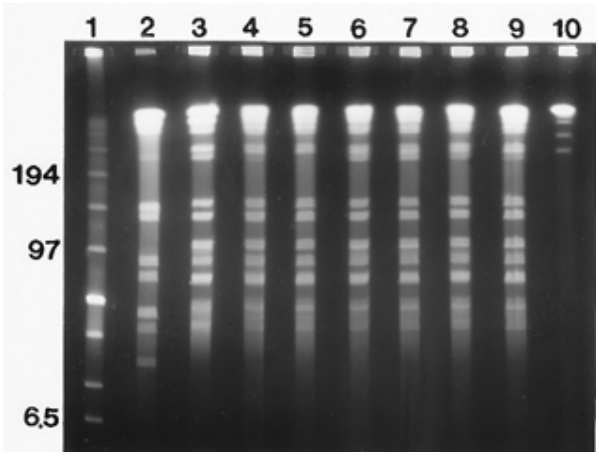


Fig. 2. DNA fragments after pulsed-field gel electrophoresis (PFGE) of *Spe*I digests of genomic DNA of donor 85-13 (Kan^rStr^r), recipient 82-8 (Rif^rNal^r), and transconjugants ($Kan^rRif^rNal^r$). Lane 1, low-range PFGE marker; lane 2, donor; lane 3, recipient; lanes 4 to 9, transconjugants; and lane 10, DNA size standards from *Saccharomyces cerevisiae*. The ramped pulse times were 1 to 13 s for 12 h at 14°C and 200 V. The gel was 1.0% Seakem-GTG agarose in 0.5× Tris-borate-EDTA buffer. Molecular sizes of marker bands are indicated in kilobases.

occurred on NA medium containing streptomycin, copper, nalidixic acid, and rifamycin. Similarly, when strain 85-13 Kan^rStr^r was used as the donor, putative transconjugants were not found on NA medium containing streptomycin, kanamycin, nalidixic acid, and rifamycin. However, if streptomycin was omitted from the medium, a low number (approximately 1×10^{-9} putative transconjugants per donor cell) were obtained.

Copper-resistant or kanamycin-resistant mutants of the recipient could account for growth of putative transconjugants on the selective medium, because resistance to only one additional compound was needed for growth of the recipient on the antimicrobial medium. However, in repeated tests, colonies of the recipient strain were not found on the selective media on which putative transconjugants were able to grow. Furthermore, no mutants of the recipient developed on NA medium plates with copper or kanamycin when approximately 10^9 cells were spread over the agar surface. Nevertheless, to confirm that mobilization of the chromosome from donor to recipient occurred, plasmid profiles, PFGE, and Southern hybridizations were performed as described below.

To determine if conjugation was the mechanism responsible for movement of kanamycin resistance into the recipient, nalidixic acid, which inhibits DNA replication, was added to the medium during mating of the donor and recipient, because DNA synthesis would be required in the donor. When the donor and recipient were sensitive and resistant, respectively, to nalidixic acid, no putative transconjugants were detected. However, when the donor and recipient were resistant and sensitive, respectively, to nalidixic acid, a low number (approximately 1×10^{-10} putative transconjugants per donor cell) were identified that were resistant to kanamycin.

Analysis of plasmid profiles of donor, recipient, and putative transconjugants. The plasmid profiles of the two donor strains and the recipient strain were different. The donor strain, XvP26 Cop^rStr^r, contains a 15-kb plasmid, and the donor strain, 85-13 Kan^rStr^r, has two plasmids of 300 and 40 kb in size. The recipient strain, 82-8 Rif^rNal^r, has three plasmids in size of about 120, 44, and 33 kb. The putative transconjugants $Cop^rRif^rNal^r$ and $Kan^rRif^rNal^r$ had the same plasmid profile as the recipient (data not shown).

Another possibility for resistance to antimicrobial compounds in the putative transconjugants would be the movement of a megaplasmid between strains that contained Cop^r or Kan^r. Two lines of evidence point to the lack of a megaplasmid. First, no megaplasmid was detected by the plasmid extraction procedure used. Second, large plasmids (approximately 300 kb) were detected by PFGE of undigested DNA in strains E3 and 75-3 of *X. axonopodis* pv. *vesicatoria*, but none were detected in donor strain XvP26, recipient, or putative transconjugants in the PFGE tests (data not shown).

