Stability of Plasmid-Borne Antibiotic Resistance in Xanthomonas vesicatoria in Infected Tomato Leaves

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ABSTRACT

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Plasmids RP4 and RK2 were maintained by *Xanthomonas vesicatoria* in infected tomato leaves which were kept up to 6 mo under a variety of environmental conditions. Only a small percentage (0 to 12%) of bacteria recovered from leaves of plants kept at 27 C or from detached leaves which were kept moist at 5 C for up to 6 wk after inoculation had lost the plasmids. Similary, 94 to 100% of the surviving bacteria in dried leaves stored at 5, 20, 35, and 45 C for up to 6 mo, retained the plasmids. In contrast, the plasmids were lost with high frequency from bacteria kept on slants at the above temperatures. These results indicate that the epidemiological stability of R plasmids in plant pathogenic bacteria may not be easily predictable on the basis of their stability in culture media. The apparent stability of R plasmids in plants infected with bacterial pathogens or plant debris in the absence of antibiotic selective pressure can contribute to their long-term maintenance.

Additional key words: bacterial leaf spot of tomato, bacterial genetics.

The R plasmids, a class of self-replicating extrachromosomal deoxyribonucleic acid (DNA) molecules that often are transmissible between bacterial cells by conjugation, play a major role in the development of antibiotic resistant strains of bacteria (14, 15, 18). Transmission of several different R plasmids to plant pathogenic Gram-negative bacteria in vitro as well as in plant tissues has been reported (2, 3, 4, 5, 9, 10, 11, 17). Similarly, naturally occurring streptomycin-resistant strains of certain phytopathogenic bacterial species have been isolated in the field (8, 13, 19). In one case, such strains have been found to contain one or more plasmids (S. Hua and N. J. Panopoulos, *unpublished*), but a relationship between these plasmids and streptomycin resistance has not been demonstrated.

Certain antibiotics are currently used for plant disease control (8, 13, 19, 20). Antibiotics prevent frost damage in plants by reducing the populations of epiphytic bacteria on leaf surfaces (12) and this may result in wider use of these compounds. The ability of plant pathogenic bacterial species to acquire R plasmids by conjugation and the prevalence of such plasmids, raise the question whether or not they may become a permanent genetic component in natural populations of phytopathogens. To answer this question we need to know, among other things, how stable different R plasmids are in phytopathogens under natural conditions. Laboratory experience indicates that plasmids are lost spontaneously from plant pathogenic bacteria in culture in the absence of antibiotic selection, but this varies widely depending on the plasmid-bacterium combination (2, 3, 4, 6, 8, 10, 17). It is not known, however, to what extent stability of R plasmids in culture and in nature are correlated.

Previous experience has shown that plasmids RP4 and RK2 are lost at moderate to high frequencies from *Xanthomonas vesicatoria* XV5113-1 upon storage on antibiotic-free medium (10). The purpose of this study was to investigate the stability of these two R plasmids in this bacterium following host inoculations and storage of infected plant materials under a variety of environmental conditions.

MATERIALS AND METHODS

Media and antibiotics.—Difco nutrient agar (NA) was used throughout the study. Streptomycin sulfate (Eli Lilly and Co., Indianapolis, IN 46206), was added to NA at 500 μg/ml (SNA). Disodium carbenicillin (Pfizer, Inc., New York, NY 10016) was filter-sterilized and added to autoclaved and cooled NA or SNA at 500 μg/ml to make carbenicillin nutrient agar (CNA) and double antibiotic medium (DAM), respectively. Cultures were stored on yeast dextrose carbonate agar (YDC) containing 10 g/liter yeast extract, 20 g/liter glucose, 20 g/liter $CaCO_3$ and 20 g/liter agar, and DAM slants. Novick-Richmond 0.3 CY medium (16) was used for identification of penicillinase-producing clones. Penicillin G (Calbiochem., San Diego, CA 92037) was used in the penicillinase assay.

