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A Relative of the Broad-Host-Range Plasmid RSF1010 Detected in *Erwinia amylovora*

E. L. PALMER,[1] B. L. TEVIOTDALE,[2] AND A. L. JONES[1]*

Department of Botany and Plant Pathology and Pesticide Research Center, Michigan State University, East Lansing, Michigan 48824-1312,[1] and Department of Plant Pathology, Kearney Agricultural Center, Parlier, California 93648[2]

Received 5 March 1997/Accepted 30 August 1997

Streptomycin- and sulfonamide-resistant *Erwinia amylovora* CA3R from California contained an 8.7-kb plasmid, pEa8.7, with a *sulII–strA-strB* resistance region; furthermore, PCR, sequencing, hybridization, and restriction analyses showed that pEa8.7 was closely related or identical to broad-host-range plasmid RSF1010. Although RSF1010 has been found in a variety of bacteria, this is the first report of its presence in plant pathogenic bacteria.

The use of streptomycin in plant agriculture over the past 40 years to control populations of phytopathogenic bacteria has been followed by the appearance of streptomycin-resistant strains of some important bacterial plant pathogens including *Erwinia amylovora*, the causative agent of fire blight in apples, pears, and other rosaceous plants. Analysis of streptomycin-resistant *E. amylovora* isolated from apple orchards in Michigan revealed two distinct genetic determinants of resistance: the tandem *strA-strB* streptomycin-modifying genes located in Tn*5393* (2, 8) and a point mutation in the *rpsL* gene which renders the ribosome insensitive to streptomycin (3). The presence of *strA-strB* in *E. amylovora* has thus far been detected only in Michigan; in all other regions streptomycin resistance has been associated with mutations. Genetically distinct variants of Tn*5393* have been found in other plant pathogens including *Pseudomonas syringae* pv. papulans (2), *P. syringae* pv. syringae (16), and *Xanthomonas campestris* pv. vesicatoria (17), usually associated with large, conjugative plasmids. Many streptomycin-resistant bacteria isolated from plant surfaces also contain sequences of *strA-strB* and Tn*5393* (2, 9, 12, 15, 17). However, thus far *Erwinia herbicola* is the only other species which has been found to harbor the variant of Tn*5393* found in *E. amylovora* (2).

Although Tn*5393* appears to be restricted to plant-associated bacteria, the *strA-strB* genes of Tn*5393* are identical to the streptomycin resistance genes found worldwide in at least 14 genera of gram-negative animal and human pathogens (18). In these clinical strains streptomycin resistance is usually linked to sulfonamide resistance on small broad-host-range plasmids like RSF1010 and pBP1 or large self-transmissible R plasmids like pGS05 and pCJ004. In these plasmids, the *strA-strB* genes are often linked with the *sulII* gene (10, 13, 20). Plasmids with a *sulII–strA-strB* resistance region have not been reported in plant-associated bacteria.

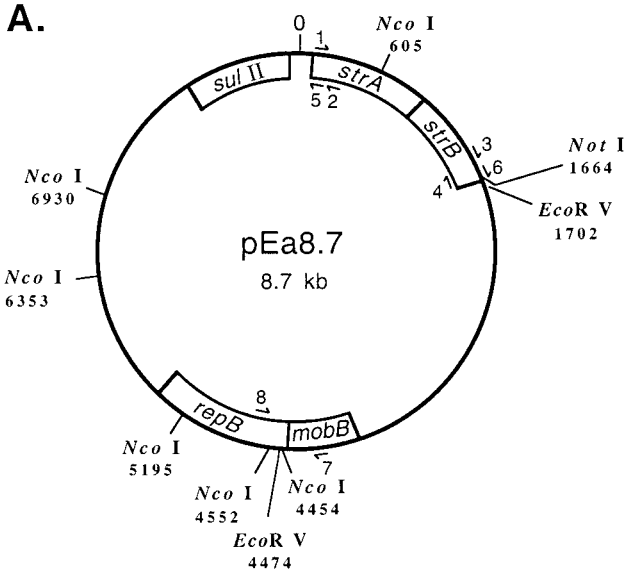
In a study of streptomycin-resistant *E. amylovora* isolated in several parts of the United States and in New Zealand, a group of strains (CA3R, CA4R, and CA5R) from an apple orchard in California was found to have a moderately resistant streptomycin phenotype and to harbor a small plasmid which hybridized to a *strA* probe (8). This observation was unique because *strA-strB* had not been previously found in *E. amylovora* outside of Michigan and because the identified plasmid was much smaller than plasmids associated with *strA-strB* in *E. amylovora* or any other plant-associated bacteria. We therefore sought to characterize this plasmid.

