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Genetic Analysis of Nonpathogenic *Agrobacterium tumefaciens* Mutants Arising in Crown Gall Tumors

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Little is known about the effect of the host on the genetic stability of bacterial plant pathogens. Crown gall, a plant disease caused by *Agrobacterium tumefaciens*, may represent a useful model to study this effect. Indeed, our previous observations on the natural occurrence and origin of nonpathogenic agrobacteria suggest that the host plant might induce loss of pathogenicity in populations of *A. tumefaciens*. Here we report that five different *A. tumefaciens* strains initially isolated from apple tumors produced up to 99% nonpathogenic mutants following their reintroduction into axenic apple plants. Two of these five strains were also found to produce mutants on pear and/or blackberry plants. Generally, the mutants of the apple isolate D10B/87 were altered in the tumor-inducing plasmid, harboring either deletions in this plasmid or point mutations in the regulatory virulence gene *virG*. Most of the mutants originating from the same tumor appeared to be of clonal origin, implying that the host plants influenced agrobacterial populations by favoring growth of nonpathogenic mutants over that of wild-type cells. This hypothesis was confirmed by coinoculation of apple rootstocks with strain D10B/87 and a nonpathogenic mutant.

The virulence (*vir*) and transferred (T) regions of *Agrobacterium tumefaciens* Ti plasmids contain genes directly involved in plant transformation. Expression of the *vir* genes by the bacterium is induced through the action of the *virA-virG* two-component regulatory system in response to plant phenolic compounds such as acetosyringone. The T-DNA, produced from the T region at the direction of the *vir* genes, becomes integrated into plant chromosomal DNA, where it determines the synthesis of plant growth hormones responsible for crown gall tumor development. The T-DNA also confers on tumor cells the capacity to produce unusual metabolites called opines. One of these opines, nopaline, is synthesized through the reductive condensation of arginine and α-ketoglutaric acid. Other Ti plasmid-encoded genes, which are not transferred to plant cells, enable the bacterial pathogen to utilize opines for growth and tumor colonization (for reviews, see references 5, 6, and 20). In spite of the enhanced colonization potential associated with expression of the opine catabolic genes, pathogenic *Agrobacterium* strains are difficult to recover from certain types of tumors in which nonpathogenic, opine-utilizing agrobacteria often prevail (4, 14). To account for the frequent occurrence of nonpathogenic agrobacteria, it is proposed here that some plant infections with *A. tumefaciens* result in the modification of the bacterial genome. Conceivably, nonpathogenic mutants produced by *A. tumefaciens* in association with the host plant could be altered either in the *vir* or T region of the Ti plasmid or in the chromosome. Chromosomal genes that are required for efficient plant transformation include *chvE*, which encodes a protein that potentiates the effect of plant phenolic compounds on *vir* gene induction (11).

Our recent observations of the effects of acetosyringone and host plants on the occurrence of nonpathogenic mutants lent credence to the hypothesis of a plant-mediated effect on genetic stability of *A. tumefaciens* populations. In defined medium, acetosyringone inhibited the growth of *A. tumefaciens* C58 and T37 and favored the selection of opine-utilizing, nonpathogenic mutants. These mutants had lost the capacity to show the *vir* gene induction response, and their growth was no longer inhibited by acetosyringone. In most of the C58 mutants that were examined, *virA* or *virG* was inactivated by the integration of the endogenous insertion sequence element IS*426* (7, 8). To determine if the production of nonpathogenic mutants could also be observed in planta, various strains of *A. tumefaciens* were inoculated on apple plantlets grown aseptically in tissue culture. In a preliminary report, we showed that one of these bacteria, the apple isolate D10B/87, produced a high proportion of nonpathogenic mutants under such conditions (3). While these initial experiments suggested that the plant host influenced the genetic stability of associated agrobacterial populations, they provided no indication of the generality of this phenomenon and the nature of the observed mutations. The object of this study was to determine if other *A. tumefaciens* strains besides D10B/87 have the capacity to produce mutants in planta, investigate the nature of the D10B/87 mutants, and finally examine the possible role of plant-mediated selection in the appearance of nonpathogenic mutants.

