Genotyping of *Serratia marcescens* Strains Associated with Cucurbit Yellow Vine Disease by Repetitive Elements-Based Polymerase Chain Reaction and DNA-DNA Hybridization

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ABSTRACT

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The bacterium that causes cucurbit yellow vine disease (CYVD) has been placed in the species *Serratia marcescens* based on 16S rDNA and *groE* sequence analysis. However, phenotypic comparison of the organism with *S. marcescens* strains isolated from a variety of ecological niches showed significant heterogeneity. In this study, we compared the genomic DNA of *S. marcescens* strains from different niches as well as type strains of other *Serratia* spp. through repetitive elements-based polymerase chain reaction (rep-PCR) and DNA-DNA hybridization. With the former, CYVD strains showed identical banding patterns despite the

fact that they were from different cucurbit hosts, geographic locations, and years of isolation. In the phylogenetic trees generated from rep-PCR banding patterns, CYVD strains clearly were differentiated from other strains but formed a loosely related group with *S. marcescens* strains from other niches. The homogeneity of CYVD strains was supported further by the DNA relatedness study, in that labeled DNA from the cantaloupe isolate, C01-A, showed an average relative binding ratio (RBR) of 99%, and 0.33% divergence to other CYVD strains. Used as a representative strain of CYVD, the labeled C01-A had a RBR of 76%, and a 4.5% divergence to the *S. marcescens* type strain. These data confirm the previous placement of CYVD strains in *S. marcescens*. Our investigations, including rep-PCR, DNA-DNA hybridization, and previous phenotyping experiments, have demonstrated that CYVD-associated strains of *S. marcescens* cluster together in a group significantly different from other strains of the species.

Cucurbit yellow vine disease (CYVD) was first observed in squash (*Cucurbita maxima*) and pumpkin (*C. pepo*) in 1988 in Oklahoma and Texas (7), but is now known to affect other cucurbits, including watermelon and cantaloupe. The disease has been confirmed in Arkansas (J. C. Correll, *personal communication*), Tennessee (4), Massachusetts (26), Kansas (N. A. Tisserat, J. Fletcher, and B. D. Bruton, *unpublished data*), Colorado (B. D. Bruton, *unpublished data*), and Nebraska (R. M. Harveson and B. D. Bruton, *unpublished data*). Affected plants exhibit characteristic symptoms of yellowing, stunting, gradual decline, and phloem discoloration. Losses can range from less than 5 to 100% in affected fields.

Disease symptoms consistently are associated with the presence in the phloem of a rod-shaped, gram-negative bacterium, detected using transmission electron microscopy (7). The bacterium was cultured, and Koch's postulates were completed by mechanical inoculations and by transmission via an insect vector, the squash bug, *Anasa tristis*, confirming that this bacterium is the causal agent of CYVD (6).

Two CYVD pathogenic strains, W01-A and Z01-A, were originally isolated from diseased watermelon and zucchini, respectively. Sequence analysis of 16S rDNA and *groE* gene fragments of these two strains indicated that they shared more than

97% sequence similarity with the type strain of *Serratia marcescens* (19). The possible identity of the CYVD bacterium as *S. marcescens* was unexpected. Different strains of this species can assume roles as soil saprophytes (17), plant endophytes (24), insect pathogens (8), and even opportunistic human pathogens (25), but the only previous reports of plant pathogenicity for this species, in sainfoin (21) and alfalfa (13), were made when the tools of bacterial identification were less definitive than those of today.

Biological characterization of Z01-A and W01-A using BIOLOG and fatty acid profiling showed that, despite the strong similarity of their 16S rDNA and *groE* genes to those of other *S. marcescens*, CYVD strains were quite unique in some other respects, lacking a number of metabolic functions and possessing different lipid complements than those present in *S. marcescens* strains from other niches (19).

This apparent disparity led us to seek a more definitive confirmation of the identity of CYVD strains and to examine the taxonomic position of the CYVD bacteria based on techniques that take into consideration the sequence and organization of the entire chromosome. In addition, the broad geographic distribution and potential devastation of this disease make it important to better understand the genetic relationships among CYVD strains, because these may provide clues as to their site of origin, pattern of spread, and disease epidemiology.