Analysis of total genome profiles of donor, recipient, and putative transconjugants by PFGE. The fragment profiles of the donor and recipient strains differed after digestion of genomic DNA with *Spe*I and PFGE. The fragment profiles of the transconjugants (Cop^r Rif^r Nal^r) were different from genomic profiles of the donor strain or recipient strain in the range of 225 to 2,000 kb in size (Fig. 1), but they were like the recipient strain in the range of 100 to 225 kb in size (data not shown). A fragment at approximately 500 kb was present in donor and transconjugants, but not in the recipient parent. Similarly, the putative Kan^r transconjugants (Kan^r Rif^r Nal^r) mainly shared a similar genomic profile with the recipient in the range of 225 to 2,000 kb (data not shown), but they were different from the donor and recipient in the range of 50 to 225 kb in size (Fig. 2). However, an approximately 100-kb fragment was present in recipient and transconjugants, but not in the donor parent.

Southern hybridization of genomic DNA with genes for copper resistance and Tn*5* sequences. Hybridization of the genomic DNA, separated by PFGE after digestion with *Spe*I, with DNA from clones carrying copper resistance genes and *Tn5* sequences, which contained the kanamycin resistance gene, located the resistance genes. Copper resistance was located on a nearly 500-kb DNA fragment of the donor strain, XvP26, and on fragments of approximately 450-kb DNA in the putative transconjugants. No hybridization of copper genes occurred with DNA of the recipient strain (Fig. 3). There was little or no variation in fragment size within the transconjugants. The Tn*5* sequences inserted in the chromosome of donor strain 85-13 were detected on a fragment of 86.8 kb in the donor and putative transconjugants by using the probe of Tn*5* sequences. No hybridization occurred with the recipient (Fig. 4).

Matings performed in pepper plants. Experiments on linkages of copper resistance and kanamycin resistance to other chromosomal genes were limited by the low frequency of conjugation on agar media. Matings made in pepper leaves increased the frequency of transconjugants obtained with kanamycin resistance approximately 75-fold over that obtained in vitro (data not shown). Transconjugants increased in pepper leaves with increasing mating time (Table 2). A subset of putative transconjugants selected after matings in pepper leaves were determined to be true transconjugants by comparisons with donor and recipient DNA fragment

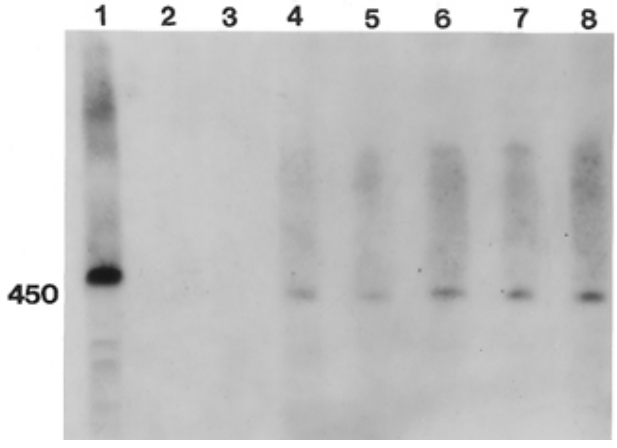


Fig. 3. Hybridization of labeled DNA of pXv26Cu containing the copper gene cluster of strain XvP26 of *Xanthomonas axonopodis* pv. *vesicatoria* to genomic DNA (450-kilobase fragment) of donor XvP26 (Cop^rStr^r), recipient 82-8 (Rif^rNal^r), and transconjugants ($Cop^rRif^rNal^r$). Lane 1, donor; lane 2, recipient; lanes 3, empty; and lanes 4 to 8, transconjugants. Genomic DNA was restricted with *Spe*I and separated by pulsed-field gel electrophoresis. The ramped pulse times were 5 to 45 s for 22 h at 14°C and 200 V. The gel was 1.0% Seakem-GTG agarose in 0.5× Tris-borate-EDTA buffer.