MATERIALS AND METHODS

Bacteria. Agrobacteria were isolated from naturally occurring tumors on apple (for strains listed in the first column of Table 1) or cherry or blackberry (for the strains mentioned in footnote *a* to Table 1) plants. These bacteria were isolated on selective media or media containing opines (4) and characterized by standard techniques (12). Bacterial cultures were maintained on potato dextrose agar medium (Difco). Their purity was regularly verified by confirming 100 isolated colonies for pathogenicity on tomato plants (see below). Bacterial cultures in acidic (pH 5.8) minimal medium containing 60 μM acetosyringone were initiated from 10^7 cells, as described before (8). Minimal media used to grow the biotype 2 strain D10B/87 were supplemented with 0.1 μg of biotin per ml. Spontaneous mutants of strain D10B/87 with combined resistance to rifampin (Rif^r) (100 μg/ml), streptomycin (Sm^r) (500 μg/ml), and spectinomycin (Sp^r) (100 μg/ml) were obtained by sequential selection for growth in potato dextrose agar medium containing one of these antibiotics.

Plant tissue culture and inoculations. Micropropagated plants in tissue culture were apple (*Malus*) rootstocks 'Malling7', 'Mark', 'Ottawa3', 'P106', and 'Bud116'; cherry (*Prunus avium*) 'Mazzard'; wild Himalayan blackberry (*Rubus*

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discolor); peach (*Prunus persica*) 'Lovell'; and pear (*Pyrus communis*) 'Old Home Farmingdale'. Tissue culture medium was varied for the different plant species. Murashige and Skoog (MS) medium (13) was used for peach cultures, and a modification of MS medium containing 0.4 mg of thiamine per liter was used for blackberry cultures. For apple, pear, and cherry cultures, the medium used contained the macronutrients of Quoirin et al. (16) along with MS medium micronutrients except that 10.5 mg of $ZnSO_4$ per liter, 0.016 mg of $CuSO_4$ per liter, and 2.5 mg of thiamine per liter were included. Axenic plants were maintained in a growth chamber at 25°C with a 15-h photoperiod. Uninoculated plants grown in tissue culture were systematically examined for the absence of contamination by depositing parts of these plants on a solid medium suitable for general bacterial growth. This medium was based on the minimal medium supplemented with 10 g of mannitol per liter of Hooykaas et al. (10) but was modified by omitting KNO_3 and including 2 g of glutamate per liter and 0.4 g of yeast extract per liter. For inoculation of the plants grown in tissue culture, 10^8 to 10^9 cells of the test agrobacterial strain were recovered from a bacterial colony with a needle and introduced in the stem by wounding. Inoculated plants were returned to in vitro incubation conditions for 1 to 2 months. Two methods, designated conservative sampling and destructive sampling, were used alone or sequentially to sample the tumors which had developed during the incubation period. For conservative sampling, a tumor was rinsed twice with 150 μl of physiological saline (0.85% NaCl). In destructive sampling, the same tumor was excised and crushed in 0.5 ml of physiological saline. The two resulting suspensions, obtained by conservative and destructive sampling, were diluted separately, and appropriate dilutions were plated on bacterial medium. For each particular suspension, 100 of the colonies which subsequently developed were tested for pathogenicity on tomato plants. All mutants examined in this work and recovered from a given rootstock-bacterium combination were obtained from the same tumor. For some inoculations with strain D10B/87, a spontaneous Rif^r Sm^r mutant was used, which facilitated verification of origin of the bacterial clones recovered from the sampled plants. For conciseness, further mention of the spontaneous antibiotic resistances harbored by certain derivatives of strain D10B/87 will be made only when this information will be experimentally relevant.

In planta competition experiments involved inoculation of Ottawa3 apple rootstocks in tissue culture with a mixed inoculum containing two different bacteria. Both test bacteria were resistant to rifampin and streptomycin, but one could be distinguished from the other on the basis of additional spectinomycin resistance. Cultures of the two test strains grown overnight were prepared in potato dextrose broth (Difco) and adjusted to an optical density at 600 nm of 1.2. The two cultures were mixed in the desired proportion (the spectinomycin-resistant bacterium representing about 50, 90, or 99% of the total cell population), and the resulting suspension was centrifuged. The pellet was resuspended in 50 μl of potato dextrose broth. The resulting bacterial paste was used for stem inoculations with a sterile needle. Two apple plants were inoculated with each of the dual bacterial suspensions. At intervals, inoculated plants were sampled by the conservative sampling method described above. Appropriate dilutions of the initial inoculum and washings from conservative samplings were plated on solid medium, and in each case, the resistance of 100 colonies to rifampin, streptomycin, and spectinomycin was verified. Results from the two plants inoculated with the same bacterial combination were pooled. As a control, dual bacterial suspensions (in which the spectinomycin-resistant strain represented 50% of the total cell population) were deposited on tissue culture medium in the absence of a host plant. In this case, samples were taken by recovering part of the bacterial mass with a loop.