Repetitive elements-based polymerase chain reaction (rep-PCR) generates DNA fingerprints by amplifying different-sized DNA fragments lying between the repetitive elements in a genome. The amount of differentiation among the tested strains depends on the

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primer used; however, overall, the rep-PCR banding patterns of strains from the same bacterial species can be quite diverse and often reveal relationships not seen by other methods. This method has been used successfully to fingerprint clinical isolates of *S. marcescens* (18). DNA-DNA hybridization, on the other hand, has the advantage of comparing organisms at the whole-chromosome level. The goals of this work were to type CYVD strains, based on repetitive elements, and to determine the DNA relatedness among CYVD strains as well as between plant-pathogenic strains and those from other niches.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. Tested bacteria included type strains of several *Serratia* spp., several *S. marcescens* strains from different ecological niches, and several CYVD isolates from different cucurbit hosts, geographical locations, and years of isolation (Table 1). Bacteria were stored at -80°C in aliquots in 1.5 ml of Luria-Bertani (LB) broth containing 15% glycerol. For use in experiments, bacteria were streaked onto LB agar (20) and incubated at 28°C for 24 h.

TABLE 1. Strains of cucurbit yellow vine disease-associated and nonassociated *Serratia marcescens* and other *Serratia* spp. used in this study^a

Strain designation	Alternative	Host	Location	Collection date	rep-PCR	DNA-DNA hybridization
CYVD strains ^b						
W01-A to W01-J	...	Watermelon	Texas	1999	Y	Y
W04-A	...	Watermelon	Arkansas	2000	Y	ND
W05-A	...	Watermelon	Arkansas	2001	Y	Y
W08-A	...	Watermelon	Oklahoma	1997	Y	Y
W09-A	...	Watermelon	Oklahoma	1997	Y	Y
Z01-A	...	Zucchini	Oklahoma	1999	Y	Y
Z01-B	...	Zucchini	Oklahoma	1999	Y	ND
Z02-A	...	Zucchini	Oklahoma	1999	Y	ND
Z02-B	...	Zucchini	Oklahoma	1999	Y	Y
P01-A	...	Pumpkin	Oklahoma	1999	Y	Y
P01-B	...	Pumpkin	Oklahoma	1999	Y	ND
P02-A	...	Pumpkin	Oklahoma	1999	Y	Y
P02-B	...	Pumpkin	Oklahoma	1999	Y	ND
P03-A	99-2515	Pumpkin	Oklahoma	2000	Y	ND
P07-A	00B058	Pumpkin	Massachusetts	2001	Y	ND
P08-A	00B060	Pumpkin	Massachusetts	2001	Y	Y
C01-A	...	Cantaloupe	Oklahoma	1999	Y	Y
C01-B	...	Cantaloupe	Oklahoma	1999	Y	ND
C02-A	...	Cantaloupe	Oklahoma	1999	Y	Y
C02-B	...	Cantaloupe	Oklahoma	1999	Y	ND
Endophytic strains						
R01-A ^c	IRBG 501	Rice	Philippines	Unknown	Y	Y
R02-A ^c	IRBG 502	Rice	Philippines	Unknown	Y	Y
R03-A ^c	IRBG 505	Rice	Philippines	Unknown	Y	ND
G01 ^d	90-166	Cotton	Alabama	Unknown	Y	Y
G02 ^d	JM-965	Cotton	Unknown	Unknown	Y	Y
G03 ^d	JM-983	Cotton	Unknown	Unknown	Y	Y
Animal pathogenic strains						
I02-A ^e	0-8-12	Insect	California	1961	Y	ND
I06-A ^e	56-1-1	Insect	California	1960	Y	ND
I09-A ^e	73-1-1	Insect	California	1961	Y	ND
H01-A ^f	...	Human	Oklahoma	1999	Y	Y
H02-A ^f	...	Human	Oklahoma	1999	Y	Y
V01-A ^f	94100675	Feline	Oklahoma	Unknown	Y	ND
Saprophytic strains						
S01-A ^d	CP01(4)CU	Rhizosphere	Unknown	Unknown	Y	Y
S02-A ^d	98A-742	Soil	Florida	Unknown	Y	Y
S03-A ^g	ROX6	Soil	Oklahoma	2001	Y	ND
<i>Serratia</i> spp.						
<i>S. fonticola</i> ^h	...	Spring water	ATCC 29844	Unknown	Y	Y
<i>S. grimesii</i> ^h	...	Unknown	ATCC 14460	Unknown	Y	Y
<i>S. liquefaciens</i> ^h	...	Milk	ATCC 27592	Unknown	Y	Y
<i>S. marcescens</i> ^h	...	Water	ATCC 13880	Unknown	Y	Y
<i>S. plymuthica</i> ^h	...	Water	ATCC 183	Unknown	Y	Y
<i>S. rubidaea</i> ^h	...	Unknown	ATCC 27593	Unknown	Y	Y
<i>S. ficaria</i> ^h	...	Unknown	ATCC 33105	Unknown	ND	Y
<i>S. proteamaculans</i> ^h	...	Unknown	ATCC 19323	Unknown	ND	Y
<i>S. odorifera</i> ^h	...	Unknown	ATCC 33077	Unknown	ND	Y