Bacterial strains.—*Escherichia coli* strain C-3, defective for DNA restriction and modification (r^-, m^-) and requiring arginine (Arg^-) was obtained from R. Calendar, Department of Molecular Biology, University of California, Berkeley, CA 94720. All other strains used in this work were derived from *Xanthomonas vesicatoria* XV5113, a wild-type strain isolated from tomato. Strains XV5113(RP4) and XV5113(RK2), carrying the plasmids

RP4 and RK2, respectively, which specify resistance to carbenicillin (Cb^r), neomycin (Nm^r)/kanamycin (Km^r), and tetracycline (Tc^r), were obtained by conjugation (mating) as described elsewhere (10). A streptomycin-resistant strain XV5113-1, presumably due to a mutation on the chromosome, was developed by irradiating wild-type cells with ultraviolet light as described previously (10). This mutant, like its parent strain XV5113, was pathogenic on tomato, hydrolyzed starch, and produced yellow-mucoid colonies on NA medium. Matings between XV5113(RP4) or XV5113(RK2) and XV5113-1 resulted in transconjugants XV5113-1(RP4) and XV5113-1(RK2), resistant to the above antibiotics and to streptomycin. All R^+ cultures were maintained on DAM.

Plant inoculations.—Three-wk-old tomato plants *(Lycopersicon esculentum* L. 'Ace') grown in 13-cm diameter pots containing UC mix (1) at 22-28 C in the greenhouse, were inoculated with 48- to 72-hr cultures of XV5113-1(RP4) and XV5113(RK2) grown on DAM. For inoculation, the bacteria were suspended from 24-hr slants in 5 ml of sterile distilled water at a concentration of 10^8 to 10^9 cells/ml and applied with a cotton swab on the upper surface of leaves dusted with Carborundum. The inoculated plants then were placed under mist (15 sec at 5-min intervals) for 4 hr each day. Small water-soaked lesions which became small brown-to-black scabby spots surrounded by a yellow zone (typical of bacterial spot) developed on the inoculated leaves 7-10 days after inoculation.

Storage of plant material and slant cultures.—The stability of the R plasmids was tested as follows: (i) infected leaves were left intact on growing plants in the greenhouse; (ii) infected leaves were excised from the plants, wrapped in a moist paper towel, placed in a plastic bag, and stored at 5 C; (iii) infected leaves were excised from the plants, air-dried overnight at room temperature, and stored in paper bags at 5, 20, 35, and 45 C. Cultures of XV5113-1(RP4) and XV5113-1(RK2) were stored on slants with (DAM) and without (YDC) antibiotics at the above temperatures for in vitro stability tests. In each case, samples were taken weekly for assaying.

Transmission of (Cb^r) from nonsegregating clones obtained from lesions.—Carbenicillin-resistant clones obtained from dried leaves were mated with restriction-defective *Escherichia coli* C-3 as follows: one drop of cell suspension of donor and recipient strain, each containing approximately 10^7 to 10^8 cells/ml, were spotted together but not spread on selective CNA agar plates. The plates were allowed to dry (1-2 hr) and then were transferred to a 42 C incubator. These conditions counterselect the donors by their inability to grow at 42 C while allowing Cb^r *E. coli* recipients to grow.

Transconjugant colonies were further examined for penicillinase production as described later.

Recovery of bacteria from plant tissue.—Bacterial cell viability as well as R plasmid retention were assayed as follows: leaves maintained under different conditions were sampled at weekly intervals by excising single lesions. The excised lesions were ground in a sterile mortar in 2 ml of sterile distilled water, and serial dilutions were prepared to yield 100 to 500 colony-forming units (cfu) per plate of SNA medium. Bacterial colonies also were examined for penicillinase activity on Novick-Richmond medium (10, 16) supplemented with 500 μg/ml streptomycin to minimize contamination. After incubation at room temperature for 48-72 hr, plates were flooded with 1.5 ml of a 0.25% (w/v) solution of N-phenyl-1-napthylamine-azo-*o*-carboxybenzene (PNCB, Gallard-Schlesinger Chem. Mfg. Corp., Long Island, NY 11514) prepared in N,N-dimethylformamide with 6% (v/v) 1.0 N NaOH, allowed to dry, and flooded again with 0.1 to 0.2 M solution of penicillin G (10). Penicillinase-producing clones turned the acid-base indicator (PNCB) around them from orange to purple, whereas penicillinase-negative clones remained orange. The change in color was taken as presumptive evidence for plasmid retention. Spot checks for Km and Tc resistance and for transmissibility of the resistance markers by conjugation also were conducted using *E. coli*, C-3.