Characterization of nonpathogenic mutants. Induction of a *virB*::*lacZ* fusion was monitored in the presence of 60 μM acetosyringone as described previously (8). The Ti plasmid was transferred from derivatives of strain D10B/87 to the Ti plasmid-less *Agrobacterium* recipient C58C1 by conjugation (8). Ti plasmid deletions were detected by separation of linear DNA from plasmid DNA on an agarose gel after lysis in wells (8). These deletions were analyzed by separate digestions of total bacterial DNA with *Eco*RI, *Hin*dIII, and *Bam*HI, and probing by Southern hybridization with overlapping cosmid clones from the *vir* and T regions of pTiC58 (9). The ability to be complemented in *trans* for pathogenicity by cosmid clones containing wild-type *virA* or *virG* was examined as previously described (7). The identity of the complementing locus was confirmed with clones carrying *virA* or *virG* inactivated by transposon insertion (7). *virA* mutants were further characterized by Southern transfer of *Eco*RI-digested total DNA and probing with a 2.2-kb DNA fragment containing part of wild-type *virA*. *virG* mutants were further examined by DNA sequencing (see below). Putative *chvE* mutants were characterized by examining their ability to be complemented for pathogenicity on tomato plants by a strategy already developed for *virA* and *virG* mutants (see above). The clones used in this complementation analysis contained the chromosomal *chvE* region from strain C58, either intact in pMWH146 or with *chvE* inactivated by a transposon insertion in pMWH102 (11).

DNA and RNA analyses. pTiD10B/87 *virG* DNA was amplified by using PCR and oligonucleotide primers complementary to the pTiC58 sequence corresponding to positions 14,387 to 14,407 and 15,520 to 15,540 of the *vir* region (18). The *virG* PCR product was purified with Magic PCR Prep (Promega) before sequencing with the AmpliTaq cycle sequencing kit (Perkin-Elmer Cetus). *virG* sequencing reactions were initiated from a set of eight custom primers allowing sequence determination on both strands.

TABLE 1. Characteristics of *A. tumefaciens* strains used in this study and abilities to produce nonpathogenic mutants following inoculation on plants

Strain designation[a]	Opine utilization[b]	% of nonpathogenic mutants[c] on plants		
		Apple	Pear	Blackberry
D10B/87[d]	Nop	90	4	0
F27/93	Nop, Mop	69	21	2
S12/93	Nop	21	ND	ND
S120/93	Nop	69	ND	ND
J5/75	Nop	7	ND	ND
S155/93	Nop	0	ND	ND
B49C/83	Nop, Mop	0	0	0
AR16K/71	Nop, Mop	0	ND	ND
A432/75	Nop	0	ND	ND

[a] Unless stated otherwise, agrobacteria used in this work are from the collection of L. W. Moore. Strains listed in the first column of this table are biotype 2 apple isolates. In addition to these bacteria, the following *A. tumefaciens* strains were inoculated on apple, pear, blackberry, peach, and cherry plants but did not produce mutants: strain C58 (biotype 1, nopaline-utilizing cherry isolate obtained from J. Tempé), strains I11/85 and I22/85 (biotype 2, nopaline-utilizing cherry isolates), and strains B209B/85 and B230/85 (blackberry isolates).

[b] Nop, nopaline; Mop, mannopine.

[c] The test bacterium was inoculated on Mark apple rootstock and other hosts as indicated. After 2 months, the proportion (percentage) of nonpathogenic mutants in a tumor was determined by recovering and examining 100 clones. ND, not determined.

[d] In addition to reactions given in this table, it was found that strain D10B/87 did not produce mutants on peach or cherry plants.

