Plasmid-Mediated Resistance to Streptomycin in *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

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Fifty-five percent of 548 strains of *Xanthomonas campestris* pv. *vesicatoria* from pepper and tomato grew on media containing 100 μg/ml of streptomycin sulfate. A DNA library of resistant strain BV5-4a was constructed in the cosmid vector pLAFR3. A clone containing an insert of 17 kb conferred resistance to streptomycin after conjugative transfer to a sensitive strain of *X. c. vesicatoria*. The 4.9-kb insert DNA of a subclone encoding wild-type resistance to streptomycin was labeled and used as a probe in Southern hybridization analyses. The streptomycin-resistance locus was found to be on a plasmid of 68 kb. The subclone DNA hybridized with enzyme-digested genomic or plasmid DNA of three of 17 other resistant strains of *X. c. vesicatoria* as well as with the DNA from streptomycin-resistant strains Psp 34 and Psp 36 of *Pseudomonas syringae* pv. *papulans*. No hybridization occurred with DNA from *Erwinia amylovora* strain UCBPP 829 or *P. cichorii* strain Pc 83-1, which also are resistant to streptomycin.

The use of the antibiotic streptomycin to control plant diseases caused by prokaryotes began in the late 1950s and was soon followed by the development of streptomycin resistance in target pathogens. Resistance to streptomycin has been reported in strains of the fire blight pathogen *Erwinia amylovora* (2,7,20), in several pathovars of *Pseudomonas syringae* (5,11,13,19,26), and also in strains of *Xanthomonas campestris* pv. *dieffenbachiae* (16). Some of the earliest reports describing the occurrence, frequency, and distribution of streptomycin resistance in a plant-pathogenic bacterium are concerned with the causal organism of bacterial spot disease in pepper and tomato, *X. c. vesicatoria* (21,23,24). Strains of streptomycin-resistant *X. c. vesicatoria* were able to grow in media amended with streptomycin sulfate at concentrations ranging from 100 to 400 μg/ml, and no significant control of the bacterial spot disease was observed when plants inoculated with resistant bacteria were sprayed with the antibiotic at these concentrations (21,24).

Resistance to streptomycin develops in one of two general ways at the cellular level—either by alteration of the binding affinity of ribosomal proteins for the antibiotic by chromosomal mutations, or by modification or destruction of the antibiotic by periplasmic enzymes commonly encoded by plasmid-borne genes (10,14). Evidence exists for both chromosomal (20) and plasmid-mediated (5) streptomycin resistance in plant-pathogenic prokaryotes.

The purpose of this study was to characterize a diverse collection of strains of *X. c. vesicatoria* with respect to frequency and levels of resistance to streptomycin and to obtain a basic understanding of the genetic mechanisms associated with the resistance phenotypes present.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Initially, 548 strains of *X. c. vesicatoria* isolated from many sources in different years and obtained from various geographic regions (Table 1) were screened for resistance to streptomycin. Bacteria were recovered from long-term storage in sterile tap water or lyophilized skim

milk. All strains were tested for pathogenicity to pepper (*Capsicum annuum* L.) or tomato (*Lycopersicon esculentum* Mill.) by infiltration of leaves with suspensions of approximately 10^8 colony-forming units (cfu)/ml in sterile tap water with a hypodermic needle and syringe. Strain UCBPP 829 of *E. amylovora* was supplied by Dr. M. N. Schroth, University of California, Berkeley, and *P. s. papulans* strains Psp 34 and Psp 36 were supplied by Dr. T. J. Burr, New York Agricultural Experiment Station, Geneva. Bacteria were subcultured on nutrient agar (NA) and tested for streptomycin resistance on NA supplemented with filter-sterilized streptomycin sulfate at a concentration of 100 μg/ml.

Levels of resistance to streptomycin were determined for a subset of strains of *X. c. vesicatoria* chosen from the initial collection, along with several other streptomycin-resistant plant pathogenic bacteria (Table 2). Fresh suspensions of bacteria in sterile tap water at concentrations of 3–5 × 10^8 cfu/ml were incubated at room temperature without shaking for 16 hr and then spread evenly (50 μl/plate) onto the surface of NA plates containing streptomycin sulfate at concentrations ranging from 25–1,000 μg/ml. The plates were incubated at 28 C for 3–5 days and rated for bacterial growth compared to that on unamended NA.