^a Designation = strain designation used in this study. Alternative = alternate strain designation, if any. rep-PCR = repetitive elements-based polymerase chain reaction. Y = test performed and ND = not done.

^b CYVD = cucurbit yellow vine disease. For the cucurbit isolates, the strains were named as follows: for W01-A, W = type of host plant, 01 = number assigned to the plant from which the strain was isolated, and A = number assigned to one particular strain isolated from that plant. W01-A to W01-J represents 10 isolates; all other lines list only single isolates. Strains isolated from the same host, state, and year were from the same field.

^c Provided by J. K. Ladha, International Rice Research Institute, Los Banos, Philippines.

^d Provided by J. Kloepper, University of Alabama, Auburn.

^e Provided by A. H. Purcell, University of California, Berkeley.

^f Provided by D. Adamson, Medical Arts Laboratory, Oklahoma City, OK.

^g Provided by S. P. Deng, Oklahoma State University, Stillwater.

^h Provided by the American Type Culture Collection, Manassas, VA. All the strains listed are the type strain of the respective species.

DNA isolation. Single colonies were transferred to 5 ml of LB broth and incubated at 28°C, with shaking at 220 rpm, for 18 h. DNA was extracted using a modified version of the hexadecyl trimethyl ammonium bromide (CTAB) method (2). Briefly, 5 ml of bacterial culture were centrifuged for 5 min at 10,000 × g and 4°C, the supernatant was removed, and the pellet was resuspended in 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The mixture was incubated 30 min at 60°C, then 100 µl of 5 M

NaCl and 80 µl of CTAB solution (10% CTAB in 0.7 M NaCl) were added and mixed, and the suspension was incubated at 60°C for 10 min. The solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was mixed with 0.6 vol of isopropanol, and DNA precipitated at -20°C for at least 15 min. DNA was collected by centrifugation (20,000 × g for 15 min at 4°C) and washed with 70% ethanol. The DNA pellet was air dried at room temperature for 15 min, and then dissolved in 100 µl of TE buffer (pH 8.0). The DNA solution was stored at -20°C until use.

Rep-PCR. The BOX primer (14), polymorphic GC-rich repetitive sequences (PGRS) primer (18), and enterobacterial repetitive intergenic consensus (ERIC) primers (11) were synthesized by the Oklahoma State University Recombinant DNA-Protein Resource Facility. Amplification was performed with each of three primer sets: BOX/BOX, PGRS/PGRS, and ERIC1R/ERIC2. Each reaction consisted of 5 µl of 1× Gitschier buffer (16.6 mM [NH₄]₂SO₄; 67 mM HCl, pH 8.8; 6.7 mM MgCl₂; 6.7 mM EDTA; and 30 mM β-mercaptoethanol), 10% bovine serum albumin, 10% dimethyl sulfoxide (vol/vol; Fluka Chemical Corp., Ronkonkoma, NY), 50 pmoles of primer, 125 mM each dNTP, 2 units of *Taq* polymerase (Promega Corp., Madison, WI), and 1 µl of the bacterial DNA solution. The total volume per reaction was 25 µl. Amplification was carried out in a DNA thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, CT). Reaction conditions were slightly modified from previous studies (18). With all three primer sets, each round of amplification began with initial incubation at 94°C (3 s) and 92°C (30 s). This was followed by 52°C (1 min) and 65°C (8 min) for the BOX primer, 50°C (1 min) and 65°C (8 min) for the ERIC primers, or 54°C (1 min) and 72°C (8 min) for the PGRS primers. A total of 35 cycles was used. An 8-µl aliquot of the PCR product was analyzed on a 1.5% agarose gel, electrophoresed at 70 V (4 V/cm) for 5 h in 1× Tris-acetate EDTA buffer (pH 8.3). Gels were stained with ethidium bromide at 0.5 µg/ml and differences in DNA fingerprint patterns were assessed visually.