Total RNA was extracted from bacteria incubated in glycerol induction broth (1) containing 10 mM glucose and 60 μM acetosyringone. The guanidium thiocyanate method was used for total RNA extraction and Northern dot blot analysis was performed under high stringency conditions (2). The 555-bp *virG* DNA fragment used as probe for Northern dot blot hybridizations covered nucleotides 14,678 to 15,232 of the C58 *vir* sequence (18). A 312-bp PCR fragment, generated from a conserved portion of 16S rRNA genes by using primers Y1 and Y2 (22) and *Rhizobium meliloti* A3 DNA, was used as a control probe to verify that equal amounts of RNA had been blotted. Primer extension (2) was carried out by using total RNA from acetosyringone-treated bacteria and primer +40−, corresponding to positions 14,747 to 14,767 of the *vir* region (18).

Nucleotide sequence accession numbers. The complete *virG* sequence of strain D10B/87 has been submitted to GenBank under accession number U16142. The *virG* sequence of strain C58 has been deposited by Rogowsky et al. in EMBL and GenBank data libraries under accession number J03320 (18).

RESULTS

Observations of pathogenicity of bacterial clones recovered from inoculated plants in tissue culture. Agrobacteria obtained from naturally occurring tumors (origins of these strains are indicated in Table 1) were introduced into the stems of axenic plants in tissue culture. Bacteria were later isolated from the resulting experimental crown gall tumors by the destructive sampling method and examined for pathogenicity. Strains which had initially been obtained from tumors on cherry or blackberry plants as the natural host remained pathogenic following their reisolation from experimentally induced tumors and hence were not considered further. However, five of the nine apple isolates tested produced nonpathogenic mutants following their introduction to an axenic apple plant (Table 1). Two of these five strains generating mutants on apple plants, strains D10B/87 and F27/93, were also verified on other hosts. Strain D10B/87 produced a few mutants on pear plants but none on blackberry, peach, or cherry plants. The second apple strain, F27/93, generated mutants on pear and blackberry plants in addition to apple plants. This second strain was not tested on peach or cherry plants (Table 1). The proportion of nonpathogenic mutants among the D10B/87 and F27/93 clones recovered from apple plants increased with time

of association with the host. The proportions of strain D10B/87 nonpathogenic mutants recovered after 1 and 2 months were 35 and 90%, respectively, while the corresponding values for strain F27/93 were 13 and 69%. Strain D10B/87 did not produce nonpathogenic mutants when inoculated on tissue culture medium alone. This observation, coupled to that of a differential response according to the host plant (see Table 1), suggested that the massive recovery of D10B/87 mutants following apple inoculation was dependent on the host plant.

Further experiments involved the inoculation of five different types of apple rootstocks (Malling7, Mark, Ottawa3, P106, and Bud116) with strain D10B/87rif^{r}sm^r. In all cases, 68 to 99% of the clones recovered after 2 months, by either conservative or destructive sampling, were nonpathogenic but still utilized nopaline. Bacteria recovered from the plants grown in tissue culture always expressed the spontaneous antibiotic resistances of the parent strain.

Characterization of nonpathogenic mutants of strain D10B/87. A total of 16, 15, and 17 D10B/87 mutants recovered from inoculations on P106, Mark, and Ottawa3 apple rootstocks, respectively, were characterized in detail. All of these bacteria failed to show *vir* gene induction in the presence of acetosyringone. Conjugative transfer of the Ti plasmid from the mutants to a Ti plasmid-less recipient, followed by an examination of the pathogenicity of the transconjugants, showed that all the mutants but one had altered Ti plasmids. The only exception, a mutant recovered from Mark rootstock, was complemented for pathogenicity by clone pMWH146 (containing *chvE* from the strain C58 chromosome) but not by the related clone pMWH102 (in which *chvE* is inactivated by a Tn*5* insertion [see Materials and Methods]). This result established that the only chromosomal mutant to have been recognized among the 48 nonpathogenic clones examined had an altered *chvE* gene.

