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

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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 Tn5393 (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 Tn5393 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 Tn5393 (2, 9, 12, 15, 17). However, thus far Erwinia herbicola is the only other species which has been found to harbor the variant of Tn5393 found in E. amylovora (2).

Although Tn5393 appears to be restricted to plant-associated bacteria, the strA-strB genes of Tn5393 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 pGSO5 and pCJ104. 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 Tn5393 (2). Reaction mixtures (50 μl) consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM deoxyribonucleoside 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 H2O, and making a 1:100 dilution in H2O. 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% 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 (NH4)2SO4, 1.75 mM MgCl2, 2.6 U of Expand Long Template enzyme mix, 0.3 μM each primer, 0.35 μM deoxyribonucleoside triphosphates and 1 μl of CA3R plasmid DNA (Wizard Miniprep DNA Purification System, Promega, Madison, Wis.). The reaction was performed according to the manufacturer’s instructions in a model PTC-100HB.
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 NotI-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 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. 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.

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).

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 (HindIII-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).
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 mg 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 EcoRV and NcoI 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. AF017391) 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 Tn5393-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

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Fragment size (kb)</th>
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<tbody>
<tr>
<td></td>
<td>RSF1010</td>
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<tr>
<td>EcoRV</td>
<td>4.319</td>
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<td></td>
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<td></td>
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<td>0.577</td>
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<td>0.098</td>
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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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**REFERENCES**

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