Some strains were screened for resistance to other aminoglycoside-aminocyclitol antibiotics by placing antibiotic test disks (Sensi-Disc, BBL Microbiology Systems, Cockeysville, MD) onto 1% water-agar overlays of the test bacteria on NA and recording the presence or absence of inhibition zones after incubation of the plates. Additional antibiotics tested and their concentrations were kanamycin (5 and 30 μg/ml), neomycin (5 and 30 μg/ml), gentamycin (10 μg/ml), tobramycin (10 μg/ml), and spectinomycin (25 μg/ml).

Isolation of DNA and recombinant-DNA techniques. Indigenous plasmid profiles for bacterial strains were determined by agarose gel electrophoresis of native plasmid DNA extracted by a modification of the alkaline lysis method of Kado and Liu (15). Lysis of bacterial cells was performed at 30 C for 20 min in a solution of 50 mM Tris containing 0.57 M sodium chloride, 0.04 M sodium hydroxide, and 3% (w/v) sodium dodecyl sulfate. The subsequent cell lysates containing the DNA were extracted with two volumes of phenol-chloroform-isoamyl alcohol (25:24:1) prior to electrophoresis. Plasmids were sized based on relative mobility in 0.5% agarose gels in Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) at 5 V/cm (18) with the plasmids of *Erwinia stewartii* strain SW2 (6) as molecular weight markers.

Plasmid DNA for restriction enzyme analysis, cloning, and hybridization experiments was obtained by alkaline lysis extraction and purification (18). Total genomic DNA for construction of a cosmid library and for hybridization analyses was prepared by described techniques (4). DNA from *X. c. vesicatoria* strain BV5-4a was partially digested with *Sau*3A. Fragments of 15–25 kb were then purified from an agarose gel by the freeze-squeeze method (25) and ligated into the *Bam*HI cloning site of the cosmid cloning vector pLAFR3 (22). The ligated recombinant DNA was packaged in vitro with a DNA-packaging kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and then transduced into *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, MD). The library was subcultured at 37 C and stored at 4 C on plates of Luria-Bertani agar (18) containing 10 μg/ml of tetracycline. Subsequent restriction enzyme analysis and cloning manipulations were accomplished by standard techniques (18).

Clones of DNA maintained in *E. coli* were mobilized individually into a rifamycin-resistant mutant of strain 75-3 of *X. c. vesicatoria* by triparental matings on NYGA media (9) with the helper plasmid pRK2013 (12). Transconjugants were transferred individually to plates of NA containing 100 μg/ml of streptomycin sulfate to screen for clones carrying the gene(s) for resistance to this antibiotic.

Hybridization and Southern blot analyses. Unrestricted plasmid DNA and restriction enzyme-digested plasmid and total genomic DNAs were electrophoresed in 0.5% agarose gels and transferred to BA85 nitrocellulose membrane filters (Schleicher and Schuell, Inc., Keene, NH) for hybridization by standard techniques (18). Sizes of DNA fragments and hybridized bands in Southern analyses were based on relative mobilities of *Eco*RI-*Hind*III digested bacteriophage λ DNA as markers. Insert DNA used for labeling was obtained from the clone by enzyme digestion and extraction from gels by the freeze-squeeze method after electrophoresis (25). Nonradioactive DNA labeling and detection were accomplished with the Genius kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) by following the manufacturer's protocol. Random primed linear DNA of clones and sized insert fragments were labeled with digoxigenin-dUTP and hybridized to target DNA immobilized on the nitrocellulose membranes. DNA hybrids were detected by enzyme immunoassay. Hybridizations were performed at 68 C for 18–24 hr after an initial 4-hr prehybridization of the membranes at the same temperature. Two final posthybridization washes of the membranes were completed at 65 C in a solution of 0.5× SSC (18), containing 0.1% (w/v) sodium dodecyl sulfate.

Self-transmissibility of plasmids. Streptomycin-resistant strains BV5-4a and 85-10 were mated with streptomycin-sensitive, rifamycin-resistant mutants of strains 75-3 and 87-7 in nutrient broth cultures and on NA plates. Approximately equal numbers of donor and recipient bacterial cells were mixed together as overnight broth cultures or as patches on plates and allowed to grow for 24 hr at 28 C. Suspensions and dilutions of bacteria from the matings were spread onto plates of NA containing both 100 μg/ml of streptomycin sulfate and 50 μg/ml of rifamycin to select for transconjugants. Transfer of plasmids by conjugation was determined by comparing the plasmid profiles of suspected transconjugants with those of the parent strains.