Most or all Ti plasmid mutants originating from the same crown gall tumor were of a unique type or closely related. However, differences between mutants obtained from different tumors were seen. Ti plasmids of nonpathogenic mutants recovered from a tumor on rootstock P106 had deletions which encompassed part of the *vir* region or, alternatively, the entire *vir* region and at least part of the T region (Fig. 1A). Nonpathogenic mutants recovered from Ottawa3 and Mark rootstocks were complemented in *trans* by *virG* and harbored a point mutation located at position −63 relative to the translational start site and in the C-terminal coding region of *virG*, respectively (Fig. 1B). Three observations made on bacteria recovered from the Ottawa3 tumor supported the interpretation that the mutation noted upstream of the *virG* coding sequence conferred a nonpathogenic phenotype. First, clone DOVS91, which was capable of *vir* gene induction and pathogenic, showed no nucleotide change at position −63. Second, cells of the nonpathogenic DOVS3 did not produce *virG* mRNA (Fig. 2A), confirming that the mutation in this strain was regulatory in nature. Third, primer extension analysis of strain D10B/87 mRNA (Fig. 2B) located the *virG* transcriptional start sites downstream of the A-to-C transition identified in DOVS3 and the other Ottawa3 nonpathogenic mutants. This analysis further suggested that the point mutation in these bacteria was proximal to the −10 regulatory region (Fig. 1C). Other examples of promoter-down point mutations located immediately upstream of the −10 consensus region are known (21).

Observation of competition in planta between the wild-type D10B/87 and a nonpathogenic mutant. Identity of or similarity between mutants recovered from the same tumor suggested that these mutants were of clonal origin and had benefited from a selective advantage over wild-type cells in the plant

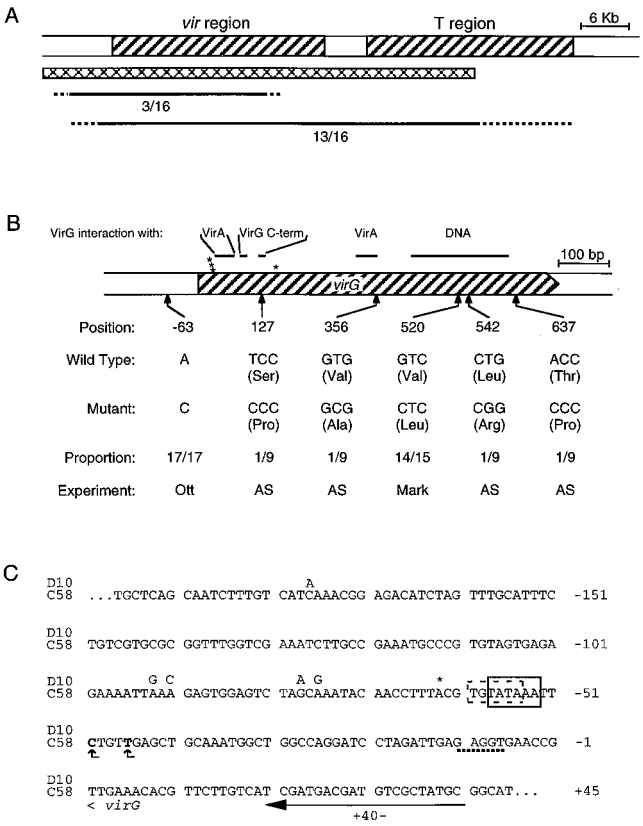

FIG. 1. Genotypes of nonpathogenic D10B/87 mutants recovered from apple crown gall tumors and acetosyringone-treated cultures, and nucleotide sequence analysis of the *virG* promoter region. (A) Deletions in the Ti plasmid of mutants recovered from tumor on rootstock P106. The *vir* and T regions of pTiC58 are shown at the top of panel A. The cross-hatched bar in the middle of panel A represents a segment of pTiD10B/87 exhibiting a high degree of similarity to pTiC58 DNA on the basis of hybridization patterns of digested DNA. The lines at bottom of panel A indicate the extents of the observed deletions. Minimal and maximal possible extents of the deletions are indicated by solid and dotted lines, respectively. The proportion of mutants showing each deletion is indicated by the fractions. (B) Point mutations in *virG* nonpathogenic mutants either obtained from cultures treated with acetosyringone (AS) or recovered from tumors on rootstocks Ottawa3 (Ott) and Mark. The results from different nonpathogenic mutants, each mutant carrying a single mutation, are pooled in one diagram for conciseness. The bar in the upper section of panel B shows *virG* coding (hatched area) and flanking sequences. Gene segments coding for particular functional domains of the VirG protein are shown above the bar. The role of each protein domain, defined as interaction with VirA, C-terminal region of VirG (VirG C-term), and regulatory DNA sequences of the *vir* region (DNA), respectively, is indicated. The positions of codons specifying aspartic acid residues at the active site are given by asterisks (15, 17, 19). The positions of the point mutations, each mutation occurring in a different clone, are indicated by the arrows. The following information is provided below the arrows: the nucleotide coordinates of the mutation (with respect to *virG* translational start site), the codon (and corresponding amino acid) in the wild-type and mutated genes, the number of mutants with indicated nucleotide change to the total number of nonpathogenic mutants examined, and the experiment in which these mutants were obtained. (C) Nucleotide sequence of *virG* promoter region from two wild-type strains, C58 and D10B/87 (D10). The complete sequence is presented for strain C58 (18). For the wild-type strain D10B/87, only the nucleotide changes with respect to the C58 sequence are presented. The beginning of the *virG* coding sequence is indicated (<) below the first nucleotide of the TTG start codon. The asterisk indicates the position of the A-to-C transition identified in 17 (of 17 analyzed) nonpathogenic mutants recovered from a tumor on rootstock Ottawa3 (see also panel B). Solid and dotted boxes indicate the putative −10 *virG* regions, and a dotted line is placed below the potential Shine-Dalgarno sequence. Vertical arrows prolonged by a horizontal line (↑_) are placed below the two transcriptional start sites, which are given in boldface type. These sites were determined by extension of RNA extracted from strains C58 and D10B/87 (Fig. 2B), using primer +40− as indicated by the horizontal arrow. Nucleotides are numbered relative to *virG* translational start site (+1). The *virG* coding sequences (of which only the 5′ end is shown) for pTiD10B/87 and pTiC58 are identical.