RESULTS

Presence of the carbenicillin resistance (Cb^r) character in X. vesicatoria recovered from intact and from detached moist-stored leaves.—The frequency of penicillinase-positive (Cb^r) bacteria in single lesions was high throughout the 6-wk period, fluctuating between 96 to 100% for XV5113-1(RK2) and between 90 to 100% for XV5113-1(RP4) (Table 1). Survival of bacteria in the lesions also was high (Table 1) but the total number of viable bacteria differed greatly from one lesion to another and accurate estimates of viability during the period of observations could not be made. Similar results both as to frequency of Cb^r cells and viability were obtained with lesions from leaves stored at 5 C for 6 wk. The frequency of penicillinase-positive cells in this case varied from 95 to 100% (61/64 at 2 wk to 120/120 at 5 wk) for XV5113-1(RK2) and 88 to 96% (161/182 at 4 wk to 107/114 at 3 wk) for XV5113-1(RP4). The differences observed between the $RK2^+$ and $RP4^+$ strains in these experiments are not considered significant.

Presence of the Cb^r character in X. vesicatoria recovered from detached, dried, infected leaves stored at different temperatures.—Strains $RP4^+$ and $RK2^+$ retained the Cb^r character throughout the entire test period. The frequency of penicillinase-negative colonies ranged from 0-6%, with the higher values recorded during the first 3 wk of testing. The number of cfu per lesion from leaves stored at 5 and 20 C remained high throughout the testing period (2.2×10^4 to 1.8×10^7) (Table 2) but bacterial viability dropped significantly after 2-3 wk in lesions on leaves stored at 35 and 45 C (7.5×10^3 to 1×10^1 cfu/lesion).

Although bacterial populations dropped by 90-99% in lesions from leaves stored at 45 C after 2 to 3 wk, surviving bacteria were virtually 100% penicillinase-positive. No viable bacteria could be recovered from leaves which were stored for 26 wk at 35 and 45 C. At 20 C viable bacteria were recovered from leaves infected with XV5113-1(RK2), but not from those infected with XV5113-1(RP4), although recovery of both strains from leaves stored at 5 C was relatively high (2.2×10^3 to 2.6×10^4 cfu/lesion).

Stability of the Cb^r character in vitro.—Cultures of XV5113-1(RP4) and XV5113-1(RK2) stored in vitro on YDC and DAM under the same temperatures as the dried leaf material also were tested weekly for 6 wk and then at

26 wk for frequency of spontaneous loss of penicillinase. As expected, cultures stored on DAM exhibited no spontaneous loss of penicillinase. However, cultures stored at 45 C were dead after only 2 wk of storage and those stored at 35 C were dead after 6 wk of storage. Cultures stored on YDC agar slants exhibited the same viability characteristics as those stored on DAM, but frequencies of penicillinase-negative colonies were higher. During the first 2 wk, plasmid loss frequencies were similar to those obtained from samples taken from

TABLE 1. Loss of R plasmid in *Xanthomonas vesicatoria* in infected plants in the greenhouse at 27 C

Time after lesions develop (wk)	Plasmid	Colonies examined[a] (no.)	Colonies without R plasmid (%)	Survival (cfu[b] per lesion)
1	RP4	293	6	2.9×10^7
	RK2	262	4	2.6×10^6
2	RP4	494	10	4.9×10^6
	RK2	1563	3	1.5×10^7
3	RP4	248	6	2.1×10^5
	RK2	160	0	1.6×10^5
4	RP4	567	4	5.6×10^6
	RK2	1036	1	1.0×10^7
5	RP4	832	3	8.3×10^6
	RK2	303	2	3.0×10^6
6	RP4	38	0	3.8×10^5
	RK2	...	...	...