RESULTS

Resistance of bacterial strains to streptomycin. All strains of *X. c. vesicatoria* used were pathogenic on their respective host plants. In the initial screening for resistance to 100 μg/ml of streptomycin sulfate, 55% of the 548 strains tested were resistant. The frequency of resistant cultures varied relative to geographic region of isolation (Table 1). A low percentage of streptomycin resistance occurred among strains isolated from Australia, New Zealand, and Taiwan, whereas the percentages of streptomycin-resistant isolates obtained from the South American countries of Argentina and Brazil were higher, although not as high as was noted for Florida.

A wide range of resistance phenotypes was observed in a

TABLE 1. Frequency of streptomycin-resistant cultures in a diverse collection of *Xanthomonas campestris* pv. *vesicatoria* strains relative to geographic origin

Origin of strains[a]	No. tested	Percentage resistant[b]
USA		
Florida	379	60
Ohio	16	100
Georgia	9	22
California	4	75
Louisiana	2	0
Pennsylvania	1	100
Indiana	1	0
Hawaii	1	0
Other countries		
Argentina	87	48
Taiwan	29	6
Brazil	9	33
Australia	6	0
New Zealand	1	0
Venezuela	1	0
El Salvador	1	0
Tonga	1	100
Total Collection	548	55

[a] Cultures of *X. c. vesicatoria* were obtained from many sources from 1961 to present.

[b] Percentage of strains resistant to 100 μg streptomycin sulfate per milliliter of nutrient agar.

representative group of strains (Table 2). No growth was evident when streptomycin-sensitive strains were incubated on plates of NA with 25 μg/ml of streptomycin sulfate. In general, strains resistant to the antibiotic could be classified into three phenotypic groups based on degree of resistance to streptomycin at various concentrations. Resistance levels of the phenotypes were designated as low (<400 μg/ml), intermediate (400–800 μg/ml), and high (>800 μg/ml) (Table 2).

With one exception, no definite pattern of association was evident between the occurrence of the resistance phenotype in strains and the host, location of original isolation, or plasmid profile. A direct correlation between resistance or sensitivity to streptomycin and the presence or absence, respectively, of a unique plasmid was observed in a group of strains, prefixed BV, which were isolated in Bella Vista, Argentina, in 1987. Based on these initial observations, strain BV5-4a was chosen as the source of DNA for the construction of our cosmid clone library.

Genomic library and hybridization analyses. Two of 943 transconjugants from triparental matings of the BV clone library with the recipient strain of *X. c. vesicatoria* conferred resistance to streptomycin. These were designated pBV924 and pBV932. The 17-kb insert in clone pBV924 was partially digested with *Sau*3A, and fragments of various sizes were ligated into the *Bam*HI cloning site of pLAFR3. A 4.9-kb subclone (pBV10) coded for resistance to streptomycin in strain DH5α of *E. coli* (25 μg/ml) and strain 75-3 of *X. c. vesicatoria* (300 μg/ml), but did not code for resistance to any of the other aminoglycoside-aminocyclitol antibiotics.

The labeled insert DNA of pBV10 hybridized with a 6.3-kb *Eco*RI-*Hind*III fragment of digested plasmid DNA isolated from strain BV5-4a (Fig. 1, Lane B). Hybridization also occurred with the *Eco*RI-digested total genomic DNA of three of 17 resistant and none of four sensitive strains of *X. c. vesicatoria* (Table 2). One of the three strains, 79-2, was isolated in Argentina in 1979 and was identical in terms of plasmid profile to BV5-4a, which was isolated in 1987. Homologous DNA to the probe occurred in both plasmid and chromosomal DNA in strain 85-10. Two fragments of 6.7 and 3.1 kb hybridized in the *Eco*RI-*Hind*III plasmid DNA digest (Fig. 2, Lane B), whereas an additional hybrid band of 4.9 kb appeared in the total genomic DNA digest with the same restriction enzymes for this strain (Fig. 2, Lane C).