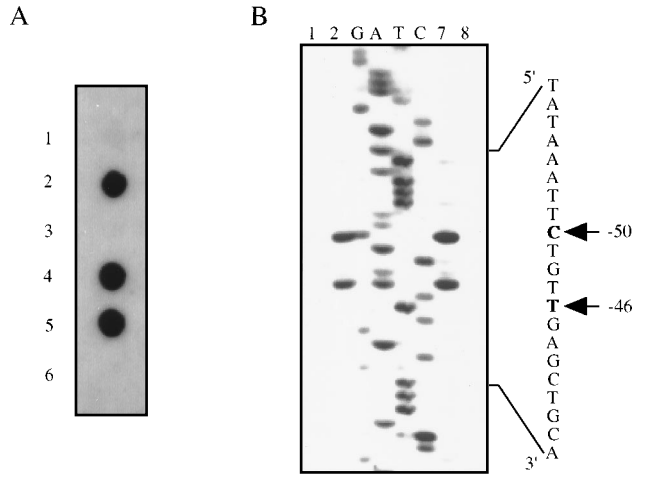

FIG. 2. Analysis of *virG* transcription in *A. tumefaciens* strains. (A) Dot blot of total RNAs from *Escherichia coli* DH5α (dot 1), pathogenic clone DOVS91 (dot 2), virG mutant DOVS3 (dot 3), wild-type strains D10B/87 (dot 4) and C58 (dot 5), and the Ti plasmidless strain C58C1 (dot 6) hybridized with the *virG* probe. (B) Primer extension analysis of *virG* transcript. Total RNAs from strains C58C1 (lane 1), C58 (lane 2), D10B/87 (lane 7), and DOVS3 (lane 8) were extended by using the synthetic primer +40− (Fig. 1C). The reaction products were separated along a pTiD10B/87 *virG* sequencing ladder (presented in the four lanes labeled G, A, T, and C), prepared with the same radioactive primer. The sequence of the complementary strand is indicated to the right of the panel, and nucleotides corresponding to deduced transcriptional start sites are given in boldface type and identified by the arrows. The positions relative to the *virG* translational start site (+1) are given at the extreme right of the panel.