To verify that strain CA3R contained both *strA* and *strB*, PCR experiments were performed with primers which would amplify a 301-bp region of the *strA* gene (Fig. 1B, primers strA-F and strA-R) and a 303-bp region of the *strB* gene (Fig. 1B, primers strB-F and strB-R). Control strains were streptomycin-resistant and -sensitive *E. amylovora* CA11 and Ea110, respectively. Both strains were originally isolated in Michigan (1, 11); CA11 contained linked *strA-strB* genes in transposon Tn*5393* (2). Reaction mixtures (50 μl) consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1 μM each primer, 0.1% (vol/vol) Tween-20, 2.5 U of *Taq* DNA polymerase (Gibco/BRL, Gaithersburg, Md.), and 10 μl of bacterial sample. Bacterial samples were prepared by centrifuging 0.5-ml overnight cultures grown in Luria-Bertani medium, resuspending the pellet in 25 μl of H_2O, and making a 1:100 dilution in H_2O. Cycling parameters were 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplifications were performed in a model PTC-150-16 minicycler (MJ Research, Inc., Watertown, Mass.), and amplification products were analyzed by electrophoresis on 1.5% agarose gels run in 1× Tris-borate-EDTA buffer (7). Streptomycin-sensitive strain Ea110 did not yield an amplification product; streptomycin-resistant strains CA3R and CA11 both yielded the expected 301-bp and 303-bp amplification products (Fig. 2).

To analyze the plasmid containing the *strA-strB* genes in strain CA3R, we used the Expand Long Template PCR System (Boehringer Mannheim, Indianapolis, Ind.) and primers designed to amplify outward from the *strA-strB* region around the rest of the plasmid (Fig. 1B, strA-out, strB-out). Previously sequenced plasmid RSF1010 (13) (GenBank accession no. M28829) was provided in *Escherichia coli* CB613 by M. Bagdasarian (Michigan State University, East Lansing) and was included for comparison. The PCR mixture contained 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 2.6 U of Expand Long Template enzyme mix, 0.3 μM each primer, 0.35 μM deoxynucleoside triphosphates and 1 μl of CA3R plasmid DNA (Wizard Minipreps DNA Purification System, Promega, Madison, Wis.). The reaction was performed according to the manufacturer's instructions in a model PTC-100HB

* Corresponding author. Michigan State University, 103 Pesticide Research Center, East Lansing, MI 48824. Phone: (517) 355-4573. Fax: (517) 353-5598. E-mail: 22620alj@msu.edu.

A.



B.

	Primer	5'-3'	Nucleotide Position (in RSF1010)
1	strA-F	TGAATCGCATTCTGACTGGTT	89-109
2	strA-R	AAGTTGCTGCCCCATTGA	389-372
3	strB-F	GGAACTGCGTGGGCTACA	1357-1374
4	strB-R	GCTAGATCGCGTTGCTCCTCT	1659-1639
5	strA-out	AACCAGTCAGAATGCGATTCA	109-89
6	strB-out	AGAGGAGCAACGCGATCTAGC	1639-1659
7	RSF1010-F	GGTACTGGAAGCTATGG	4266-4285
8	RSF1010-R	AGGTCAAACTCGCTGAGGTCG	4658-4638

FIG. 1. (A) Location and orientation of primers and of the *sulII–strA-strB* resistance region on a restriction map of plasmid pEa8.7 from *E. amylovora* CA3R. Numbering of restriction sites was based on RSF1010 sequence data (13). (B) Oligonucleotide primers used to amplify portions of the *strA* and *strB* genes, plasmid sequences outward from the *strA-strB* genes, and the *repB-mobB* region of plasmid pEa8.7.