TABLE 2. Levels of streptomycin resistance and hybridization of cloned genes for streptomycin resistance to xanthomonads and other bacteria

Strain[a]	Origin	Host	Resistance phenotype[b]	Hybridization[c]
Xanthomonas campestris pv. *vesicatoria*				
Streptomycin-resistant				
BV5-4a	Argentina	tomato	low	+p
88-4	Brazil	tomato	high	−
87-80	Florida	tomato	low	−
87-48	Taiwan	pepper	int.	−
87-77	Ohio	tomato	high	+g
86-2	Florida	tomato	low	−
85-10	Florida	pepper	high	+g,p
83-12	Florida	tomato	low	−
79-2	Argentina	tomato	low	+p
77-8	Florida	tomato	int.	−
76-2	Florida	pepper	high	−
73-1	Florida	tomato	high	−
70-10	Florida	tomato	high	−
69-13	Florida	tomato	high	−
67-11	Florida	tomato	low	−
65-11	Florida	tomato	high	−
61-38	Florida	tomato	high	−
61-3	Florida	tomato	low	−
Streptomycin-sensitive				
89-8	Australia	pepper		−
87-7	Florida	pepper		−
82-8	Florida	pepper		−
75-3	Florida	tomato		−
Other pathogens				
UCBPP 829	California	pear	high	−
Psp 34	New York	apple	low	+p
Psp 36	New York	apple	low	+p
Pc 83-1	Florida	Hibiscus	low	−

[a] Cultures of *X. c. vesicatoria* were from the University of Florida collection; *Erwinia amylovora* strain UCBPP 829 was supplied by Dr. M. N. Schroth; and *Pseudomonas syringae* pv. *papulans* strains Psp 34 and Psp 36 were contributed by Dr. T. J. Burr.

[b] Phenotypes represent the concentration of streptomycin sulfate at which the first noticeable inhibition of cell growth occurred, to the concentration where growth was completely inhibited. Low levels were <400, intermediate levels (int.) were between 400–800, and high levels were >800 μg streptomycin sulfate per milliliter of nutrient agar.

[c] Hybridization observed (+) or not observed (−) when plasmid and total genomic DNA (p and g, respectively) were probed with the labeled 4.9-kb insert DNA of pBV10, which contains the gene(s) for streptomycin resistance in strain BV5-4a.

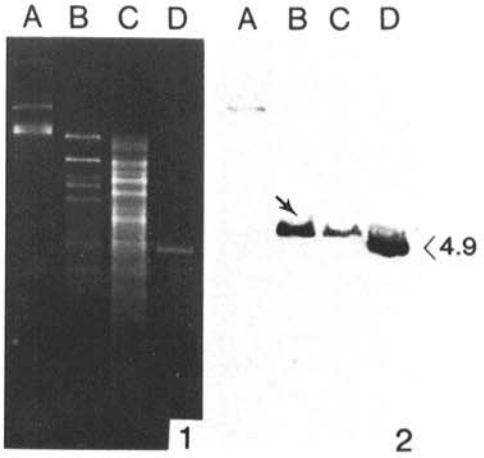

Fig. 1. Hybridization of the 4.9-kb *Eco*RI-*Hind*III insert DNA of pBV10 with DNA from streptomycin-resistant strain BV5-4a of *Xanthomonas campestris* pv. *vesicatoria*. **1,** Ethidium bromide-stained gel showing the unrestricted plasmid profile of strain BV5-4a (lane A), *Eco*RI-*Hind*III digested plasmid DNA (lane B), *Eco*RI-*Hind*III digested total genomic DNA (including plasmid DNA) (lane C), and the purified *Eco*RI-*Hind*III insert of pBV10 (lane D). **2,** A Southern blot of the gel in panel 1 probed with labeled pBV10 insert DNA. The 6.3-kb hybridization band found in strain BV5-4a is marked with an arrow for references to size.