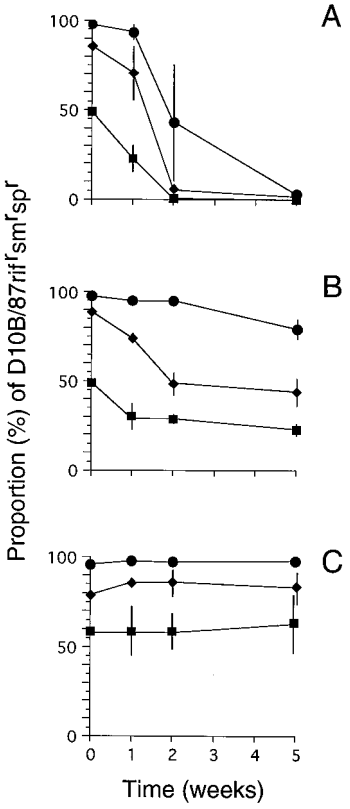

FIG. 3. Abilities of D10B/87 derivatives with inactive or active *virG* to compete with the pathogenic strain D10B/87rif^{r}sm^{r}sp^r during dual culture in apple stems. In addition to the spectinomycin-resistant strain D10B/87rif^{r}sm^{r}sp^r, three spectinomycin-sensitive bacteria were used in the competition assays. These three Sp^s bacteria were two clones recovered from an Ottawa3 apple tumor, namely, the *virG* mutant DOVS3rif^{r}sm^{r}sp^s and the pathogenic clone DOVS91rif^{r}sm^{r}sp^s, and their parent strain, the pathogenic D10B/87rif^{r}sm^{r}sp^s. The spectinomycin-resistant strain D10B/87rif^{r}sm^{r}sp^r was mixed with DOVS3rif^{r}sm^{r}sp^s (A), DOVS91rif^{r}sm^{r}sp^s (B), or D10B/87rif^{r}sm^{r}sp^s (C), and the resulting dual inoculum was used to infect Ottawa3 apple stems in tissue culture. Strain D10B/87rif^{r}sm^{r}sp^r and the three Sp^s competing strains were mixed in different proportions, so that strain D10B/87rif^{r}sm^{r}sp^r represented about 50 (■), 90 (◆), or 99% (●) of the initial inoculum. Two apple plants were inoculated with each bacterial mixture. The proportions of D10B/87rif^{r}sm^{r}sp^r cells in the dual cultures were then determined at intervals. The datum points represent the means of the results obtained with the two plants inoculated with a given mixture, and the vertical bars correspond to the range of individual values for each plant. Vertical bars are not visible for some of the datum points because they are smaller than the datum point symbols.

environment. To test this hypothesis, a series of in planta dual cultures were conducted in which a common competing strain, the pathogenic strain D10B/87rif^{r}sm^{r}sp^r, was cocultivated with an Sp^s test strain or one of two Sp^s control bacteria. To prepare the test dual cultures, the pathogenic competing strain D10B/87rif^{r}sm^{r}sp^r and a nonpathogenic *virG* mutant, DOVS3rif^{r}sm^{r}sp^s, were mixed in different proportions and coinoculated on apple stems. Two additional bacterial combinations were prepared for control inoculations. The first control combination consisted of the competing D10B/87rif^{r}sm^{r}sp^r and the pathogenic clone DOVS91rif^{r}sm^{r}sp^s, which had been recovered from the apple tumor that also yielded the nonpathogen DOVS3rif^{r}sm^{r}sp^s (see above). This control was included because it seemed possible that a variety of adaptive mutations, related or not to pathogenicity, could arise in bacteria subjected to the particular growth conditions prevailing in apple plantlets grown in tissue culture. The second control combination was composed of strains D10B/87rif^{r}sm^{r}sp^r and D10B/87rif^{r}sm^{r}sp^s, which differ solely by the presence or absence of the Sp^r spontaneous determinant. This particular combination was used to detect a possible side effect of the additional Sp^r determinant carried by strain D10B/87rif^{r}sm^{r}sp^r. For each of the test and two control combinations, three suspensions were prepared, containing a proportion of about 50, 90, or 99% of the pathogenic competing strain D10B/87rif^{r}sm^{r}sp^r. Thus, each particular bacterial combination was represented by a set of three dual cell suspensions differing according to the relative proportion of the two component strains.

The pathogenic competing strain D10B/87rif^{r}sm^{r}sp^r had become almost undetectable after 5 weeks of dual culture in apple stems with the *virG* mutant strain DOVS3rif^{r}sm^{r}sp^s, even when this pathogenic strain initially represented 99% of the mixed inoculum (Fig. 3A). By contrast, after 5 weeks, the same pathogenic strain, D10B/87rif^{r}sm^{r}sp^r, still represented a large proportion of the bacteria present in the control dual cultures, which had been established by coinoculation of this competing strain and one of two pathogenic bacteria, strain DOVS91rif^{r}sm^{r}sp^s (Fig. 3B) and D10B/87rif^{r}sm^{r}sp^s (Fig. 3C), respectively. Thus, a comparison of results obtained for these various bacterial combinations confirmed that inactivation of *virG* conferred a strong selective advantage on DOVS3rif^{r}sm^{r}sp^s inoculated on apple plants. Expression of this advantage was dependent on the presence of the host plant, since the *virG* mutant did not outcompete the pathogenic Sp^r strain when a bacterial mixture containing these two strains was deposited on tissue culture medium alone. In this case, the proportion of D10B/87rif^{r}sm^{r}sp^r in the dual culture remained virtually unchanged for the first 2 weeks of incubation. No viable bacteria were recovered after 5 weeks of incubation on tissue culture medium alone (data not shown).