thermocycler (MJ Research, Inc.) with the following cycle conditions: 94°C for 2 min; 10 cycles of 94°C for 10 s, 61°C for 30 s, and 68°C for 8 min; 15 cycles of 94°C for 10 s, 61°C for 30 s, and 68°C for 8 min plus 20 s additional per cycle; and 68°C for 7 min. The resulting PCR product was approximately 7 kb (Fig. 3A). Given that the predicted inward distance between the *strA* and *strB* primers is 1.5 kb, the whole plasmid, pEa8.7, was estimated to be between 8 and 9 kb in length. Southern analysis of *Not*I-digested CA3R plasmid DNA using a digoxigenin-labelled *strB* PCR fragment made with primers strB-F and strB-R (PCR DIG Probe Synthesis Kit; Boehringer Mannheim) yielded a single band of approximately 9 kb (data not shown).

The 7-kb PCR product was purified with the Wizard PCR Preps DNA Purification System (Promega), and the ends were partially sequenced at the Michigan State University Plant Research Lab Sequencing Facility. The resulting sequence was nearly identical to the reported *strA-strB* flanking regions of the 8.7-kb broad-host-range plasmid RSF1010 (13) corresponding to positions 8250 to 88 and 1661 to 2114 in this sequence. The only discrepancy between the sequences was the presence of an additional base in the pEa8.7 sequence corresponding to the position after nucleotide 8547 in the reported RSF1010 sequence, which results in a change in the C-terminal amino acid

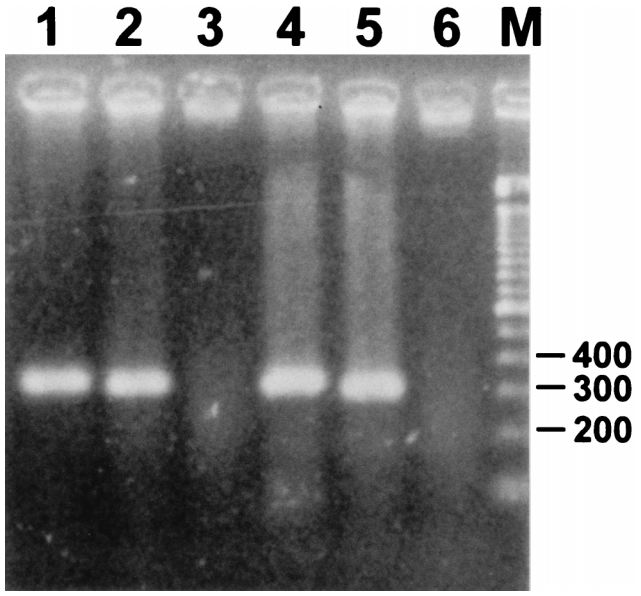


FIG. 2. PCR amplification of *strA* DNA (lanes 1 to 3) and *strB* DNA (lanes 4 to 6) from *E. amylovora*. Lanes 1 and 4, strain CA11 from Michigan containing *strA-strB* (2); lanes 2 and 5, streptomycin-resistant strain CA3R from California; lanes 3 and 6, streptomycin-sensitive strain Ea110; lane M, size markers (100-bp DNA ladder; Gibco/BRL).

sequence for the predicted *sulII* gene product. Because the extra base was also present in the *sulII* gene sequences found in several other plasmids (4, 6, 21), we suspected that there was simply a base missing from the reported RSF1010 sequence.