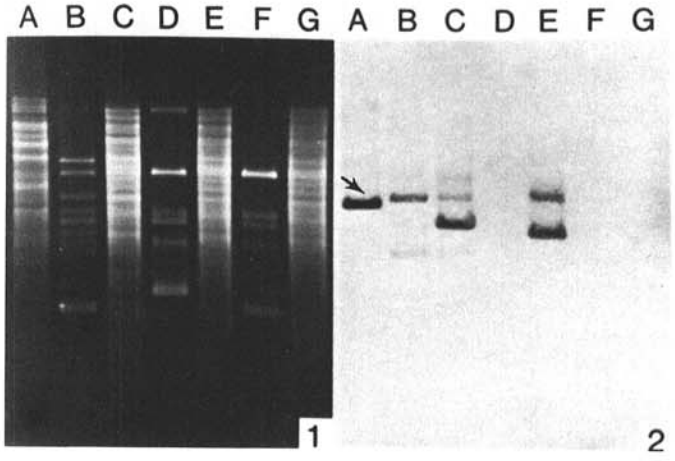

Fig. 2. Hybridization of the 4.9-kb *Eco*RI-*Hind*III insert DNA of pBV10 with DNA from various streptomycin-resistant (str^r) and streptomycin-sensitive (str^s) strains of *Xanthomonas campestris* pv. *vesicatoria*. **1,** Ethidium bromide-stained gel showing *Eco*RI-*Hind*III digestions of total genomic DNA (including plasmid DNA) of strain BV5-4a (str^r) (lane A), plasmid DNA (lane B) and total genomic DNA (lane C) of strain 85-10 (str^r), plasmid DNA (lane D) and total genomic DNA (lane E) of strain 87-77 (str^r), and plasmid DNA (lane F) and total genomic DNA (lane G) of strain 75-3 (str^s). **2,** A Southern blot of the gel in panel 1 probed with labeled pBV10 insert DNA. The 6.3-kb hybridization band found in strain BV5-4a is marked with an arrow for references to size.

When the unrestricted plasmid DNA of strains BV5-4a and 85-10 were probed, the gene(s) for resistance to streptomycin were localized to plasmids of 68 and 15.9 kb, respectively. No detectable hybridization occurred between the labeled probe and the *Eco*RI-*Hind*III restricted plasmid DNA of strain 87-77 (Fig. 2, Lane D), but two fragments of 6.7 and 4.1 kb hybridized in the total genomic DNA digest of the same strain (Fig. 2, Lane E).

The DNAs of streptomycin-resistant strains of other plant pathogenic bacteria were probed with the labeled DNA of pBV10. Four hybridizing fragments were found in *Eco*RI-*Hind*III digests of both the plasmid and total genomic DNA of two strains of *P. s. papulans* (Psp) (Fig. 3). Three fragments of 9.7, 7.1, and 3.3 kb were common to both strains, while unique fragments of 10.9 and 8.0 kb hybridized in strains Psp 34 and Psp 36 (Fig. 3, Lanes B/C and D/E, respectively). Southern blot analysis with unrestricted plasmid DNA of these strains revealed that the pBV10 probe hybridized with three and two plasmids, respectively, including the conjugative plasmid pCPP501 previously associated with streptomycin resistance in this pathogen (5). No hybridization was observed when the *Eco*RI-*Hind*III digests of total genomic DNA of *E. amylovora* strain UCBPP 829 or *P. cichorii* Pc 83-1 were probed (Fig. 3, Lanes G and F, respectively). No indigenous plasmids were evident in these strains.

Self-transmissibility of *X. c. vesicatoria* plasmids. Transmission of the plasmids carrying the gene(s) for resistance to streptomycin in strains BV5-4a and 85-10 was not achieved in matings when both strains were used as donors with either strain 75-3 or 87-7 as the recipient. The limits of detection for self-transmission were near 10^{-9} transconjugants per donor cell. The mutation frequency for streptomycin resistance in a strain of *X. c. vesicatoria* was earlier determined to be near 1.9×10^{-9} per cell per division (8).

DISCUSSION

As part of an extensive study to characterize a 30-yr collection of cultures of *X. c. vesicatoria* with respect to pathogenicity, race found on pepper and tomato, resistance to copper, and indigenous plasmid profile, a total of 548 pathogenic strains were tested for resistance to streptomycin. Although no definite associations were observed between streptomycin resistance and host, year of isolation, or plasmid profile among strains from Florida and other states in the United States, the frequency of streptomycin-resistant strains did appear to vary according to geographic location. In regions of the world where the use of streptomycin as a control agent has been relatively intense, such as Florida and Argentina, a high percentage of the strains tested were resistant to the antibiotic.