Comparisons of DNA fingerprint patterns were performed by measurement of band positions of PCR products. The presence or absence of each band in each bacterial strain was converted into binary data (1 for presence and 0 for absence). These data then were used as an input file for the MIX parsimony program of PHYLIP (10) to generate a phylogenetic tree. In cases of multiple data sets, the sets were combined to generate a consensus tree using CONSENSE (9). Finally, all the trees were visualized using the TREEVIEW program (16). The reliability of the dendrogram was assessed by a bootstrap analysis with the SEQBOOT program (9). In all, 1,000 repeated samplings with replacement were made in each analysis. The frequencies with which each group was formed in repeated cycles of dendrogram construction were used as a measure of the relative reliability of the clusters of the strains.

DNA-DNA hybridization. Purification of genomic DNA was performed using the method of Brenner et al. (5). Briefly, the bacterial cells were lysed with 0.05 M TE buffer containing 0.5% sodium dodecyl sulfate (SDS) and pronase at 50 µg/ml, followed by phenol extraction. DNA from the aqueous phase then was precipitated with 95% ethanol and washed with 70% ethanol. The resulting crude DNA solution then was incubated overnight at 37°C with RNase at 50 µg/ml containing SDS (0.5% final concentration) and pronase (50 µg/ml final concentration). The DNA was extracted once with phenol and twice with chloroform, followed by three precipitations with ethoxyethanol. DNAs were sheared by sonication at 4°C (9).

The procedures used for the determination of DNA relatedness by hybridization in free solution by the hydroxyapatite method have been described previously (5). The DNAs were labeled enzymatically in vitro with [³²P]dCTP by nick translation. DNA-DNA hybridization experiments were performed at 60°C for optimal DNA reassociation and also at the stringent temperature of 75°C. The percent divergence of hybrids was calculated as decrease of

Fig. 1. Agarose electrophoresis gels of repetitive elements-based polymerase chain reaction (rep-PCR) products generated from cucurbit yellow vine disease (CYVD) strains, *Serratia marcescens* from various niches, and other *Serratia* type strains. **A**, Polymorphic GC-rich repetitive sequences primer, **B**, enterobacterial repetitive intergenic consensus (ERIC) primers, and **C**, BOX primer. Lanes labeled M were run with the DNA ladder (DirectLoad; Sigma-Aldrich, St. Louis), sizes of fragments are listed on the right. Lane 1, C01-A; lane 2, P01-A; lane 3, W01-A; lane 4, Z01-A; lane 5, P03-A; lane 6, R01-A; lane 7, R02-A; lane 8, R03-A; lane 9, S01-A; lane 10, S02-A; lane 11, H01-A; lane 12, H02-A; lane 13, G01-A; lane 14, V01-A; lane 15, I06-A; lane 16, I09-A; lane 17, *S. marcescens*; lane 18, G02-A; lane 19, G03-A; lane 20, *S. grimesii*; lane 21, *S. plymuthica*; lane 22, *S. rubideae*; lane 23, *S. liquefaciens*; and lane 24, *S. fonticola*.

1°C in thermal stability of a heterologous DNA duplex, which correlates to approximately 1% unpaired bases within related DNA. The guidelines of Wayne et al. (23) were used in defining hybridization groups. These guidelines indicate that strains within the same species should have a DNA relatedness, as measured by relative binding ratio (RBR), of $\geq 70\%$ and a divergence of $\leq 5\%$. The experiments of DNA-DNA hybridization were performed twice.