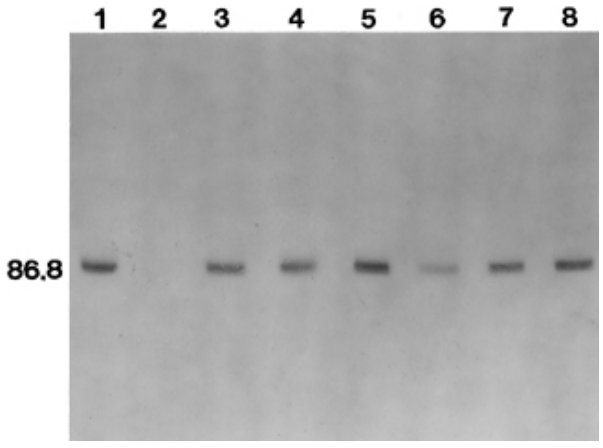


Fig. 4. Hybridization of labeled DNA of clone pRZ102 containing Tn*5* sequences to genomic DNA (86.8-kilobase fragment) of donor 85-13 (Kan^rStr^r), recipient 82-8 (Rif^rNal^r), and transconjugants ($Kan^rRif^rNal^r$). Lane 1, donor; lane 2, recipient; and lanes 3 to 8, transconjugants. Total genomic DNA was restricted with *Spe*I and separated by pulsed-field gel electrophoresis. The ramped pulse times were 1 to 13 s for 12 h at 14°C and 200 V. The gel was 1.0% Seakem-GTG agarose in 0.5× Tris-borate-EDTA buffer.

profiles after digestion with *Spe*I and separation by PFGE and by subsequent probing with copper and kanamycin resistance genes (data not shown). The numbers of transconjugants obtained were sufficient to test linkages of these genes with other chromosomal genes.

Transfer of pigmentation genes. The kanamycin-resistant donor was mated in pepper leaves with the mutant recipient that was deficient in production of pigment. Of 400 transconjugants, 90% (those colonies that grew on Kan^r Rif^r Nal^r) were yellow. Ten transconjugants were randomly selected, and they had exactly the same plasmid profile as the recipient strain. The total genomic profiles of transconjugants (Kan^r Rif^r Nal^r pig^+) after *Spe*I digestion of total DNA and PFGE were different from the donor and recipient strains. Tn*5* sequences were detected by Southern hybridizations on DNA fragments of the donor and transconjugants, but were not detected on fragments of the recipient. The pigmentation genes and Tn*5* sequences of the donor and transconjugants were located on the same 86.8-kb fragment as determined by Southern hybridization (data not shown). In recipient controls, 468 colonies were counted on 30 plates and all had the pig^- phenotype. Furthermore, at the next lower dilution, no pig^+ colonies were evident.

Horizontal transfer of *hrp* genes. The kanamycin-resistant donor was mated in pepper leaves with the Hrp^- recipient, 82-8H. A group of putative transconjugants (Kan^r Rif^r Nal^r) were confirmed to be transconjugants by plasmid profiles, PFGE fragment profiles, and Southern hybridizations using Tn*5* sequences as a probe. Of the 400 transconjugants inoculated into pepper leaves, 5% were pathogenic. Thus, horizontal transfer of chromosomal *hrp* genes to the recipient occurred as determined by recombination and restoration of pathogenicity.

In the investigation of the total genome profiles of donor, recipient, and transconjugants, the DNA of the 86.8-kb *Spe*I fragment of the donor and transconjugants, but not the recipient, again hybridized with the Tn*5* clone. The *hrp* genes were located on a 610-kb *Spe*I fragment of the donor, recipient, and transconjugants by Southern hybridization using the *hrp* genes as a probe (data not shown).

In a test designed to determine if hrp^+ revertants occurred, no typical lesions were observed on inoculated leaves at $\leq 10^8$ CFU/ml. To discount inhibition of the Hrp^+ donor by high populations of recipient strain 82-8H, mixtures consisting of 10^3 CFU of the wild type per ml and 10^6 or 10^7 CFU of strain 82-8H per ml were injected into pepper leaves. Lesions were observed on leaves infiltrated with the mixtures at both concentrations of strain 82-8H, although significantly fewer were evident at the higher concentration of strain 82-8H.