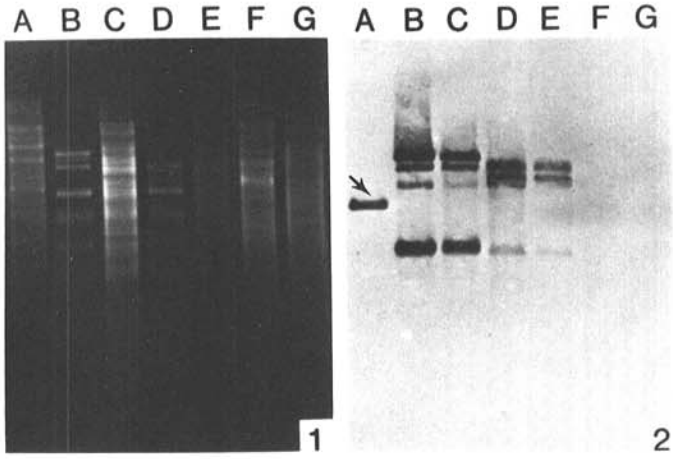

Fig. 3. Hybridization of the 4.9-kb *Eco*RI-*Hind*III insert DNA of pBV10 with DNA of various streptomycin-resistant plant-pathogenic bacterial strains. **1,** Ethidium bromide-stained gel showing *Eco*RI-*Hind*III digestions of total genomic DNA (including plasmid DNA) of *Xanthomonas campestris* pv. *vesicatoria* strain BV5-4a (lane A), plasmid DNA (lane B) and total genomic DNA of *Pseudomonas syringae* pv. *papulans* (Psp) strain Psp 34 (lane C), plasmid DNA (lane D) and total genomic DNA of strain Psp 36 (lane E), and total genomic DNA of *P. cichorii* strain Pc 83-1 (lane F) and *Erwinia amylovora* strain UCBPP829 (lane G). **2,** A Southern blot of the gel in panel 1 probed with labeled pBV10 insert DNA. The 6.3-kb hybridization band in strain BV5-4a is marked with an arrow for references to size.

In an examination of streptomycin-resistant strains of *X. c. vesicatoria* isolated in Bella Vista, Argentina, the locus for resistance to this antibiotic was determined to be maintained on a unique 68-kb plasmid in the bacterial population. Plasmid-mediated resistance is common in naturally occurring bacteria associated with animals, particularly in areas where the antibiotic is used extensively (14). However, in plant-associated bacteria plasmid-mediated resistance to streptomycin has only recently been reported (5). In the majority of cases studied, plasmid-mediated resistance to aminoglycoside-aminocyclitol antibiotics, such as streptomycin, is due to synthesis of inactivating enzymes and is at a lower level than resistance by single-step chromosomal mutations which encode for altered ribosomes (10). Low-, intermediate-, and high-level chromosomal resistance to streptomycin nevertheless was reported in natural and laboratory-derived mutants of *E. amylovora* (2,7,20).

At the present time it is difficult to associate plasmid or chromosomal genes with the level of resistance to streptomycin in strains of *X. c. vesicatoria*. The DNA of some strains with low levels of resistance did not hybridize with our probe, and at least one strain with a high level of resistance did hybridize with our probe. Although in the future it may be possible to relate a level of streptomycin resistance to plasmid or chromosomal genes, the genes conferring the other types of resistance need to be cloned and their loci determined before attempting this relationship.

Southern blot analyses with labeled insert DNA of pBV10 revealed that at least two genetic-based systems of resistance to streptomycin exist among strains of *X. c. vesicatoria*. Further experiments are necessary to determine if the resistance to streptomycin in strains which did not hybridize with our probe is due to chromosomal mutations, to changes in cell permeability, or perhaps to the synthesis of a different antibiotic-modifying enzyme. The cloning of additional resistance genes from DNA libraries of other strains is essential for a definitive clarification of the systems involved with streptomycin resistance in *X. c. vesicatoria*.

Homologous DNA in both plasmid and chomosomal DNA of strain 85-10 and in the chromosomal DNA of strain 87-77 might be evidence for integration of plasmid-based streptomycin resistance into the chromosome. Genes for resistance to the aminoglycoside-aminocyclitol antibiotics, two reported for streptomycin, have been located on transposable elements (1,17). Further genetic analyses of the cloned gene(s) are needed to determine the exact base sequence of the functional unit and possible relationship with previously characterized transposable elements.

The locus for gene(s) that determines streptomycin resistance in strains BV5-4a and 87-77 can be associated with the plasmid and chromosomal DNA, respectively, but a definite determination cannot be made concerning strain 85-10 with the information gathered in our study. Whether the active gene(s) are localized on the plasmid, chromosome, or on both in strain 85-10 may be determined in future gene (marker) exchange experiments.

It was interesting to find that the DNA of pBV10 hybridized with the conjugative plasmids carrying streptomycin resistance in *P. s. papulans* strains Psp 34 and Psp 36, as well as with an additional plasmid in each strain of an undetermined conjugative nature. Whereas the plasmids carrying the streptomycin resistance gene(s) in strains BV5-4a and 85-10 appeared to be nonconjugative in our experiments, it is highly probable that these genes occur in the natural population of *X. c. vesicatoria* in association with other plasmids of a conjugative nature.

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