RESULTS

rep-PCR. A total of 29 CYVD-associated *S. marcescens* strains, 16 CYVD-nonassociated *S. marcescens* strains, and 5 other *Serratia* type strains were tested by rep-PCR. Each reaction was repeated at least three times and only the repeatable banding patterns were used for phylogenetic analysis. The PCR products of all 29 CYVD-associated *S. marcescens* strains, regardless of the geographic location, plant host species, or collection date, showed a homogeneous pattern (data not shown). Five of these strains finally were selected to compare with other strains (Fig. 1A to C, lanes 1 to 5) and to generate phylogenetic trees (Fig. 2). *S.*

marcescens isolates from other ecological niches, as well as the type strains of different *Serratia* spp., showed a variety of patterns, all of which were different from those of CYVD isolates (Fig. 1A to C, lanes 6 to 24).

Distinguishable banding patterns were generated in all PCR primer combinations. For reactions using PGRS and BOX primers, the size of the amplicons ranged from 0.5 to 6 kb. Two rice endophyte strains, R02-A (IRBG 502) and R03-A (IRBG 505), showed identical patterns using both PGRS and BOX primers. Two insect pathogenic strains, I06-A and I09-A, also were identical with each other. Two bands generated from PGRS PCR, with sizes of 1.2 and 1.4 kb, respectively, were shared by all the *S. marcescens* strains. In BOX-primed PCR, one band of 0.6 kb was shared by all the *S. marcescens* strains. Twenty-seven bands were amplified using ERIC primers, and the size of amplified DNA ranged from 0.3 to 5 kb. Although fewer bands were generated from ERIC primers, the banding patterns of *S. marcescens* appeared to be as diverse as those from BOX and PGRS reactions.

The banding patterns of BOX, PGRS, and ERIC PCR products each were scored separately as binary data. Phylogenetic trees were generated for each of the three rep-PCR reactions. The three

Fig. 2. Consensus phylogenetic tree of the relationships among *Serratia* spp. and strains, compiled from binary data based on the banding patterns of three different repetitive elements-based polymerase chain reaction sets. Branches with bootstrap values less than 500 were collapsed.

trees were similar in that the CYVD strains grouped together within a large branch containing the *S. marcescens* type strain (data not shown). When the data from all three PCR reactions were combined, the consensus phylogenetic tree was similar to the trees generated from individual reactions, except that branches with low bootstrap value were collapsed. All the *S. marcescens* strains tested fell into two groups.

DNA-DNA hybridization. DNA-relatedness studies were conducted using labeled reference DNA from C01-A, the *S. marcescens* type strain, G03-A (JM-983), the *S. proteamaculans* type strain, and G02-A (JM-965), respectively (Table 2). As a representative CYVD strain, C01-A, from cantaloupe, was hybridized to all the strains listed in Table 2 at both 60 and 75°C, while the other labeled reference strains were hybridized only at 60°C to a limited number of strains, as shown. At the optimal reassociation temperature (60°C), labeled C01-A DNA showed an average RBR of 99% (range from 94 to 100%) and 0.33% divergence (from 0.0 to 1.0%) to other CYVD strains. At the same temperature, C01-A hybridized with the *S. marcescens* type strain with a 76% RBR and 4.5% divergence. In addition, C01-A DNA had an RBR of 73 to 90% relative to all the other *S. marcescens* reference strains, with an average percent divergence of 2.1 (range from 1.0 to 3.5%). In contrast, the relatedness values between C01-A and type strains of other *Serratia* spp. were well below 70%, and the percent divergences were 10.5 to 13.5%. Labeled DNA from the *S. marcescens* type strain hybridized to unlabeled C01-A DNA with a 69% RBR, slightly lower than the 76% RBR obtained when labeled C01-A DNA reacted with the type strain. However,

the percent divergences obtained from both reactions were <5.0%. According to the criteria of species definition (23), our data clearly illustrate that C01-A and other CYVD strains group together and belong to species *S. marcescens*. In more stringent hybridization reactions carried out at 75°C, labeled C01-A DNA showed an average RBR of 72% (range from 62 to 88%) to *S. marcescens* DNA from different niches, which reflects the reliability of results obtained at 60°C.

DISCUSSION

As a PCR-based fingerprinting method, rep-PCR has the advantages of convenience and sensitivity. In our study, 29 CYVD strains showed 100% similarity by rep-PCR despite the fact that they were collected from different plant species, in different years, and at different geographic locations. This method clearly discriminated CYVD-associated strains from strains of *S. marcescens* not associated with CYVD.