[a]Figures represent the total number of colonies examined from single lesions after serial dilutions.
[b]Abbreviation: cfu = colony-forming unit. Tests were replicated once and the figures reported are from tests yielding the greatest number of countable colonies per plate.

TABLE 2. Relation of temperature to loss of RP4 plasmid and cell viability of *Xanthomonas vesicatoria* in infected tomato leaves held in storage

Storage time (wk)	Storage temp (C)	Colonies examined[a] (no.)	Colonies without RP4 plasmid (%)	Survival (cfu[c] per lesion)
1	5	183	1	1.8×10^7
	20	211	2	2.1×10^6
	35	192	1	1.9×10^6
	45	136	0	2.5×10^5
3	5	161	1	1.6×10^7
	20	56	0	5.6×10^5
	35	520	2	5.0×10^6
	45	63	3	6.3×10^3
6	5	130	0	1.3×10^6
	20	60	0	6.0×10^6
	35	299	1	3.4×10^4
	45	40	1	4.0×10^2
26	5	218	4	2.2×10^4
	20	NG[b]	...	...
	35	NG	...	...
	45	NG	...	...

[a]These figures represent the total number of colonies examined from single lesions after serial dilutions. Tests were replicated once and the figures reported are from tests yielding the greatest number of countable colonies per plate.
[b]No growth.
[c]Colony-forming unit.

the various leaf materials and from the DAM slants (1% to 4%), but after 3 wk the frequencies of penicillinase loss increased significantly reaching 10% to 76% at 6 wk. Loss of penicillinase was virtually complete after 26 wk of storage on YDC.

Transmissibility of Cb^r from survivors.—Twenty-four colonies each of XV5113-1(RK2) and XV5113-1(RP4) recovered from dried leaf lesions donated Cb^r readily to *E. coli*, C-3. Transconjugants from each of the crosses produced penicillinase. The transmissibility of the Cb^r character from these cultures indicated that the original plasmids or portions thereof were maintained as such.

DISCUSSION

This is the first report concerning the genetic stability of R plasmids in phytopathogenic bacteria in plant tissues under natural conditions. Our studies showed that only a small percentage of *X. vesicatoria* cells recovered from infected tomato leaves lost the R plasmids either during growth and lesion development or during subsequent storage. The apparent stability of the plasmids in diseased tissue was in sharp contrast to the relatively high frequency of spontaneous loss of the plasmid by bacteria kept on YDC slants for the same period of time. It is important to note that the same strains were used in both series of experiments. It also may be significant that in culture, loss of the plasmids became greater with time, whereas in plant tissues the opposite was true. It is possible that these differences reflected different survival rates for R^+ and R^- cells under the conditions used.

Measurements of plasmid stability on the basis of maintenance of the Cb^r character would be open to question if the segment of plasmid DNA carrying the Cb^r gene(s) had transposed itself onto the chromosome. Since this situation is common with antibiotic resistance genes carried by plasmids [including the penicillinase gene found in RP4 (7)] we examined 48 cultures obtained from stored leaves for the ability to transmit carbenicillin resistance. If transposition had taken place, we would have expected the Cb^r character to become less efficient in transmissibility. Our findings, however, are consistent with the interpretation that Cb^r was maintained as part of the original plasmid.

Stability of R plasmids in *X. vesicatoria* in leaf tissues would not have been accurately predicted from their behavior in culture. It is important to understand how R plasmids will behave under natural conditions so that proper measures can be implemented in the event that a naturally-occurring pathogen containing an R plasmid is discovered. If the plasmid is not stable under natural conditions, then there is little need for unusual measures. However, if the plasmid is stable, as demonstrated in these experiments, and antibiotics are to be used in bacterial plant disease control, then management practices should be employed so that acquisition of R plasmids and season-to-season survival of R^+ strains may be kept to a minimum.

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