DISCUSSION

In this study, we confirmed chromosomal transfer via conjugation for strains of *X. axonopodis* pv. *vesicatoria*. The plasmid profiles confirmed that the putative transconjugants were derived from the recipient. The recipient strain did not have copper or kanamycin resistance; therefore, probing with DNA containing those genes confirmed that they had been transferred to recipient cells from the donor. The transfer of the copper genes resulted in little or no polymorphism among the transconjugants in the *Spe*I fragment that contained the copper genes. Thus, it appears that the same size fragment from the donor recombined into the recipient chromosome after conjugation. The *Spe*I fragment that contained the Tn*5* transposon (kanamycin resistance) in the donor was relatively small (86.8 kb), and Tn*5* was integrated into the same size *Spe*I fragment in the recipient chromosome after conjugation. Interestingly, the genes for pigmentation and the Tn*5* insertion sites were located on the same *Spe*I fragment; therefore, selection for kanamycin resistance also selected for pigmentation complementation of recipients in 90% of the transconjugants. The two characters were thus closely linked. Furthermore, it does not appear that Tn*5* hopped on and off a plasmid, thereby only transferring Kan^r. Selection for kanamycin-resistant transconjugants only resulted in linkage with *hrp* genes in 5% of the transconjugants. Thus, the Tn*5* insertion site was not as closely linked to the *hrp* cluster as it was to the *pig* genes, which was supported by the fact that the genetic determinants for pigmentation and *hrp* functions were located on different *Spe*I fragments. The two determinants are separated by at least one *Spe*I restriction site, resulting in the two fragments being separated after *Spe*I digestion of genomic DNA. This is further evidence that a region of the chromosome containing the genes for kanamycin resistance, pigmentation, *hrp*, and specific *Spe*I restriction sites was occasionally recombined into the recipient chromosome.

A very low frequency of chromosomal transfer ws detected after matings on NYGA medium. The frequency was sufficient to obtain evidence for horizontal transfer of chromosomal DNA, but the frequency was not sufficient to obtain enough colonies for linkage studies. Fortuitously, the frequency of chromosomal transfer after matings in pepper leaves increased nearly 75-fold. The numbers of transconjugants obtained were sufficient to test for linkages of markers with other characteristics. The increase in conjugation in pepper leaves compared with artificial media may mean that conjugation is induced in planta, as it is for the Ti plasmid of *Agrobacterium tumefaciens* (11). Further study of this phenomenon could lead to the discovery of a plant product that could increase chromosomal transfer in vitro.

Two strains of *X. axonopodis* pv. *vesicatoria* were used as donors for this work. The two strains were obtained from widely different locations (Florida and Taiwan), but both belong to the A group of strains (27). One of the strains, XvP26, had only one plasmid of 15 kb in size. The other strain, 85-13, had two resident plasmids. The role of the resident plasmids in the movement of chromosomal DNA between the strains of *X. axonopodis* pv. *vesicatoria* was not completely determined, but their role is thought to be minor. The presence of Tn*5* in both a plasmid and chromosome has been reported to result in plasmid integration into the chromosome and transfer of chromosomal genes (4). In this study, Tn*5* only occurred in the chromosome of strain 85-13, so integration of a resident plasmid into the chromosome should not have been aided by the Tn*5* transposon. The horizontal transfer of chromosomal DNA with these strains was accomplished without the use of introduced plasmids or phages that aid in chromosomal movement, as previously reported for chromosomal transfer among other strains of plant-pathogenic bacteria (5).

Chromosomal transfer resulted in polymorphism of DNA fragments obtained by endonuclease digestion of genomic DNA. Thus, chromosomal transfer may account for the extensive polymorphism seen among genomic fragments of closely related strains within the genus *Xanthomonas* (10). If so, extensive polymorphism within closely related strains may be indicative of extensive horizontal transfer of genes. The procedure may be useful to test for chromosomal movement between pathovars of the same species and between species of *Xanthomonas*.

The movement of chromosomal DNA will help in mapping the chromosomal genes of *X. axonopodis* pv. *vesicatoria*. A primitive

TABLE 2. Transfer of chromosomal Tn*5* sequences from a donor to recipient strain of *Xanthomonas axonopodis* pv. *vesicatoria* after matings in planta for different times

Mating time (h)	Frequency of transconjugants[a]
0	0
8	0
16	1.5×10^{-7}
24	4.7×10^{-7}
32	8.7×10^{-7}
40	2.0×10^{-6}
48	2.6×10^{-6}

[a] Values were calculated by determining the number of donor cells and transconjugants per donor cell at the end of the mating period.

physical map has been developed (13), but numerous genes that are important in plant pathogenicity and taxonomy have not been placed on the map. Groups of linked genes may be established by randomly inserting Tn*5* into the chromosome and determining movement of the genes along with kanamycin resistance. Possibly the linked genes could aid in the sequential location of DNA fragments after digestion of genomic DNA by rare-cutting endonucleases and Southern hybridization with the genes. This procedure aided in the sequential location of two important gene clusters in this work.

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