Rep-PCR, based on repetitive elements such as ERIC, BOX, and PGRS, has been used to study the epidemiology of a wide array of microorganisms. These three families of unrelated repetitive DNA sequences vary in location throughout the chromosome and show different discriminatory ability depending on the species examined. In this study, ERIC PCR generated fewer bands than did BOX and PGRS, similar to the observation of Patton et al. (18) for *S. marcescens* but in contrast to a previous study by Liu et al. (12) using the same species. Nevertheless, the phylogenetic trees generated in our study from PCR patterns amplified by all

TABLE 2. DNA relatedness disclosed by DNA-DNA hybridization, with respect to strains of cucurbit yellow vine disease (CYVD)-associated and nonassociated strains of *Serratia marcescens* and other *Serratia* spp.

Source of unlabeled DNA	Source of labeled DNA ^a									
	C01-A		<i>S. marcescens</i>		G03-A		<i>S. proteamaculans</i>		G02-A	
	RBR 60°C	%D	RBR 75°C	%D	RBR 60°C	%D	RBR 60°C	%D	RBR 60°C	%D
CYVD strains										
C01-A	100	0.0	100	69	3.5	50	11	...	20	...
C02-A	100	0.0	95	71	3.0	...	...	...	...	...
P01-A	94	0.0	95	...	...	...	...	...	...	...
P02-A	100	0.5	100	...	...	...	...	...	...	...
P04-A	100	0.5	...	...	...	...	...	...	...	...
P08-A	99	1.0	100	...	...	...	...	...	...	...
W01-A	97	0.5	...	...	...	...	...	...	...	...
W05-A	99	0.5	...	...	...	...	...	...	...	...
W08-A	100	0.0	100	...	...	...	...	...	...	...
W09-A	100	0.0	100	...	...	...	...	...	...	...
Z01-A	100	0.5	99	...	...	...	...	...	...	...
Z02-B	100	0.5	100	...	...	...	...	...	...	...
<i>S. marcescens</i>										
R01-A	82	1.5	79	...	...	...	...	...	...	...
R02-A	90	1.5	88	...	...	...	...	...	...	...
H01-A	73	3.0	63	87	0.5	...	...	...	...	...
H02-A	82	1.0	77	...	...	...	...	...	...	...
G01-A	76	1.0	75	...	...	...	...	...	...	...
S01-A	75	3.5	65	93	2.0	...	...	...	...	...
S02-A	73	3.5	62	91	1.0	...	...	...	...	...
Strains to be identified										
G02-A	15	13.0	...	...	...	22	...	...	100	0.0
G03-A	46	9.5	...	...	...	100	...	74	6.1	22
<i>Serratia</i> type strain										
<i>S. marcescens</i>	76	4.5	66	100	0.0	44	...	...	18	...
<i>S. ficaria</i>	66	10.5	...	...	...	52	...	...	29	...
<i>S. plymuthica</i>	51	11.0	...	...	...	61	...	64	11.2	22
<i>S. liquefaciens</i>	49	12.5	...	...	...	74	8.5	65	9.1	21
<i>S. proteamaculans</i>	48	13.5	...	...	...	79	6.5	100	0.0	21
<i>S. odorifera</i>	42	13.0	...	...	...	41	...	...	22	...
<i>S. grimsii</i>	41	12.0	...	...	...	58	...	65	9.7	16
<i>S. rubidaea</i>	40	12.0	...	...	...	42	...	...	...	...
<i>S. fonticola</i>	39	12.5	...	...	...	40	...	...	...	...

^a Relative binding ratio (RBR) is determined by the equation (percent bound to hydroxylapatite in heterologous reactions)/(percent DNA bound in homologous reactions) × 1,000. Percent divergence (%D) assumed that a 1°C decrease in thermal stability of a heterologous DNA duplex compared with that of the homologous duplex was caused by 1% unpaired bases.

three primer sets were very similar. The final consensus phylogenetic tree generated from rep-PCR banding patterns also was similar to the trees generated from 16S rDNA and *groE* sequence analysis (19). All the CYVD strains were identical to each other and fell into a group containing CYVD nonassociated *S. marcescens* strains.