Production of nonpathogenic mutants by strain D10B/87 in

the presence of acetosyringone. Nonpathogenic mutants produced by strain D10B/87 inoculated on apple plants carried deletions in the Ti plasmid or mutations in *virG* (see above) and in this respect resembled the mutants generated by strain C58 incubated in minimal medium with acetosyringone (7, 8; see introduction). Thus, it seemed possible that strain D10B/87 would also yield mutants when incubated under laboratory *vir* gene-inducing conditions. As expected, strain D10B/87 exhibited growth inhibition and generated up to 40% nonpathogenic mutants when incubated in minimal induction medium with acetosyringone. Nine mutants of strain D10B/87, all recovered from the same experiment, were examined and found not to show *vir* gene induction in the presence of acetosyringone. The pathogenicity of five of these nine mutants was restored in *trans* by wild-type *virA*. At least two classes of mutations were represented among these nonpathogenic *virA* mutants, since Southern hybridization indicated that additional DNA had become inserted in *virA* from two of these five bacteria. The remaining four mutants obtained in the acetosyringone-treated culture were complemented by *virG*. Each of these four bacteria harbored a single nucleotide change in the *virG* coding sequence, the location of which differed between mutants (Fig. 1B).

DISCUSSION

Point mutations in the *virG* coding sequence of nonpathogenic mutants recovered from Mark rootstock and acetosyringone-treated culture were distributed over different gene segments coding for various functional domains of the VirG protein. Three of the five mutations identified, including the G-to-C transversion harbored by bacteria recovered from Mark rootstock, were located in the gene segment coding for the VirG DNA-binding domain (Fig. 1B). A previous study (19) on chemical mutagenesis of *virG* showed that point mutations inactivating protein function were distributed randomly over the coding sequence.

The high proportion of nonpathogenic mutants recovered from acetosyringone- and plant-treated cultures is most probably the outcome of a dual process, involving mutagenesis followed by selection of noninducible derivatives. In acetosyringone-treated cultures, the selection component of this process is revealed by the direct correspondence between *vir* gene induction and bacterial growth inhibition (7, 8; see also results described above for strain D10B/87). In apple-treated cultures, the selective advantage of noninducible mutants is indicated by similarity or identity of most nonpathogenic mutants isolated from the same tumor. Hence, these nonpathogenic mutants appear to be of clonal origin, suggesting that they are present in small numbers at the time of tumor induction but later benefit from a selective advantage over wild-type cells. Competition studies in apple stems confirmed that inactivation of the *virA-virG* regulatory system confers such a selective advantage. These competition experiments further indicated that the presence of a well-developed tumor is not required for the selection of nonpathogenic mutants. Indeed, in most instances, the *virG* mutant had already outcompeted the wild type 2 weeks after inoculation, when no tumor was as yet visible at the inoculation site.

To our knowledge, the results presented here provide the first example of host-induced loss of pathogenicity in populations of a bacterium infectious to plants. Identifying specific plant and bacterial factors stimulating the appearance of nonpathogenic mutants may help assess the impact of this phenomenon on the spread of disease and suggest new strategies for plant protection. The existence of specificity factors influencing expression of genetic instability is evidenced by three observations. First, strain D10B/87 generated only a few or no mutants when inoculated on host plants other than apple plants. Second, the acetosyringone-sensitive strain C58 did not produce mutants when inoculated on apple or other plants. Third, the capacity to generate nonpathogenic mutants was demonstrated in many, but not all, of the apple isolates tested. Previous observations on the prevalence of nonpathogenic mutants among agrobacteria isolated from naturally occurring apple tumors were experimentally reproduced here. From these results, it appears that interaction between *A. tumefaciens* and plants contributes directly to divergence of agrobacterial strains and represents a plant model for deciphering the fate of bacteria causing disease.

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