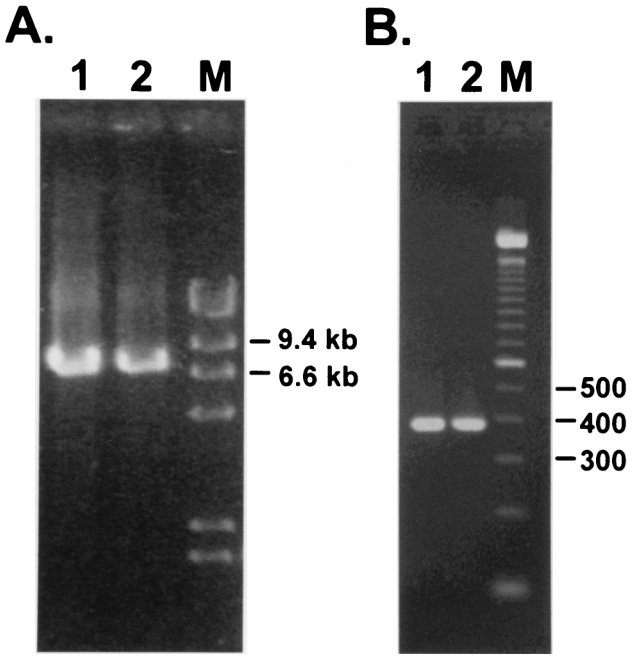


FIG. 3. (A) PCR amplification with primers strA-out and strB-out of plasmid pEa8.7. DNA from *E. amylovora* CA3R (lane 1) and RSF1010 DNA from *E. coli* CB613 (lane 2). Lane M, size markers (*Hin*dIII-digested λ DNA). (B) PCR amplification of *mobB-repB* DNA with primers RSF1010-F and RSF1010-R. Lane 1, RSF1010 DNA from *E. coli* CB613; lane 2, pEa8.7 DNA from *E. amylovora* CA3R; lane M, size markers (kb).

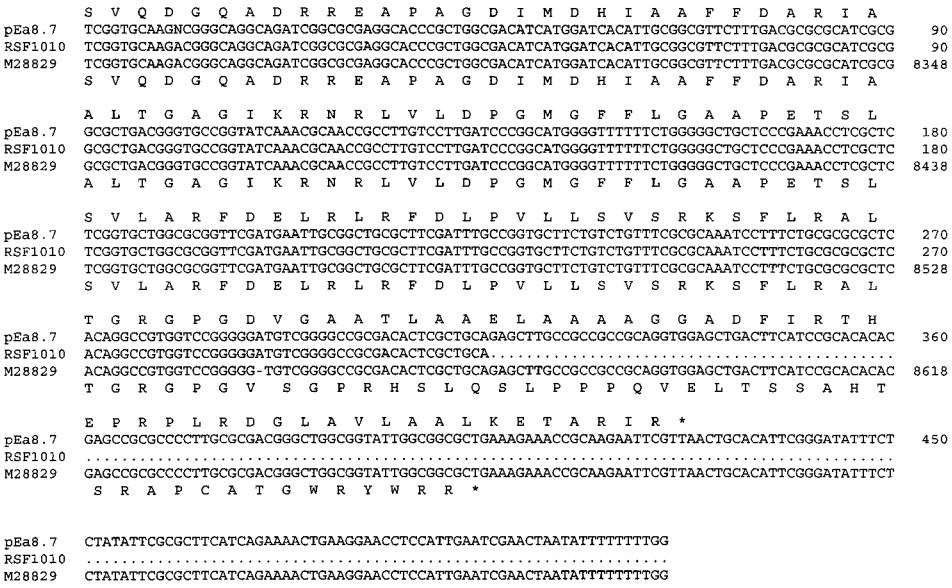


FIG. 4. Alignment of the nucleotide and predicted amino acid sequences of the 3′ ends of the *sulII* genes from pEa8.7 (this work, AF017389), RSF1010 (this work, AF017391), and RSF1010 (reference 13, M28829). The translation of the upper two nucleotide sequences is listed above the sequences and the translation of the lower sequence is listed below the sequences. *, stop codon; -, "missing" base from reported M28829 sequence; ., position for which sequence was not determined.

This was verified by sequencing the 3′ end of the *sulII* gene of RSF1010 (Fig. 4).

A digoxigenin-labelled RSF1010 PCR fragment generated with primers strA-out and strB-out (PCR DIG Probe Synthesis Kit) identified a single band on a Southern blot of total plasmid from strain CA3R (data not shown). *E. amylovora* CA3R and *E. coli* CB613, but not *E. amylovora* Ea110, were able to grow when spotted onto plates of Mueller-Hinton medium (Difco Laboratories, Detroit, Mich.) amended with 200 μg of sulfathiazole (Sigma Chemicals, St. Louis, Mo.)/ml. The establishment of streptomycin resistance in *E. amylovora* in a California orchard was presumably favored by selection pressure imposed when streptomycin was used for fire blight control. As apple growers cannot use sulfonamide antibiotics in orchards, the presence of the *sulII* gene in *E. amylovora* is an example of genetic hitchhiking.