Results from the DNA-DNA hybridization study also illustrate that CYVD strains form a closely related group. As a reference strain of this group, C01-A was more than 70% related to the *S. marcescens* type strain, with a percent divergence of <5.0. This result supported the placement of CYVD strains in the species *S. marcescens*. In a previous study (19), the biological tests BIOLOG, Vitek, API-20E, and FAME indicated that the metabolic capabilities and fatty acid composition of the CYVD strains were quite different from those of CYVD nonassociated strains, including the *S. marcescens* type strain. The approximately 25 to 30% difference at the genome level might be responsible for these phenotypic differences.

The DNA relatedness between C01-A and other CYVD strains ranged from 94 to 100%, as shown by DNA-DNA hybridization. This result is in contrast to the unanimous identity revealed in rep-PCR analysis. Considering the fact that, in rep-PCR, the amplicons may represent only a small portion of the genome, and bands with the same electrophoretic mobility were not compared for DNA sequence similarity, we believe that DNA-DNA hybridization is likely a more accurate measure than is rep-PCR of the genetic complementarities. Even the lowest relatedness measured among CYVD strains (94% RBR between C01-A and P01-A) reflects the overall similarity among pathogenic strains, and the percent divergence of 0.0% suggests that the C01-A and P01-A similarity is actually even higher than suggested by the 94% RBR. This is in contrast to the results for *S. marcescens* clinical isolates, in which the rep-PCR banding patterns of different clinical isolates were quite diverse. This result suggests to us that CYVD strains of *S. marcescens* may have diverged from an ancestor much more recently than the human isolates have.

Rascoe et al. (19) found that strain G02-A (JM-965), which had been tentatively identified as *S. marcescens*, is significantly different from the *S. marcescens* type strain by 16S rDNA and *groE* analysis. In this study, the 15% RBR and 13.0% divergence clearly demonstrated that G02-A belongs to a species other than *S. marcescens*. Strain G03-A (JM-983), which had been tentatively designated as a strain of *S. plymuthica*, has an RBR of >70% with *S. proteamaculans*, suggesting that it belongs to this species.

Both rep-PCR and DNA-DNA hybridization results showed that, among the CYVD nonassociated bacteria tested, the rice endophytic strains R02-A and R01-A were those most closely related to CYVD strains. Rice strain R03-A, which was not tested by DNA-DNA hybridization, was indistinguishable from R02-A based on BOX and PGRS PCR, a result in good agreement with data of Tan et al. (22), who characterized the rice endophytic strains more fully. Like the CYVD strains, all rice strains were nonpigmented and differed from the *S. marcescens* type strain in several key nutrient utilization characteristics. Preliminary results (B. D. Bruton, unpublished data) showed that none of the three rice strains were pathogenic to cucurbit plants. Comparing the rice endophytes with the phytopathogenic CYVD strains on the genetic level may be a starting point to the identification of virulence factors of CYVD strains.

In a separate study, Bextine et al. (3) demonstrated that the squash bug, *Anasa tristis*, is a vector of CYVD strains of *S. marcescens*. Insects of two other taxa (leafhoppers and mirids) also were able to transmit the CYVD pathogen under artificial conditions (1). However, in the work reported here, all strains isolated from field-collected insects were much more distantly related to CYVD strains than were the rice endophytes. These findings suggest the possibility that *S. marcescens* strains associated with plants may have followed two routes of evolutionary adaptation,

one as a plant pathogen and the other as a harmless and possibly even beneficial endophyte. They also indicate that different strains of *S. marcescens* have different types of relationships with insects that harbor them, some as entomopathogens and others in a vector-host relationship.

Compared with the *S. marcescens* endophyte 90-166 and clinical isolate H01-A, CYVD strains lack 30 of the 95 metabolic capabilities in the BIOLOG test (19). Whether CYVD strains fail to produce those enzymes as a result of regulatory control of gene expression or by loss of genes due to genomic rearrangements during evolution, or both, is still unclear. Ochman (15) recently suggested that changes in genome repertoire, occurring through gene acquisition and deletion, are the major events underlying the emergence and evolution of bacterial pathogens and symbionts. In the future, analysis of *S. marcescens* from other niches that are more closely related to CYVD strains of *S. marcescens* might provide clues as to the origin and the evolutionary path of plant-pathogenic strains.

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