The similarity of the two plasmids outside of the *strA-strB* resistance region was established by using restriction analysis of the PCR product obtained with primers strA-out and strB-out and amplification of the *repB-mobB* region with primers based on RSF1010 sequence data (13) (Fig. 1A). The strA-out and strB-out amplification fragments from *E. amylovora* CA3R and *E. coli* CB613 were similar in size (Fig. 3A) and produced identical *Eco*RV and *Nco*I fragments (Table 1). The PCR product amplified from *E. amylovora* CA3R, with primers RSF1010-F and RSF1010-R under the reaction conditions described above for amplifying *strA* and *strB* sequences, was identical in size to the product from *E. coli* CB613 (Fig. 3B; expected size, 393 bp). The sequence of this fragment (GenBank accession no. AF017392) was identical to the sequence of RSF1010 (M28829) in this region. Because plasmid replication genes play a role in plasmid incompatibility, the identity between RSF1010 and pEa8.7 in this region suggests that pEa8.7 is probably an IncQ plasmid like RSF1010.

Taken collectively, our PCR, sequence, hybridization, and restriction data indicate that plasmid pEa8.7 from *E. amylovora* CA3R is closely related, if not identical, to the broad-host-range plasmid RSF1010. It is interesting to speculate on the dissemination of the members of the RSF1010 plasmid family. RSF1010-like plasmids have been reported in a wide variety of clinical bacteria, but they have not previously been reported in bacteria from plants or soil. Plasmids of the same incompatibility group as RSF1010, IncQ, have been detected in soils (5), but these plasmids have not been further characterized to determine their exact relation to the RSF1010 family. Recently an RSF1010-like plasmid has been detected in *E. herbicola* isolates from apple orchards in New Zealand (19). These observations, taken collectively, suggest that IncQ plasmids, especially of the RSF1010 family, may be more common in environmental samples than was previously thought.

Previously, strains of *E. amylovora* from Michigan with moderate resistance to streptomycin were shown to harbor Tn*5393*-encoded *strA-strB* on conjugative plasmid pEa34 (2). Now, transferable, plasmid-borne streptomycin resistance has been detected in *E. amylovora* isolates from a different geographic

TABLE 1. Restriction fragments obtained by digestion of PCR fragments from plasmids pEa8.7 and RSF1010[a]

Restriction enzyme	Fragment size (kb)		
	RSF1010		pEa8.7 (observed)
	Predicted[b]	Observed	
*Eco*RV	4.319	4.3	4.3
	2.772	2.8	2.8
	0.063	ND[c]	ND
*Nco*I	2.815	2.9	2.9
	1.863	2.0	2.0
	1.158	1.2	1.2
	0.643	0.65	0.65
	0.577	0.60	0.60
	0.098	0.10	0.10

[a] Plasmids pEa8.7 and RSF1010 were amplified from *E. amylovora* CA3R and *E. coli* CB613, respectively.
[b] Based on RSF1010 sequence data (13).
[c] ND, not detected.

region. Although streptomycin resistance due to *rpsL* mutation is already a common phenomenon in California orchards (3, 14), an additional source of streptomycin resistance could make it more difficult to reduce resistance levels to where streptomycin could be reintroduced as part of a disease management plan in the future. Perhaps more seriously, an RSF1010-like plasmid has also been detected in bacteria which share the same niche as *E. amylovora* on another continent where streptomycin resistance in *E. amylovora* has not been a widespread problem. If this plasmid is transferred to *E. amylovora* and becomes established in the population, it could become much more difficult for New Zealand apple growers to control infections. The potential risk in other regions of the world is unknown at this time because the extent to which RSF1010-like plasmids are present in the environment has not been established.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been submitted to the GenBank database under accession no. AF017389, AF017390, AF017391, and AF017392.

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